CHAPTER 4

RESULTS

CHAPTER 4 : RESULTS

4.1 Isolation and Identification of Organisms

4.1.1 Isolation and Identification of Bacteria

A total of 43 bacterial isolates were collected and a detailed description of the diseased thalli from which they were obtained is described in Table 4. The colonies that appear were isolated based on frequent occurrence and different morphological characters (Kong & Chan, 1979). Most of the bacterial colonies were isolates from whitened/bleached and soft/decayed tissues. The disease symptoms most commonly found were the whitening of the thalli. A total of 24 isolates were from samples obtained in Kedah, eight from Morib, five from Sg. Pulai, Johor, three from Carey Island, and three from Kuala Setiu, Terengganu.

Two agar-degrading bacteria were isolated from 'sick' thalli obtained from the mangrove in Morib. One colony appeared purple on Marine Agar 2216E (Fig. 5) after six days of incubating a piece of diseased algal thallus swabbed across the agar surface. There was depression on the agar all along the area where the colony spread. The other isolate was dark orange in colour obtained from diluted (10^{-1}) algal homogenate forming tiny colonies (1 mm diameter) and depression in Marine Agar 2216E at the third day of incubation.

However, both strains could not be reisolated. Subculturing the strain on Marine Agar 2216E and reactivation of it in marine broth failed to produce any growth.

Table 4: Bacteria Isolated from Diseased Thalli of *G. changii.*

Isolate	Source of Collection,	Condition of Host Thalli				Condition of Seawater at Collection Site	
No.	Date and Location	white	soft	depression	other	pH	Salinity
K1B1	Ban Merbok, Kedah.	+		+		7.48	28
K1B2	Month of June.		+				
K1B3		+					
K1B4		+					
K1B5		+					
K1B6		+	+				
K1B7		+	+				
K1B8				+			
K1B9					pale pink		
K1B10				+			
K1B11				+			
K1B12		+	+				
K1B13		+					
K1B14		+					
K1B15		+					
K2B16	Ban Merbok, Kedah.				pale green	7.63	28
K2B17	Month of October.		+				
K2B18			+	+			
K2B19			+				
K2B20			+				
K2B21			+				
K2B22		+					
K2B23		+					
K2B24		+					
M1B25	Morib, Selangor.	+			pale green	7.50	30
M1B26	Month of July.	+					
M1B27		+	+				
M1B28		+					
M1B29		+	+				
M2B30	Morib, Selangor.			+		7.64	30
M2B31	Month of November.			+			
M2B32		+					
J1B33	Sg. Pulai, Johor.	+	+			7.52	35
J1B34	Month of December.	+	+				
J1B35		+					
J1B36		+	+		pale pink		
J1B37					pale pink		
C1B38	Carey Island, Selangor.	+				7.45	20
C1B39	Month of January.	+					
C1B40							
T1B41	Kuala Setiu, Terengganu.	+				7.57	22
T1B42	Month of April.	+					
T1B43		+					

+, presence of specified disease symptoms.

Table 5 : Some Characteristics of the Agar-degrading Bacteria.

Characteristics	Orange colony	Purple colony
1. Colony Morphology	small circular	circular
2. Pigmentation	orange	purple
3. Cell Morphology	long straight rods	short rods
4. Mobility	-	+
5. Gram reaction	Gram negative	Gram negative

Fig . 5. Colonisation of an agar-degrading bacteria (purple colony) on Marine Agar 2216E.

4.1.2 Isolation and Identification of Fungi

The identification of fungus was done by Professor Gareth Jones (Portsmouth University, United Kingdom) and Dr. Siti Aisah bt. Alias (University of Malaya), as well as with reference to Barron (1968) and Smith, (1969). A total of eleven isolates were collected. The isolation of the lower fungi (Chytridiomycetes, Hyphochytridiomycetes, Oomycetes) using the baiting technique did not yield any isolates. Zoospores were not found attached to the baits. Mycelial fungi presumably terrestrial were attached to the baits. It was therefore not isolated. Identification and isolation of fungi using the direct plating method is shown in Table 6.

The majority of the species isolated were terrestrial Fungi Imperfectii. Only *Cladosporium* can be described as marine (Pugh, 1974). One yeast-like fungi was isolated. Two of the isolates are designated as sterile mycelia as it did not produce fruiting bodies. A description and photographs of the isolates obtained is given.

