CHAPTER 3

MATERIALS AND METHODS

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3.1 *Gracilaria changii* Abbott, Zhang & Xia (Xia & Abbott, 1987; Abbott *et al.*, 1991; Lewmanomont, 1994; Phang, 1994)

Gracilaria changii is relatively bushy with thalli 6 - 20 cm tall; purplish brown to dark brown in colour when dry; one to many turgid branches arising from a dics-like holdfast or from a percurrent axis. Branching of two to four orders; irregular, alternate or secund; cylindrical 0.3 - 2.5 mm in diameter, abruptly constricted at the base forming a slender stipe, slightly swollen distal of stipe, tapering at the tip. Tetrasporangial and gametophytic plants similar in size and branching pattern. Fronds in transverse section consist of large rounded medullary cells (350 - 570 μm) with thick cell walls (7.5 - 20 μm), and 2 to 3 layers of small, pigmented cortical cells; transition of cells abrupt. Tetrasporangia ovoid to elongate, densely scattered over frond surface; cruciately divided. Spermatangial conceptacles verrucosa-type, oval to nearly globose, adjacent conceptacles may coalesce forming polycavernosa-type spermatangia. Cystocarps conical or semiglobose, up to 1.4 mm diameter, slightly rostrate, not constricted at base; basal absorbing filaments few, lateral and upper; carposporangia rounded or ovoid, 18 - 32.5 μm; pericarp thick consisting of rounded to oval cells and inner 7 - 9 rows of compressed cells.

Gracilaria changii grows abundantly on intertidal mudflats and attached epiphytically to mangrove roots, shells and pebbles and fish cages.

3.2 Sampling Sites

Samples were collected in clean plastic bags from the *Gracilaria changii* Abbott, Zhang and Xia farm of the Fisheries Department, Ban Merbok, Kedah. *Gracilaria changii* was cultivated in an integrated system with prawns and sea bass in a 0.5 ha earthponds at a cleared mangrove area. Wild populations of *Gracilaria changii* from the mangroves of Morib and Carey Island (Fig. 2), Selangor and Sungai Pulai, Johor were sampled. *Gracilaria changii* growing in fish cages in Kuala Setiu, Terengganu and Kukup, Johor were also sampled. The seaweeds were put in an ice-chest and were brought immediately to the laboratory for the screening of disease causing organisms.

3.3 Isolation of Disease Causing Organisms

Thalli of the seaweeds which were morphologically different from healthy thalli and displayed symptoms of being sick that is thalli bleaching (pale green, white or pale pink) as seen in Figure 3, loss of rigidity, decay, depressions/bruise along its' length, the appearance of mottled patches, green bands encircling the thallus and galls (Put-Ang, 1995; Jaffray & Coyne, 1996) were selected to further screen for causative agents of disease.

Fig. 2. Sampling site of *G. changii* on Carey Island.

Fig. 3. Thalli of *G. changii* with 'sick' symptoms. Note the discolouration of the thalli and rotting of the thalli tip.

3.3.1 Isolation of Bacteria

Thalli showing 'sick' symptoms were cut out and rinsed repeatedly with sterile seawater. Bacteria were isolated using three different techniques:

1. Discs of 'sick' thallus approximately 3 mm long were swabbed across petri plates containing Marine Agar 2216E (Difco).

2. Discs of thallus were partially inserted into plates of Marine Agar 2216E.

3. A weighed portion of thalli was rinsed with 70% alcohol, homogenised in 10 ml of sterilised seawater. One ml of the suspension was diluted to 10^{-5} in test tubes with sterile seawater. An amount of 0.2 ml homogenate from each dilution was spread on MA 2216E agar. Each dilution had 2 replicates.

4. Agar-degrader bacteria were isolated on a media which allowed a better development of agar-degrading bacteria. 'Agar-degraders' were considered to be those which either proliferated into the agar, forming depressions, or visibly liquefied it (Austin, 1988; Weinberger *et al.*, 1994). This medium contains 0.5 g peptone (Difco), no yeast extract, 15 g bacto-agar (Difco) and 0.01 g $FePO_4$.

All plates were incubated at 25°C for up to 7 days. The pH of the media was adjusted to 7.2. The bacterial colonies were isolated based on frequent occurrence and different morphological characters (e.g. shape, colour, size). Purified cultures are maintained in agar slants at 4°C.

