CHAPTER 3

MATERIALS AND METHODS

3.1 Sampling

3.1.1 Study sites

Thailand is located at the center of Southeast Asia, in the middle areas of Indo -China Peninsula. It is situated between 7-20º North and 98º-105º East (Figure 4). The climate of this area is tropical and affected by 2 types of monsoon which cause 3 seasons. The north-east monsoon causes the Cool-dry season (middle of October-middle of February) and Summer (March-May). The rainy season is influenced by the west-east monsoon which blow through this country every 3 months (http://en.wikipedia.org/wiki/Thailand#Geography). From the statistics data of Thai Meteorological Department showed the mean monthly rainfall in Thailand in 30 years periods. It was range from 16.7-252.7 mm with maximum of precipitations in September (www.tmd.go.th/en/climate.php?fileID=7). The preliminary surveys, originally, cyanobacterial samples were investigated from 6 solar salterns in different regions of Thailand which included 1) solar salterns in Ban Maung district, Sakon Nakhon province (SN), 2) salterns in Ban Dung district, Udon Thani province (UT), 3) natural hypersaline in Kham Tale So district, Nakhon Ratchasima province (NR) in Northeastern part; 4) salterns in Tha mai district, Chanthaburi province (CP) in Eastern part; 5) salterns in Ban Laem district, Petchaburi province (PP) and 6)

salterns in Mueang Samut Songkhram, Samut SongKhram province (SS) in middle part of Thailand (Figure 4-5 and Table 2).

However, from the preliminary survey on diversity of organisms from these localities, cyanobacteria were not presented in 3 sampling sites which included UT, SN and CP and the other, NR, these area was changed every times of sampling belonging to the landlord purpose. Therefore, this study will emphatically concern on salt fields in Petchaburi province (PP) and Samut Songkhram province (SS); particularly Petchaburi salterns the greatest diversity and several interested strains were found. The brief geographic positions of Petchaburi, Samut Songkhram locations were shown in Table 2, the others were included. For more information about each area will present in next chapter.

Table 2 Geographic positions and some characteristics of studied areas.

Site No.	Study sites (salter names)	Location , Coordination	Altitude (m)	Some characteristics of areas
1	Petchaburi salterns (PP)	13°30' N 100°07' E	5	-coastal saline area (man-made) -used for sea salt productions
2	Samut Songkhram salterns (SS)	13°25'12" N 100°0'0" E	7	-coastal saline area (man-made) -used for sea salt productions
3	Chanthaburi salterns (CP)	12°36'36" N 102°6'36" E	30	-coastal saline area (man-made) -used for sea salt productions
4	Udon Thani salterns (UT)	17°24'36" N 100°0'0" E	187	-inland saline soils area (man-made) -used for rock salt productions
5	Sakon Nakhon Salterns (SN)	17°10'12" N 104°9'0" E	150	-inland saline soils area (man-made) -used for rock salt productions
6	Nakhon Ratchasima salterns (NR)	14°58'28" N 102°5'3" E	253	-inland saline soils area (natural areas) -waste/wildness land