Table 6: Fungi Isolated from *G. changii.*

Sampling Sites	Specimen No.	Isolated Fungi	Isolation Media and Method
		Phylum Deuteromycota	
Morib	AF1	[a]*Cladosporium sp.*	SPDA (plating techniques); i
Morib	AF2	[b]*Cladosporium sp.* (probably a different species).	" ; ii
		Phylum Ascomycota	
Kedah	AF3	[a]*Aspergillus sp.*	" ; i
		Phylum Deuteromycota	
Kedah	AF4	[a]*Penicillium sp.* (*Eurotium* stage)	" ; i
Kedah	AF5	[a]*Penicillium sp.*	" ; i
Kedah	AF6	[a]Sterile mycelia.	" ; ii
Kedah	AF7	[c]Sterile mycelia producing chlamydospores. Oil production is also observed.	" ; ii
		Phylum Deuteromycota	
Kedah	AF8	[c]*Gliomastix sp.* (produces oxalates).	" ; ii
Kedah	AF9	[a]*Gliomastix sp.*	" ; ii
Carey Island	AF10	[b]*Pestalotia* sp.	MEA ; ii
Carey Island	AF11	[a]Yeast-like fungi.	MEA ; ii

i, seaweed thalli is sterilized by rinsing with 70 % alcohol;
ii, seaweed thalli is sterilized by soaking in 0.5 % Chlorox.
[a], fungus isolated from softened thalli; [b], fungus isolated from thalli with whitened patches;
[c], fungus isolated from decayed/fragmented thalli.

Cladosporium sp.

Description:

The culture is thick velvety, deep rich green in colour, with a reverse a characteristic greenish black. Conidia are borne singly at the apex, not coiled, spherical, in chains, non-septate, brown in colour. Synnemata and sporodochia lacking, conidiophores lack apical swelling and found developing and maturing acropetally. The other isolate identified as *Cladosporium* may be of a different species as the colour of the culture was grey green, with a reverse of opalescent blue black.

Aspergillus sp.

Description:

Culture is smoky brown in colour, with a reverse of a pale yellow. The apex of the main conidiophore axis swells up to form a vesicle on which the phialides are borne directly. Conidiophores stand solitarily with non-septate conidias persisting in chains.

Penicillium sp.

Description:

Culture is a slight green in colour with a reverse of a beige colour. Vegetative mycelium septate, conidiophore terminating in a broom-like whorl of branches (penicillus), the latter consisting of a single whorl of spore-bearing phialides.

Conidiophores solitary, with the main axis bearing a number of bottle-shaped phialides. Spores are small ovoids, in long chains and possess a rough surface .

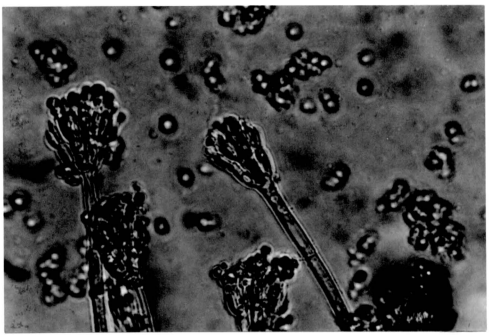

Fig. 6. *Penicillium* sp., 500X isolated from the surface of *G. changii* thalli.

Gliomastix sp.

Description:

Culture is black in colour, in reverse as well. The conidiophores are short, arising as lateral branches from trailing hyphae. The balls of dark amerospores arise from almost hyaline sporogenous cells. Non-septate conidia slimes down to

produce gloeoid balls, phialides long and tapering. The conidiophores are solitary. This strain produces crystals that might be oxalates.

Pestalotia sp

Description:

The colour of the culture is whitish with spots of black, carbonaceous fruiting bodies. This genus is easy to identify since the spores are quite characteristic in that they have four cross-septa. The lower end of the spore bears a hair-like stalk or pedicel, and the upper end is furnished with a crest of two long colourless hairs.

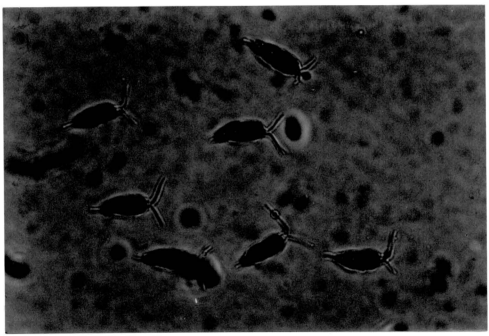

Fig. 7. Spores of *Pestalotia* sp., X200 isolated from the thalli of *G. changii.*

Yeast-like fungi

Description:

No true mycelium produced; cells reproducing by budding or binary fission.