3.3.2 Isolation of Fungi

1. Direct Plating

Parts of the'sick' seaweed thalli were surface sterilised by rinsing the thalli with (i) 70% alcohol (ii) soaking for 2 minutes with 0.5% Chlorox. Fungi were isolated using four different media: Petri plates containing Seawater Potato Dextrose Agar (SPDA) and Czapeks Dox Agar, both added with 200 $mg.l^{-1}$ Chloramphenicol, Malt Extract Agar (2% malt extract) and Corn Meal Agar (CMA) added with 0.5 $g.l^{-1}$ Streptomycin and 0.5 $g.l^{-1}$ Penicillin G, were inoculated with discs of 3 mm length surface-sterilised thalli.

2. Baiting Technique (Jones, 1971)

This technique is used to primarily isolate lower fungi, those producing zoospores as Phycomycetes rarely develop in sufficient numbers for them to be identified in the field (Jones, 1971). Sterilised petri dishes were one half filled with autoclaved seawater added with the antibiotics Streptomycin (0.5 $g.l^{-1}$) and Penicillin G (0.5 $g.l^{-1}$). Sterilised grass leaf and maize were added as baits. Algal discs were placed in the petri dish together with the baits, incubated at 20°C and examined at daily intervals from the third day to the seventh day of incubation. Fungi which grow on these baits can be isolated into pure cultures by plating them onto cornmeal agar with added antibiotics: Streptomycin and Penicillin G.

3.3.3 Screening for Virus

The detection of virus was done using transmission electron microscopy. Thalli of 0.05 grams were homogenised in 0.5 ml seawater. The homogenate was centrifuged at 5000 rpm for 5 minutes. One drop of supernatant was dropped onto a Formvar and carbon coated 300-400 mesh copper grid for 5 minutes. Excess liquid was removed by drying with filter paper. The grid was then negatively stained with 1% uranyl acetate for 5 minutes then dryed as before. Ultrathin sections of 'sick' thalli was also viewed under TEM. The grid was then viewed using Philips CM12 TEM. The remaining sample (thalli) was dessicated in a vacuum jar with silica gel. When dried, the sample was placed in Bijoux bottles layered with $CaCl_2$ and muslin cloth before storage at 0°C.

3.3.4 Isolation of Algal Epiphytes, Endophytes and Unicellular Organism

The macroalgal epiphytes were separated from the thalli using forceps whereas the microalgal epiphytes were observed under light microscope for identification purposes. Endophytes invading the cellular matrix of the seaweed was screened by making thin cross-sections of the thalli. The cross sections were stained with iodine or aniline blue. They were then observed under the microscope for a clearer view of the endophyte morphology (ie. branching of

thalli, etc). Unicellular organisms invading cells of *G. changii* were screened using transmission electron microscopy.

Specimen Preparation for Transmission Electron Microscope

Samples were prefixed in 4% glutaraldehyde in 0.1M cacodylate buffer.

↓

The samples were postfixed in 1% aqueous Osmium tetroxide for 1 h.

↓

Samples were washed 2 to 3 times in distilled water for 15 min per wash

↓

They were dehydrated in an ascending series of ethanol from 10% to 100% with 10% intervals

↓

Specimens were embedded in Epoxy resin

↓

Blocks were cut on Reichert Ultra E

↓

Ultrathin sections were stained with 50% aqueous uranyl acetate for 5 minutes followed by Reynold's Lead citrate Stain (in 0.1N NaOH) for 10 minutes at room temperature

↓

Sections were placed on 300-400 mesh copper grids and viewed on Philips CM12 TEM

3.4 Re-infection of Isolates into *G. changii* Thallus

The isolated bacteria, fungi and virus were screened for their ability to infect the seaweed *G. changii*.

3.4.1 Bacterial Infection of *G. changii*

3.4.1.1 Infection of Non-axenic Thallus with Bacteria (Seawater Medium)

The bacterial isolates were tested for their ability to infect the seaweed *G. changii*. Healthy thalli which were cleaned and freed from external epiphytes were selected and cultured in synthetic seawater medium in an orbital shaker (100 rpm: 25°C under 12:12 h LD. Six centimeter length thalli were subcultured thrice at ten days intervals into 150 ml of fresh synthetic seawater medium to ensure removal of unwanted surface organisms.