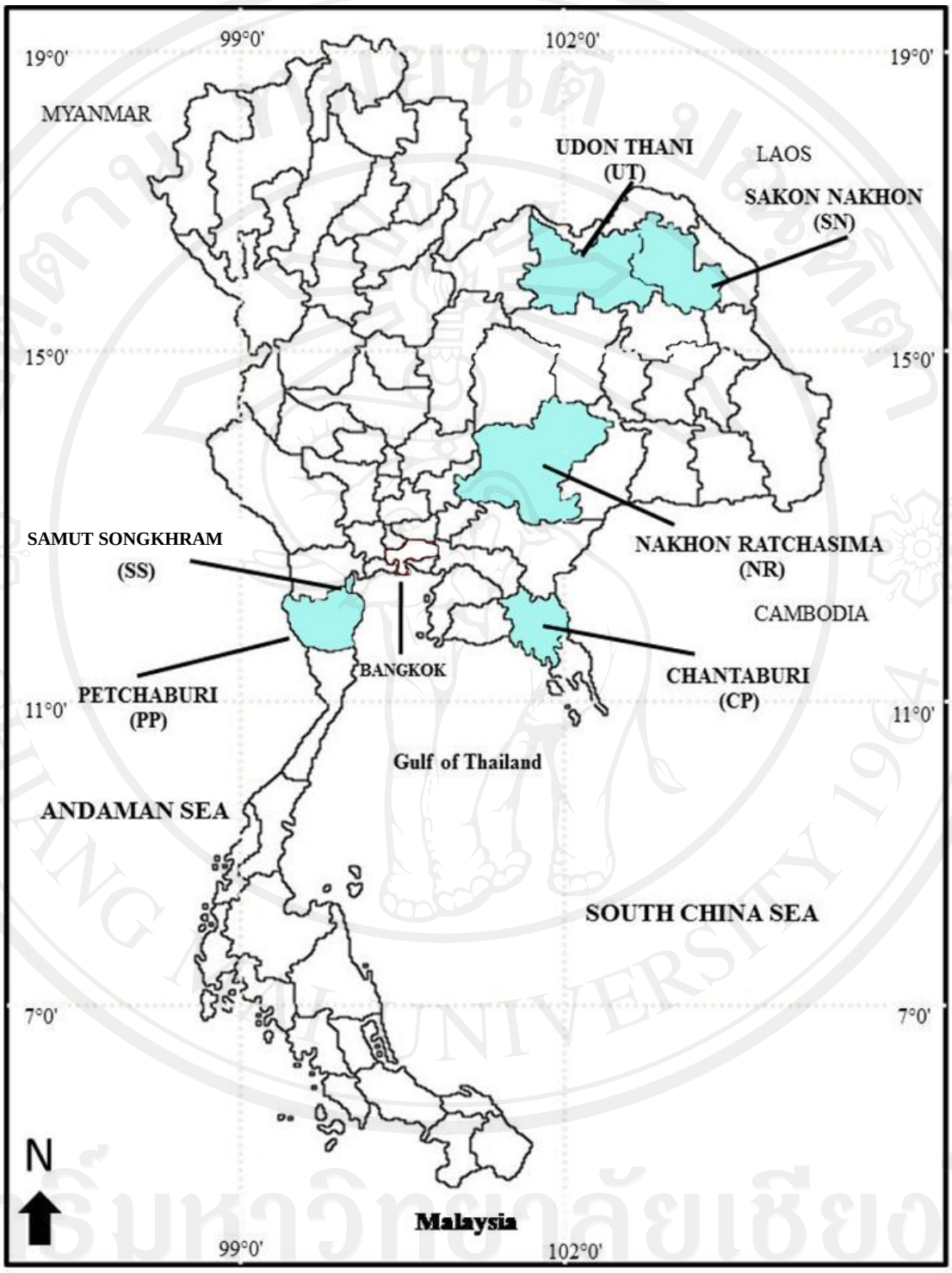

Figure 4 Map of Thailand showed 6 studied areas including salt fields in Petchaburi, Samut Songkhram, Chanthaburi, Udon Thani, Sakon Nakhon and Nakhon Ratchasima province.

Figure 5 The areas of 6 studied locations in different parts of Thailand (1-2) the areas of Petchaburi salterns (PP), (3) saltern in Samut Songkhram (SS), (4) Salterns in Udon Thani (UT), (5) natural hypersaline ares in Nakhon Ratchasima (NR), (6) NR after developed (7-8) Sakon Nakhon saltern (SN) and (9) Chanthaburi saltern (CP).

3.1.2 Cyanobacterial mat collections and preservation

At all study sites, in each area (pond) was firstly measured and classified the salinity profiles. The sampling sites, with salinity more than 35 ppt (3.5%) were chosen. Cyanobacterial mats presented on soil surface of evaporization ponds were collected during salt producing periods (February-May and October-December 2009).

Samples for isolation, cultivation and morphological study were handled by forceps and transferred to sterile Eppendorf tubes. Then, the samples were immediately stored in cool boxes (= 4 °C). The samples for morphological analysis were also preserved by 4% formaldehyde. Some data of each sampling site were recorded such as altitude, geographic position, topography and presence of other organisms, local plants and utilization of each area.

3.1.3 Soils sampling

Soils from each location were collected for the physico-chemical chacteristics analysis. The soils from approximately 10-15 points throughout each pond in each study areas were collected randomly, mixed well together and then put into steriled plastic bags. During the transportation process, all soil samples were mantained in cool boxes as well.