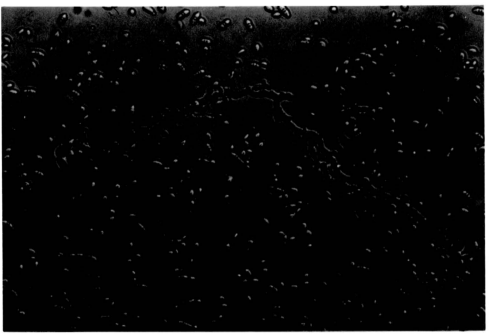

Fig. 8. Yeast-like fungi, 100X isolated from the thalli of *G. changii.*

4.1.3 Screening for Virus

The specimens obtained from the sampling site in Morib showed virus-like particles (VLPs) when viewed under the Transmission Electron Microscope. Figure 9 and 10 show rod-like particles. Figure 11 shows filamentous-like particles. Figure 12, 13 and 14 show another virus-like particle with characteristic hexagonal envelope with an electron dense core. From the twelve

specimens observed under TEM for VLPs, five showed VLP present. Details of the morphology is given in Table 7. The re-infection of thalli with the virus were not successful. VLPs were not observed in cross-sectioned thalli after the incubation period.

Table 7: Description of VLPs Observed

Sampling Sites	Specimen No.	Isolation Method	Shape	Size (nm)
Kedah	C463[c]	a	rod-like particle, anisometrical	120 x 22
Kedah	C465[c]	a	filamentous	> 468.9 x 6.2
Morib	C466	a	rod-like particle, anisometrical	130 x 18
Morib	C469	a	hexagonal, with outer envelope and electron dense core which appear granulated.	90 (o); 62.5 (i)
Carey Island	C651	a		124 (o); 84 (i)
Carey Island	C720	b		120 (o); 90 (i)

a, homogenate of 'sick' thalli exhibiting blackened spots on whitened thalli.
b, ultrathin sections via Microtome; [c], obtained from the same specimen.
o, diameter of outer envelope; i, diameter of inner dense core.

Fig. 9

Fig. 10

Fig. 9-10. Virus C466 (Fig.9) and C463 (Fig.10) from homogenate of 'sick' *G. changii* thalli (bar = 100 nm).

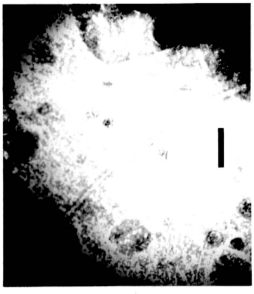

Fig. 11. Virus C465 from homogenate of *G. changii* (bar = 100 nm).

Fig. 12. VLP C469 from homogenate of *G. changii* (bar = 100 nm).

Fig. 13. VLP C651 from homogenate of *G. changii* thalli (bar = 100 nm).

Fig. 14. VLP C720 in ultrathin sectioned *G. changii* thalli (bar = 100 nm).

4.1.4 Description of Algal Epiphytes Isolated from the Surface of *G. changii*

There were many macroscopic and microscopic epiphytes found growing along with *G. changii*, attached to the thalli of *G. changii* and entangled with the thalli *of G. changii*. The epiphytes were collected from mangroves of Morib and Carey Island (Selangor), Sg. Pulai (Johor), Thailand and farm in Ban Merbok (Kedah). A detailed description is given. The identification of the algae was assisted by Dr. Phang Siew Moi of University Malaya, Malaysia. Table 8 shows the type of epiphyte, the site of collection and the salinity of the surrounding waters.