Table 3: Composition of Synthetic Seawater Medium.

a) Marine Environment* Synthetic sea-salt (Aquacraft Inc., USA)	30 g dissolved in 1 L of distilled water, stirred and filtered (Whatman No. 4 diameter 90 mm)
b) Stock solutions	per 400 ml
1. $NaNO_3$	10.0 g
2. K_2HPO_4	7.0 g
3. Vitamin solution	per 200 ml
Cyanocobalamin (Vitamin B12)	0.0008 g
Thiamine HCl (Vitamin B1)	0.0008 g
Biotin	0.0008 g
4. $NaSiO_3.5H_2O$	2.24 g
Final solution:	
Stock solution 1	10.0 ml
Stock solution 2	1.0 ml

Make up to 1 L with synthetic seawater prepared from a) Adjust to pH 8.0 with 2N HCl.

*c) Contents of Marine Environment Sea-salt

Cl	20,000 $mg.l^{-1}$
Na	10,599
SO_4^{-2}	2,506
Mg	1,360
Ca	440
K	393
Br	55
Sr	7.2
PO_4^{-3}	0.010
I	0.120
Al	0.020
Fe	0.120
Zn	0.045
Ni	0.0023
Cu	0.037
Pb	0.004
Thallium	0.0001
Cd	0.0003
Se	0.005
V	0.0003

Bacterial isolates were cultured in marine broth: (per liter distilled water) 30.0 g NaCl, 2.3 g MgCl, 0.3 g KCl, 2.0 g glucose, 5.0 g casamino acid and 1.0 g yeast extract, adjusted to pH 7.2 with 1 M NaOH. The cultures were incubated overnight at 25°C in 20 ml marine broth on an orbital shaker (Lab Line) at 100 rpm. The culture is allowed to reach an optical density (A_{600nm}) of between 1.20 and 1.50. Two hundred mililiters of bacterial suspension were injected into the end of each 6 cm length thallus to a depth of approximately 1 cm using 28G x 1/2" needle (PharmaPlast). A 6 cm length thallus injected with sterilised seawater, and an uninjected thallus, served as experimental controls. The experiment were run in triplicates. The thalli were incubated at 25°C in sterile

synthetic seawater for 14 days and scored for the appearance of disease symptoms. The infected area was cut out, rinsed repeatedly with sterile seawater, followed by 70% alcohol, then homogenised and plated on Marine Agar 2216E. The cultures were scored for the growth of bacterial strains identical to the strain originally injected into the thallus.

3.4.1.2 Infection of Axenic Thallus with Bacteria (Marine Broth Medium)

Isolates causing disease symptoms in Section 3.4.1.1 above were selected for the following experiments.

1. Generation of Axenic Thallus (Jaffray & Coyne, 1996)

Five gram (wet weight) of healthy *G. changii* which were free of algal epiphytes were immersed in 500 ml of sterile distilled water for 4h. The algae were re-immersed in 100 ml of sterile seawater (SSW) followed by sonication in a sonicating bath (Branson 1200 E1) at 50-60 Hz for 4 min. Subsequently, the algae were immersed in 100 ml Povidone-iodine (1%) for 60s and finally incubated for 24h in 100 ml SSW containing a concoction of antibiotics. The composition per litre SSW was streptomycin (500 mg), kanamycin sulphate (500 mg), Penicillin G (500 mg), cefotaxime (300 mg) and nalidixic acid (0.6 mg). The algae were rinsed thoroughly in SSW and tested for axenity by rubbing 5 to

6 cm thallus pieces across the surface of Marine Agar 2216E, adjusted to pH 7.2 with 1M NaOH. The cultures were incubated for up to 14 days at 25°C, during which time they were monitored for growth of bacterial colonies.

2. Pathogenicity Assay

The bacterial isolates that caused disease symptoms in the experiment conducted in Section 3.4.1.1, were cultured in marine broth (MB) as described previously. Six centimeter lengths of axenic *G. changii* were injected with the bacterial strains as described previously. Each injected thallus was placed in a 250 ml Erlenmeyer flask containing 150 ml MB and incubated at 25°C for 5 days. The experiment were run in three replicates.