3.2 Soil physico-chemical analysis

Some physico-chemical characteristics of soils from each sampling site were measured in the field such as salinity (used salinometer), temperature, ligh intensity etc. Other soil properties were analyzed in laboratory by Department of Soil Science and conservation, Faculty of Agriculture, Chiang Mai University. Each method for

soil physico-chemical analysis were analyzed following the standard method of soil chemical analysis of the Soil Science Society of America (1996).

All parameters of soils were examined in laboratory as follows:

The physical parameters:

2.1 Moisture content

2.2 Texture

The chemical parameters:

2.3 pH

2.4 Organic matters contents

2.5 Ammonium Nitogen (NH_4^+-N)

2.6 Nitrate – Nitrogen (NO_3^- - N)

2.7 Phophorus

2.8 Potassium

2.9 Sulfur ion

2.10 Calcium ion (Ca^{2+})

2.11 Magnesium ion (Mg^{2+})

2.12 Sodium ion (Na^+)

2.13 Chloride ion (Cl^-)

2.14 Ferric ion (Fe^{2+})

3.3 Evaluation of cyanobacterial morphotypes

Each natural specimen was observed and identified under optical microscope (Olympus BX51 with adaptation to microphoto DP 71 with monitor cell measuring) with different maxnification from 400-1,000X. The identification of each strain was conducted, based on the appaerant charcteristics of each strain such as shape, size and colour of filaments and cells, present or absent of heterocytes and akinetes, the position and shape of heterocytes and/or akinetes, branching, present/absent of sheaths and hormogonia, movements or apical cell characteristics etc. The morphological details of samples were documented by digital photographer and drawing techniques. The relevant keys and other publications include Desikachary (1959), Anagnostidis and Komárek (1985, 1988 and 1990) and Komárek and Anagnostidis (1989, 1998 and 2005) were used for identification.

During morphological observation, the abundance of cyanobacterial species was semi-quantitatively evaluated. Soils 5 gms. from each sample were diluted into 1.5 ml distilled water and 0.02 ml of suspension were used to determine the abundance. Each sample was determined 3 times. The mean of each species that occurred from each site was calculated as arithmetic mean. The abundance of cyanobacterial species was classified into 4 levels as follows:

+++ = dominant, present > 30 % of population

++ = common, present > 10-30 % of population

\+ = present 1-10 % of population

\- = not present

3.4 Isolation and cultivation procedures

The isolation and cultivation of cyanobacteria for molecular and eco-physiological studies were carried out. Small amount of soil samples approximately 5 g were placed onto BG-11 agar media and BG-11 agar media (Rippka *et al*., 1979), Zarrouk's agar medium (Zarrouk, 1966) and Castenholtz modified agar medium (Castenholtz, 1981) with various salt concentrations (0 %, 3 %, 7 %, and 10 %) (Kirkwood *et al*., 2007). Then the soils were spread throughout the whole medium. All cultures were maintained under temperature at 25 °C, 12:12 light:dark cycles at the light intensity of 28 $\mu mol.m^{-2}.s^{-1}$. After 1-2 months of cultivation each growing strain was isolated and transferred into new agar media. The isolation was done continuously until monospecific cultures were gained. This step sometimes took more than 8-9 months.

3.5 Ecophysiological studies

The ecology and some physiological parameters are important for the ecology of some strains were evaluated.

3.5.1 Growth dependence under salinity gradients

The dependence of growth under salinity gradient (modified from Kirkwood, *et al*, 2007) of most monospecific strains were studied.

3.5.1.1 The strains were transferred and cultivated in BG-11 liquid media with their original salt concentrations for 1-2 weeks until initial concentration of inoculum are between 10^4-10^5 cells/ml or A_{750} (OD) = 0.5.

3.5.1.2 Then 200 μl of each strains were inoculated into steriled 96–wells serological plates with salinity ranging from 0 to 40 % of NaCl (0, 2, 6, 10, 15, 20, 25, 30, 35 and 40 %).

3.5.1.3 The cultivation was maintained under temperature around 30 °C and light intensity 32 $\mu mol.m^{-2}.s^{-1}$ for 10 –14 days until the growth is in stationary phase.

3.5.1.4 The optical density (absorbance: OD) at 750 nm (or A_{750}) was measured daily by iEMs plate Reader (Lab Systems, Ltd, Finland).

3.5.1.5 The result of growth dependence under salt gradients were analysed and the graph of growth (OD) versus times (days) were simply constructed by Excel programme.

3.5.1.6 The morphology of each strain was examined under light microscope in 30 random filaments. All equiptments were shown in Figure 6.