Table 8 : Algal Epiphytes Found Growing with, Entangled and Attached to the Thalli of *G. changii.*

Sampling Sites	Isolated Epiphytes	Salinity at Collection Site (ppt)
Thailand	**Division Chlorophyta** Order Cladophorales Family Cladophoraceae [b]*Cladophora patentiramea* Order Ulvales Family Ulvaceae [b]*Ulva ?fasciata* **Divison Phaeophyta** Order Ectocarpales Family Ectocarpaceae [a]*Ectocarpus sp.*	35
Morib	**Division Cyanophyta** Order Hormogonales Family Nóstocaceae [b]*Scytonema ?hofmanii* [b]*Scytonema ?polysystum*	30

	Division Chlorophyta Order Cladophorales Family Cladophoraceae [b]*Chaetomorpha antennina* **Division Rhodophyta** Order Gigartinales Family Hypneaceae [b]*Hypnea sp.* [b]*Hypnea ?esperi* Order Ceramiales Family Ceramiaceae [a]*Ceramium sp.*	
Sg. Pulai	**Division Chlorophyta** Order Ulvales Family Ulvaceae [b]*Ulva sp.*	35
Ban Merbok, Kedah (farm)	**Division Cyanophyta** Order Hormogonales Family Oscillatoriaceae [b]*Lyngbya ?majuscula* **Division Rhodophyta** Order Gigartinales Family Hypneaceae [b]*Hypnea sp.*	28
Carey Island	**Division Cyanophyta** Order Hormonogales Family Nostocaceae [c]*Anabaena oscillariodes* Family Oscillatoriaceae [c]*Lyngbya sp.* [c]*Oscillatoria sp.* [c]*Microcoleus sp.* Order Ulotrichales Family Microsporaceae [c]*Microspora sp.* **Division Chlorophyta** Order Cladophorales Family Cladophoraceae [b]*Cladophora fascicularis* [a]*Cladophora flexuosa* [b]*Chaetomorpha sp.* [b]*Chaetomorpha linum*	24

Order Ulvales
Family Ulvaceae
[a]*Enteromorpha sp.*

Division Rhodophyta
Order Acrochaetiales
Family Acrochaetiaceae
[b]*Audouinella sp.*

Order Ceramiales
Family Rhodomelaceae
[b]*Polysiphonia sp.*

Division Bacillariophyta
Order Araphidineae
Family Fragilariaceae
[c]*Tabellaria sp.*

Order Monoraphidineae
Family Achnanthoideneae
[c]*Achnanthes sp.*

a, Attached to the thalli of *G. changii* ; b, Entangled with the thalli of *G. changii* ; c, Growing with *G. changii*.

Audouinella sp., an endophytic alga was observed from cross-sections of *Gracilaria changii* thalli that showed *Audouinella* sp. had infiltrated into the cortex. However, the filament inside the cortex may also include species of *Streblonema* sp. (Correa, pers. comm). This will have to be confirmed later.

Lyngbya Agardh

Description:

Chapman 1961, pg. 26.

Trichomes enclosed in a firm, distinct, colourless sheath; containing only one trichome. Trichomes olive-green; end cell rounded. Filaments forming a spongy mass.

***Lyngbya ?majuscula* (Dillwyn) Harvey**

Description:

Humm & Wicks 1980, pg. 133; Dawson 1954, pg. 381, fig. 3d; Chapman 1961, pg. 28.

Trichomes 20-40 μm in diameter, the cells 2 - 4 μm long, cells very short, 1/6 to 1/15 as long as broad, end cell rounded, without calyptra; without constriction at the nodes, the apex not tapering. Plants forming olive-brown mats on the bottom in quite, shallow water. These mats often tear loose and float, buoyed up by entrapped oxygen bubbles and may drift for long distances. Distinct colourless, firm sheath; layered with thickened end wall.

Distribution: cosmopolitan.

Habitat: Mangrove swamp, palisadoes in ponds and on reef.

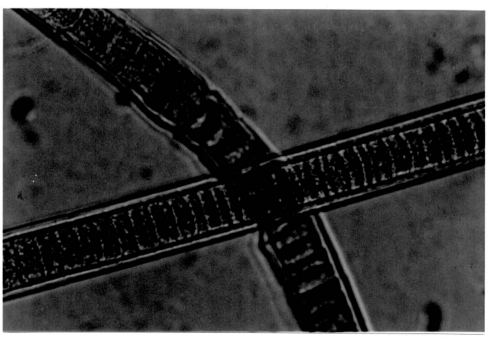

Fig. 15. *Lyngbya ?majuscula*, X500.

***Anabaena oscillarioides* Bory**

Description:

Prescott 1962, pg. 517; Humm & Wicks 1980, pg. 88.

Filaments blue-green in colour, straight, or entangled in a thin gelatinous layer or solitary. Trichomes 2-12 μm in diameter of indeterminate length. Spherical cells, diameter of 4-10 μm and 2-10 μm long. Spherical heterocyst of 13 μm diameter.

Distribution: Found worldwide in both freshwater and sea.