Thalli were scored for the appearance of symptoms after 5 days. In order to satisfy Koch's postulates, bacteria from the culture media were inoculated on Marine Agar 2216E and incubated at 25°C for 5 days. The cultures were scored for the growth of bacterial colonies identical to the strain originally injected into the thallus.

The same experiments using the strains causing disease symptoms in this experiment and experiment at Section 3.4.1.1 (infection into non-axenic thallus in SSW), were repeated on axenic thalli in SSW and marine broth.

3.4.1.3 Identification of the Bacterial Isolates Causing Disease Symptoms

Bacteria were identified using the Biolog - Bacterial Identification System (Microstation System) (Biolog, Inc. 1992).

The Biolog System

Introduction

Biolog has introduced the GN MicroPlate panel containing 95 tests for the identification of a very wide range of Gram-negative species and the complementary GP MicroPlate for Gram-positive bacteria. The YT MicroPlate was introduced for the identification of yeast and important lactic acid bacteria. With these three kits combined, over 1,100 species of aerobic bacteria and yeast can be identified. This includes virtually all human and animal pathogens, most of the important plant pathogens, and most of the environmental species. All 95 tests are carbon source utilisation tests. Utilization of a carbon source is detected as an increase in the respiration of cells in the well, leading to irreversible reduction of a tetrazolium dye. A positive utilisation reaction is indicated when a purple colour forms in a well. This "breathprint" pattern that results can be read either by eye or with an automated microplate (ELISA) reader. With 95 tests that can either be "positive" or "negative" there are 2^{95} possible "breathprint"

patterns that theoretically could result. Biolog has developed a software programme that will run on any compatible PC.

To identify an unknown bacterium, Gram stain is first performed. Gram-negative bacteria are inoculated into the GN MicroPlate and Gram-positive bacteria are inoculated into GP MicroPlate.

GN MicroPlate

The GN MicroPlate tests the ability of Gram-negative microorganisms to utilize (oxidize) the 95 carbon sources shown in the accompanying plate map in Appendix 1. The GN MicroPlate uses the Gram-negative Data Base, which includes all types of Gram-negative species: enterics, non-fermenters, and fastidious microorganisms. For inoculation of the GN MicroPlate, the bacteria are suspended in 0.85% saline.

GP MicroPlate

The GP MicroPlate tests the ability of Gram-positive microorganisms, to utilize the 95 carbon sources shown in the accompanying plate map in Appendix 1. The Gram-positive Data Base includes a broad range of Gram-positive species, and includes cocci, rods, and spore-forming rods. For inoculation of the GP MicroPlate, the micoorganisms are suspended in 0.85% saline.

Test Procedures

A) Specimen Preparation

Gram-staining of bacterial isolate is performed to separate the Gram-positive bacteria and Gram-negative bacteria. Bacterial isolates are grown on Marine Agar 2216E overnight at 25°C and prepared for inoculation in Microplate.

Reading MicroPlates with a MicroPlate Reader

MicroLog 3 computer software is used to perform automated readings. The MicroPlate lid is removed and the plate placed into the drawer of the reader. The reader is set to read at a wavelength of 590 nm. The "SIM" value shown when the plate is read ranges from 0.0 to 1.0. In order to be accepted as an identified isolate, "SIM" value should be more than 0.5.

B) Inoculum Preparation and Inoculation of the MicroPlate

Sterile tubes were filled with 18-20 ml of sterile normal saline (0.85%, pH 5.5-7.0 NaCl), prewarmed to 28-35°C

↓

MicroPlates were removed from refrigerator and prewarmed at 28-35°C (room temperature)

↓

The transmittance of the turbidimeter was set to 100% using an uninoculated saline tube (blank).

↓

Cells were removed carefully from plates using sterile swab so as not to carry over any nutrients from the MA plate into the suspension; a transmittance level of about 53-59% (3 X 10^8 cells/ml) for Gram-negative bacteria and 35-45% for Gram-positive bacteria is needed.

↓

The cell suspension was then poured into a multichannel pipet reservoir; 12 sterile yellow tips were fastened to the micropipettes

↓

Tips were filled to check that all of them are filled equally. Next the tips were primed by dispensing the first delivery back into the reservoir.