3.5.2 Crossed gradients experiments

The selected strains include *Nostoc* sp. 1 (black colony) and *Nostoc* sp. 2 (Green colony) were studied under light irradiance and temperature in the cross gradient unit of light and temperature (Kviderova and Lukavsky, 2001) (Figure 7).

3.5.2.1 Measure width and length of the cross gradients unit every 10 cm.

3.5.2.2 Measure temperature and light irradiance of the instrument and operate the unit overnight.

3.5.2.3 Then, the strains were inoculated into steriled 96-wells serological plates.

3.5.2.4 All fourty two plates of each strain were settled on cross gradients unit (Figure 7-8).

3.5.2.5 The temperature and light irradiance were applied. Temperature was ranged from 17-38 °C in the meantime light irradiance ranged from 30-750 μmol.m^{-2}.s^{-1} by Osram Dulux L55W/12-950, Italy.

3.5.2.6 The optical density (A_{750}) were recorded everyday by iEMs Plate Reader (LabSystems, Ltd., Finland).

Figure 6 All instruments used in growth dependence under salinity gradients experiment (1) all equiptments used in the experiments, (2) BG-11 liquid media with different NaCl concentration (0-40 %), (3) pre-culture prepared for this study, (4) analysis units.

3.5.2.7 After 14 days of experiment, growth rate (A_{750}) of each sample were calculated versus width and legth of the unit.

3.5.2.8 The 3 dimensional graphs of all results were concluded and constructed by Statistica Programme version 5.

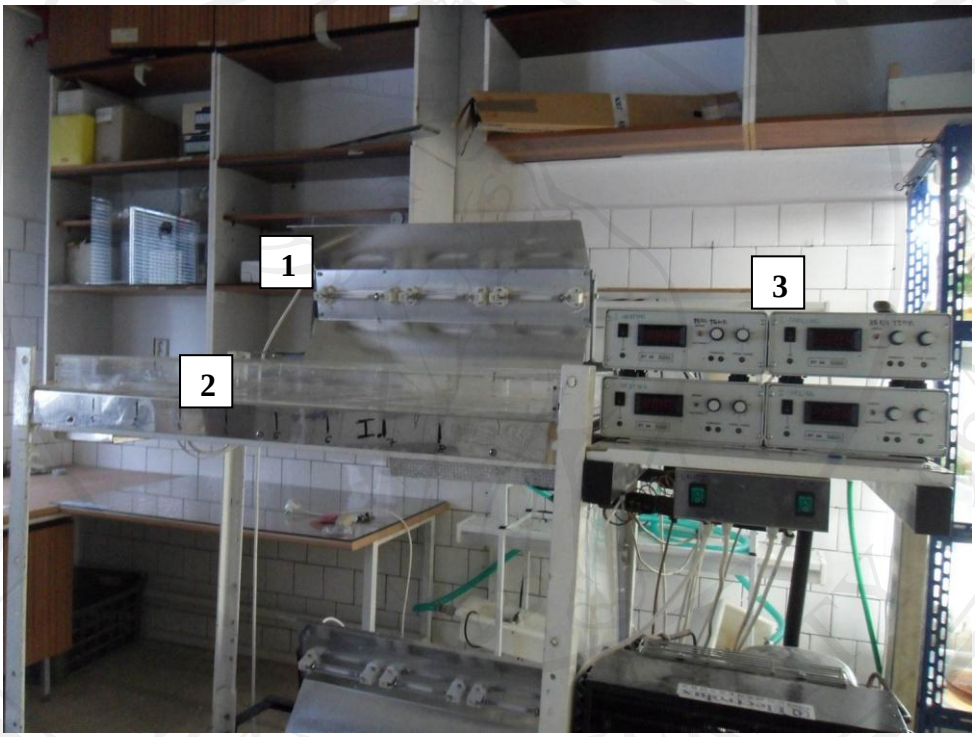

Figure 7 The crossed gradients of light and temperature unit (Kviderova and Lukavsky, 2001). (1) = light irradiance sources (Osram Dulux L55W/12-950, Italy); (2) = the settle unit; (3) = cooling and heating control unit.