***Microspora* Thuret**

Description:

Prescott 1962, pg. 106.

Plants unbranched, unattached filaments, uniseriately arranged cylindrical, swollen cells observed. Thick and lamellate cell walls. Densed chloroplast. Length of cell is twice the diameter.

***Ectocarpus* Lyngbye**

Description:

Chapman 1963, pg. 7.

Filamentous thalli, plants tufted, yellowish brown in colour, erect filaments, freely branched. Basal cells up to 30 μm in diameter. The ratio of broadness to length of cell is 1:3. Unilocular sporangium observed.

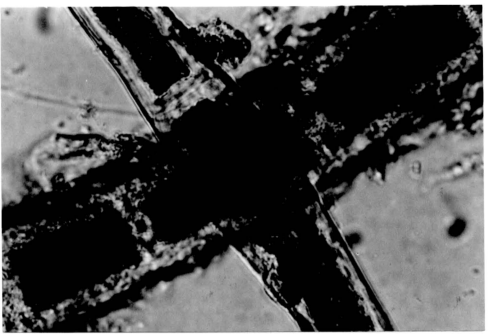

Fig. 16. *Microspora* sp., X200. Swollen cell (arrow) is shown above.

***Cladophora ?patentiramea* (Mont.) Kuetz.**

Description:

Tseng 1983, pg. 260, pl. 129, fig. 3.

Thalli light green, soft and delicate, forming entangled masses, to 3-5 cm in diameter. Main branches decumbent, irregularly bent, with rather lateral membranes and with cells 70-150 μm diameter, 1-2 diameter long. Branchlets rare, irregularly deposited, sometimes somewhat unilateral, with very thin lateral membranes and with cells blunt. Rhizoids adventitious, ending in discs, arising from anywhere, attaching to substrate or connected with others.

Distribution : Japan, Malay Archipelago, Tahiti.

***Cladophora fascicularis* (Mert.) Kuetz.**

Description:

Tseng 1983, pg. 258, pl. 128, fig. 4.

Thalli green, filamentous, large, bushy to 10-20 cm in height. Main branches stout, alternately branched, 220-300 μm diameter, 1-5 diameter long. Branchlets 70-150 μm diameter, 2-4 diameter long, somewhat pectinately arranged, but densely fascicled near the ends of the lesser branches. Rhizoids descending from thallus-bases, divided irregularly.

Distribution: Cosmopolitan in temperate, subtropical and tropical seas.

***Cladophora flexuosa* (Griffiths) Harvey**

Description:

Chapman 1961, pg. 84.

Fronds 10-20 cm long, light green; main filaments 70-110 μm diameter, regularly flexuous, with flexous, alternate branches, 40-70 μm diameter, with alternate or secund, curved and sometimes refracted ramuli; cells 6 times as long as broad below, to 2 times in the ramuli.

Distribution: Alaska, Newfoundland to Bermuda and Florida, Europe. Palisadoes; Morant Cays.

Fig.17 . *Cladophora flexuosa,* X200.

***Chaetomorpha antennina* (Bory) Kuetz.**

Description:

Dawson 1954, pg. 385 - 386, fig. 6l; Tseng 1983, pg. 262, pl. 130, fig. 2.

Filaments attached, tufted, basal cells very long, with annular constrictions near the base; upper cells much longer than broad. Dark green thalli, rigid, erect, caespitose, unbranched. Attached to substratum by rhizoids. Cell walls to 25 μm thick, clearly stratified.

Distribution : Forming scattered, dense tufts at middle levels, in Nha Trang, Vietnam; common on tropical and subtropical coasts.

***Chaetomorpha* Kuetzing**

Description:

Chapman 1961, pg. 75.

Unbranched, bright green filaments, entangled with *G. changii*. Composed of large, cylindrical, rectangular cells.

***Chaetomorpha linum* (Muell.) Kuetz.**

Description:

Tseng 1983, pg. 262, pl.130, fig. 2.

Syn. *Chaetomorpha aerea* (Dillw.) Kuetz.

Thalli light green, stiff and straight, gregarious, to about 4-10 cm in height, growing in shallow rock pools in the upper intertidal zone. Filaments slender toward the base, above to 130-180 μm diameter, cells 1-2 diameters long or shorter, cylindraceous, somewhat constricted at the septa. Basal cells subclavate, to 130-150 μm diameter at the top, about 8-10 diameters long, attached to the substratum by disc-like rhizoids.