↓

All wells were precisely filled with 150 µl very carefully as no splashing/spillage should occur

↓

MicroPlate was covered with its lid and incubated at 37°C for 24 hours in a plastic container layered with a slightly wet tissue paper underneath as a source of moisture

3.4.2 Fungal Infection of *G. changii* Thallus

Fungal isolates were grown on Seawater Potato Dextrose Agar and Malt Extract Agar at 20°C for 4 days depending on which media they were originally isolated from. One loopful fungal mycelium was added into 250 ml Erlenmeyer flasks containing axenic thalli of 6 to 10 cm length immersed in 150 ml sterile seawater medium. The cultures were incubated at 20 °C for 20 days. They were scored for the appearance of disease symptoms. Cross-sections of 'sick' portions were made and viewed under light microscopy.

3.4.3 Viral Infection of *G. changii* Thallus

An amount of 0.06 gm dessicated algal samples which were previously identified as containing putative virus particles were ground using a mortar and pestel, with few drops of sterile seawater and enough celite to make a paste.The paste was mechanically inoculated by rubbing along the entire length of the thallus and the thallus was placed into a 250 ml Erlenmeyer flask containing 150 ml sterile seawater medium. The cultures were incubated for 14 days and scored for symptoms of disease. Portions of diseased thallus were sectioned and screened through the Transmission Electron Microscope (p. 47) for the strain originally inoculated into the thallus.

3.5 The Influence of *G. changii* Extract on Bacterial Growth

3.5.1 Production of *G. changii* Extract

Hundred grams of *G. changii* thalli were washed and cleaned of algal epiphytes. The thalli were blended together with 200 ml seawater, strained, filtered with glass fibre filter, followed by 0.45 µm Millipore filter. Seaweed extract with concentrations of 0.04, 0.08, 0.16 and 0.2 $g.ml^{-1}$ were prepared and filter-sterilised using 0.22 µm Millipore filter.

3.5.2 Preparation of Bacterial Suspension.

The bacterial isolates that caused disease symptoms in Section 3.4.1.1 were cultured overnight at 25°C in Marine Broth.

Plates of Marine Agar 2216E were prepared. Hundred microliters of bacterial supension were added into 3 ml of 0.7% agar (Bacto-agar) at 45°C and overlaid on Marine Agar 2216E plates. Paper disc of 6 mm diameter were inserted in four quarters and in the center of the plate.

A total of 30 µl seaweed extract with concentrations of 0.04, 0.08, 0.16 and 0.20 $g.ml^{-1}$ respectively were pipetted onto the disc. Only seawater was

pipetted onto disc in the center of each plate. This together with the other four discs of various extract concentrations, on agar overlay void of bacterial suspension were used as control. The plates were incubated at 25°C for 10 days. The tests were run in quadriplets.

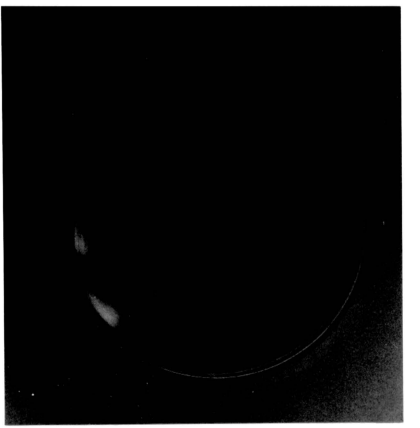

Fig. 4. Marine Agar 2216E with paper discs containing different concentrations of seaweed extract plus control (sterile seawater) in the center.

3.6 The Effect of *G. changii* Extract on Fungal Growth

Malt Extract Agar with added seaweed extracts with concentrations of 0.02, 0.04 and 0.06 $g.ml^{-1}$ were inoculated with fungal isolates which were pregrown on MEA at 20°C for 4 days. Fungal isolates were introduced onto the plates through cut-out agar discs (diameter of 3mm) of the pregrown culture. MEA without any seaweed extract were used as control. All agar plates were inoculated at 20°C for 10 days. Mycelial growth were indicated by measuring the diameter (from the centre of agar disc to the section where mycelial growth has extended) of mycelial colony. Mycelial growth were measured at the fifth day. All agar plates were added with antibiotics: 0.5 $g.l^{-1}$ Streptomycin and 0.5 $g.l^{-1}$ Penicillin G to eliminate bacterial contamination. The tests were done in replicates of four.