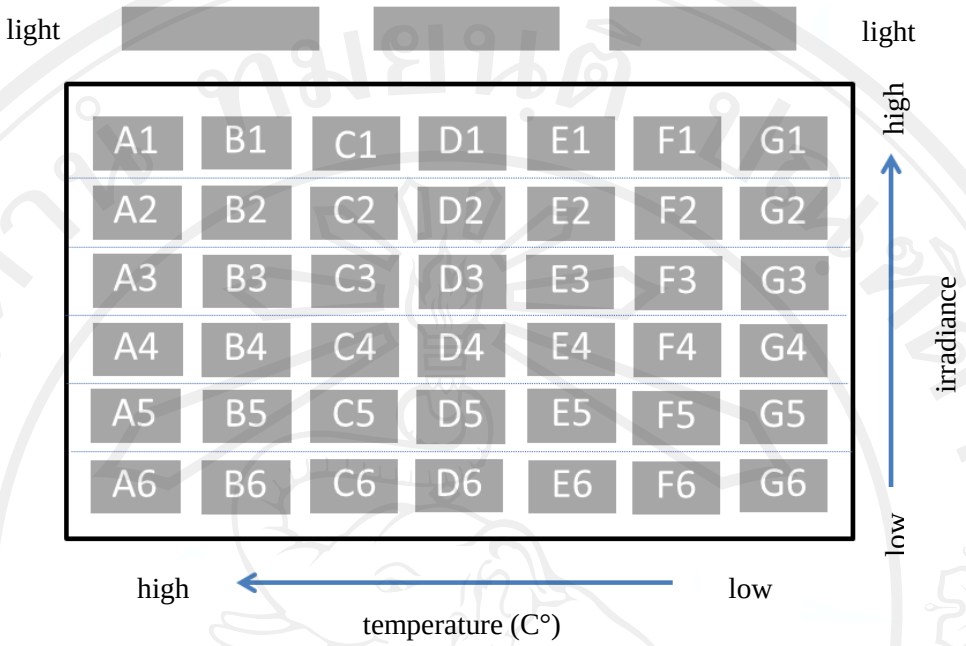

Figure 8 The model of the settle of 42 serological plates on the crossed gradients unit of light and temperature.

3.6 The intensive studies of *Oxynema thaianum* strain CCALA 960

From the observation of all cyanobacterial samples in this research, I found that the cyanophytes which previous designated as *Phormidium lloydianum* (Chatchawan *et al*., 2011) (which now known as "*Oxynema thaianum"* strain CCALA 960) was very interesting. Therefore, I selected this strain to study more in details of their ultrastructures (innercellular structures) and also the phylogenetic position. The morphology of strain *Oxynema thaianum* CCALA 960 was determined followed the traditional previous mentioned method (3) and the morphology of 2 similarly species (*Phormidium lloydianum* and *Phormidium acuminatum*) were compared by herbarium type specimens (*Oscillatoria lloydiana* PC0655991 and *Oscillatoria acuminata* PC0655992) from the Gomont herbarium (Herbarium Cryptogamie Paris-PC).

3.6.1 Study of phylogenetic position

DNA extractions:

The DNA of *Oxynema* strains CCALA960 were extracted using the modified method from Yilmaz *et al*. (2009). The protocol are as follows:

1) The cyanobacterial culture was suspended in 750 µl. of XS buffer (1 % potassium ethyl xanthogenate; 100 mM Tris-HCl, pH 7.4; 20 mM EDTA, pH 8; 800 mM ammonium acetate and 1 % SDS) in steriled eppendorf tubes with mixture 1:5 of glass beads of diameter 0.1 and 1 mm respectively.

2) All tubes were shaken and incubated at 70 °C, 1,400 rpm. for 2 hours with Thermomixer (Eppendorf, Hamburg, Germany).

3) The tubes were frozen at -70 °C for 30 minutes then thawed and shaken for 10 minutes.

4) Centrifigation at 15,000 g for 30 minutes and transferred the supernatant into new tubes.

5) The DNA were precipitated overnight in absolute ethanol (100 %) with the addition of sodium acetate (3 M, pH 5.2).

6) Tubes were centrifuged at 15,000 g for 60 minutes.

7) The supernatant was discarded and the pellet was washed with 100 µl of 70 % of ethanol and centrifuged again at 15,000 g for 15 minutes.

8) The pellets were dried and dissolved in 100 µl of steriled miliQwater.