Distribution: Cosmopolitan in temperate and subtropical seas.

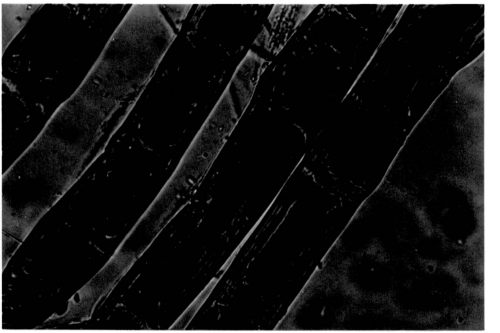

Fig. 18 . *Chaetomorpha* sp. X100.

***Oscillatoria* Vaucher**

Description:

Umezaki 1961, pg. 67.

Thalli greenish brown in colour. Plants solitary or forming layers; trichomes cylindrical with a diameter of 7-10 μm, enclosed in very thin, fragile, mucous sheaths, constricted at the cross walls, not moniliform, apices straightcurved, undulate or more or less regularly twisted in spirals, often attenuate; apical cell often with calyptra. Trichomes without a well-developed sheath, not regularly twisted.

***Hypnea* Lamouroux.**

Description:

Chapman 1963, pg. 112;Tseng 1983, pg. 98.

Thalli brownish red; irregularly branched, cylindrical, forming a mass; 5-15 cm high, 0.5 to 1 mm broad, attenuate at the apex; caespitose.

***Hypnea ?esperi* Bory**

Description:

Trono & Ganzon- Fortes 1988, pg. 176.

Plants are greenish purple in colour, soft, loosely caespitose, with non-percurrent axes. This branches are characteristically fine, terete, approximately 250-500 μm in diameter, irregularly - alternately branched. The ultimate branchlets are short and spinose, bearing stickdia near or at the apices.

This species commonly occurs as epiphytes of seagrasses and other large seaweed species.

***Polysiphonia* Greville**

Description:

Chapman 1963, pg. 135.

Multicell, branched, sporangium present; pericentral siphous reddish-brown, cylindrical, attached by surface of algae. Filaments 200-250 μm diameter; segments as long as broad.

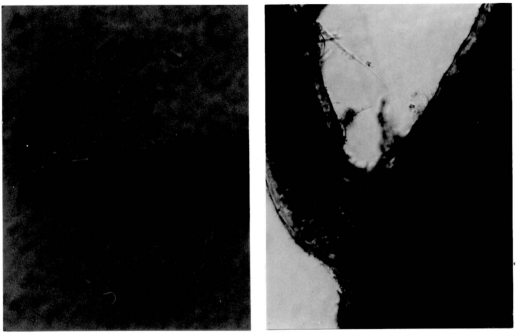

Fig. 19. *Hypnea ?esperi,* X50. A, ultimate branchlet is spinose. B, showing alternate branching.

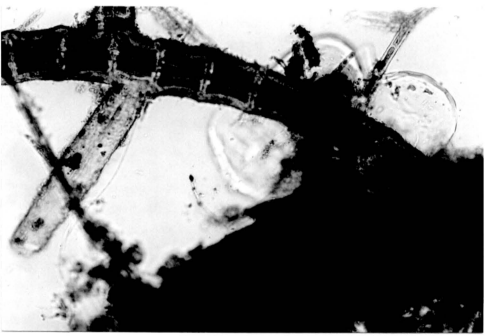

Fig. 20 . *Polysiphonia* sp., (PS), X100, attached to the surface of *G. changii* (arrow).

***Enteromorpha* Link**

Description:

Chapman 1961, pg.56; Trono & Ganzon-Fortes 1988, pg. 11.

Multiaxial, thallus unbranched, linear, cells irregularly arranged throughout; chloroplast filling the cell. Light green, dense mats of soft, delicate and hairlike filaments either attached to algae or floating. More or less regular in diameter throughout the length. Cell 5-15 μm in diameter.

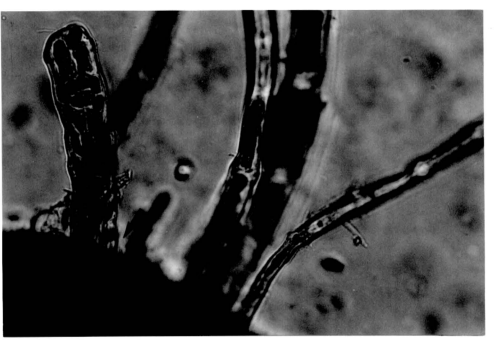

Fig. 21 . *Enteromorpha* sp., (ET), X100, attached to the surface of *G. changii* (arrow).

***Ulva* Linnaeus.**

Description:

Chapman 1961, pg. 53; Tseng 1983, pg. 256.

Thalli forming flat-sheaths. Plant broadly expanded forming a two layered membrane. Green thalli; blades irregularly lobed; 10-25 cm in height.

Distribution: In warmer waters.

***Ulva ?fasciata* Delile**

Description:

Tseng 1983, pg. 256. fig. 4; Chapman 1961, pg. 53.

Thalli dark-green, membranous 10-20 cm long. The blades irregularly dichotomously branched with margins irregularly ruffled and crenate, margin undulate. Attached to mangrove roots.

Distribution : In warmer waters

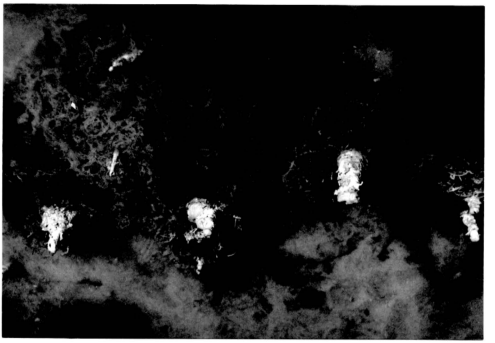

Fig. 22 . *Ulva ?fasciata,* growing together with *G. changii.*

***Microcoleus* Desmazieres**

Description:

Umezaki 1961, pg. 33; Tseng 1983, pg. 79; Prescott 1962, pg. 505.

Plants endophytic in codium or lithophytic on rocks, solitary or forming a widely expanded stratum; filaments simple, vaguely branched; sheaths hyaline, more or less cylindrical, never lamellate, in many species finally diffluent; trichomes many within a sheath, closely crowded, often funiform and contorted, the apices straight, attenuate; apical cell acute, rarely obtuse - conical.

Thalli dirty-green, trichomes inclosed by a wide, gelatinous, homogenous and sticky sheath.

Distribution : cosmopolitan.

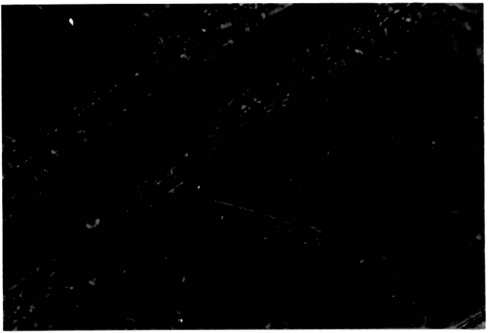

Fig. 23. *Microcoleus* sp., X200. A diatom (arrows) which is of the genus *Tabellaria* sp. is attached to the mucilage enclosing the trichomes.

***Ceramium* Lyngbye**

Description:

Liao, pers. comm. (University of San Carlos, Philippines).

Presence of banding cortical cells (pink) on the monosiphonous main axis (transparent globular cells forming a chain). Peculiar to this species is the formation of lateral rhizoids that attach to the host. When the epiphyte is viewed in relation to the host, it looks like a creeping thing but the actual orientation is vertical, with forked end to the right as the distal or upper portion of *Ceramium*.

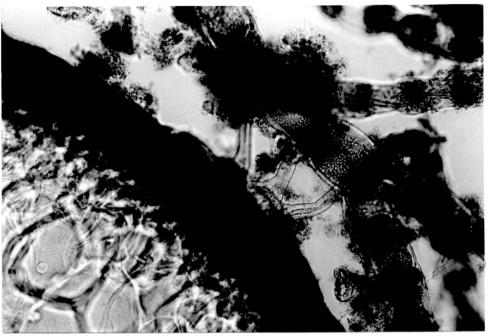

Fig. 24. *Ceramium* sp. attached by rhizoids to *G. changii.*

***Scytonema ?hofmanii* C. Agardh.**

Description:

Humm & Wicks 1980, pg. 87, fig. 30.

Trichomes 12-28 μm in diameter; trichomes somewhat swollen at tips, variations in diameter along the trichomes is observed. Distinct sheaths, filaments with false branching.

Distribution: Common in mangroves in the tropics.