The PCR amplification of cyanobacterial 16s rRNA gene:

The PCR products of 16s rRNA gene were amplified by the primers 359F (GGGGAATYTTCCGCAATGG; Y = C/T) (Nübel *et al*., 1997) and 23S30R (CTTCGCCTCTGTGTGCCTAGGT) (Wilmotte *et al*., 1993). The PCR products of expected sizes are ≥ 1,000 bp. The PCR cycles were performed with the following profiles:

Starting denaturing step	94 °C	5	minutes	
Denaturing step	94 °C	30	second	30 cycles
Annealing step	53 °C	30	second	
Extension step	72 °C	3	minutes	
Final extension	72 °C	7	minutes and cooling at 4 °C	

The PCR products were checked by 1 % of agarose gel which stained by ethidium bromide to estimated sizes, quantity and quality of products. After running gel electrophoresis PCR products were purified using QIAquick PCR purified kit.

Sequencing of PCR products:

Sequencing of PCR products were done on ABI sequencer with 6 primers that included:

1) 27F (Lane, 1991)
   (AGAGTTTGATCMTGGCTCAG; M = A/C)
2) 23S30R (Wilmotte *et al*., 1993)

(CTTCGCCTCTGTGTGCCTAGGT)

3) CYA 1064R (Strunecky *et al*, 2010)

(GATTCGCGACATGTCAAGTCTTGGTAAGG)

4) CYA783F (Strunecky *et al*, 2010)

(TGGGATTAGATACCCCAGTAGTC)

5) S17* (Wilmotte and Herdman, 2001)

(GGCTACCTTGTTACGAC)

6) ILE23F (Wilmotte and Herdman, 2001)

(ATTAGCTCAGGTGGTTAG)

The successful sequence of *Oxynema* strain CCALA 960 was submitted to GenBank under accession number JF729323.

Phylogenetic analysis:

The successful sequences with more than 1,000 bp were blast analysis against GenBank 16s rRNA database to identify species or similarity. The sequences were aligned with the closest relatives of culture and uncultured cyanobacteria in MAFFT (Katoh and Toh, 2010), minor changes were done manually with BioEdit 7.0.1 (Hall, 1999). Phylogenetic trees were constructed from the alignment sequences in Mega 5 (Tamura, *et al*., 2007) by the Maximum Likelihood method using Jukes-Cantor distance matrices. Topology was validated by Bayesian analysis in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) and unweighted maximum parsimony was implemented in PAUP* (Swofford, 2001).

3.6.2 Ultrastructure studies

The innercellular structures (thylakoid patterns, granules, cell walls, inclusions, etc.) of this strain were done using transmission electron microscope (TEM) and scanning electron microscope (SEM) (modified from Kellenberger *et al*, 1958).

3.6.2.1 Transmission electron microscope study

1) For TEM study, filaments were taken from agar cultures and fixed in two ways:

a) Osmiumfixation: 1% osmium tetroxide in 0.7 % veronal-acetate buffer, pH 6.5, with traces of sodium chloride and calcium chloride, was applied for 3 hours, following by postfixation with 0.5 % uranyl acetate in the same buffer.

b) Glutaraldehyde fixation: 3 % glutaraldehyde in 100 mM cacodylate buffer, pH 7.3, was applied at 4 °C over night, washed 3 times (10 minutes each) with the same buffer, followed by postfixation with 2 % osmium tetroxide in the same buffer for 2 hours.

2) The fixed material was washed 3 times (10 minutes each) with the respective fixation buffer and instillation into 2 % agar.

3) Dehydrated by a gradual series of ethanol baths with concentrations increasing from 30 % to 100% for three hours each.

4) The material was infiltrated with the metacrylate LR White Hard, encapsulated and polymerized at 60 °C for two days.

5) Sections were cut using a Reichert-Jung ultramicrotome Ultracut E. Ready made section on supporting grids were contrasted with 2.5 % uranyl acetate and alkaline Reynolds solution (3 % lead nitrate with 3 % sodium citrate).

6) The resulting sections were photographed at various magnifications under an FEI Morgagni 268D digital transmission electron microscope.

3.6.2.2 Scanning electron microscope study

1) A culture of *Oxynema* was fixed with 3 % glutaraldehyde and washed 3 times (10 minutes each) by 100 mM cacodylate buffer pH 7.3.

2) The biological material was dehydrated by ethylalcohol in concentration increasing from 30 % for 30 minutes to 100 % overnight.

3) The dehydrated material was dried at the critical point, mounted on supporting blocks, metal-plate by gold.

4) The samples were photographed in a TESCAN a.s. MIRA3-FEG-SEM digital scanning electron microscope.