***Scytonema ?polysystum* Bornett & Flahault**

Description:

Umezaki 1961, pg. 87, pl.15, fig. 1b.

Plants greyish green with diameter between 15-18 μm, sparsely branched, sheaths membranaceous, thin, hyaline. Trichomes 11-14 μm in diameter; cells 4-7 μm in length; heterocyst with a lenght of 10 μm present.

Distribution: Among algae in marine water, Pacific Ocean and Southeast Asia.

Diatoms

***Tabellaria* Ehrenberg**

Shamsuddin 1991, pg.177, app. 4.

Description:

Cells in zig-zag filaments; cells colonial but not in straight filaments; with pseudoraphe, cells bilaterally symmetrical.

***Achnanthes* Bory**

Description:

Shamsuddin 1991, pg. 191, app. 4.

Cells narrowly elliptic with undulate margins; bilaterally symmetrical; cells wedge shaped, attached by gelatinous stalks; bent in girdle view; cells with a raphe on one valve only.

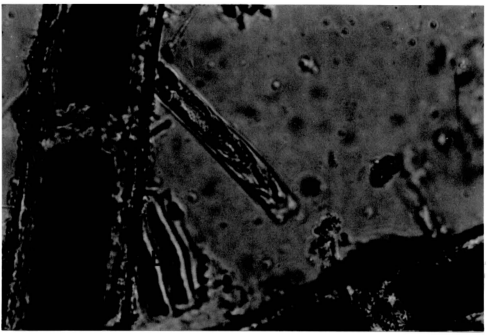

Fig. 25. The diatoms A. *Tabellaria* sp. B. *Achnanthes* sp., X200 scraped from the thalli of *G. changii.*

4.1.5 Identification of an Alga Endophytic *in G. changii*

***Audouinella* Bory**

Description:

Womersley 1996, pg. 43.

Thallus red in colour, epiphytic with endophytic base; multicellular, caespitose with septate between cells, cells cylindrical to irregularly swollen, especially at the tip. Erect filaments; sparingly branched; cells 5-25 μm long and 3-6 μm in diameter, L/D 1-5. Basal cells with curved filaments.

Fig. 26 . *Audouinella* sp. (arrows) on *G. changii.*

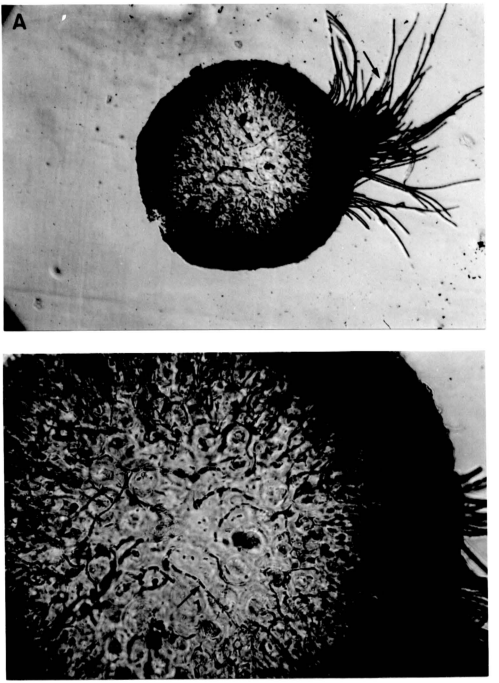

Fig. 27 . *Audouinella* sp. (arrows). A, cross-sectioned *G. changii* showing the penetration of the thallus by *Audouinella* sp., X20. B, higher magnification showing the endophyte (arrows) which may also include *Streblonema* sp., X500.

4.1.6 Identification of an Unicellular Organism.

Some thalli specimens suffered whitening and fragmentation after two weeks of growth in flasks with seawater under laboratory conditions. Sections obtained from the softened lesions of laboratory-infected, diseased *G. changii* showed that the cortex was filled with irregularly shaped 1.7 - 2.7 μm, unicellular organisms. Observations showed that these organisms had cell shape from elongate to spherical. The cells were eucaryotic and had membranous organel (looks like chloroplast) .The cells contained starch-like granules and an electron dense globule which might contain lipid. Structures resembling cilia were observed along the outer region of the organism. Based on the structure, it resembled a microalga. Cell wall of the infected thallus showed perforation and destruction, at the site where the unicellular organism agglomerated. Once in the host and to advance from one cell to the adjacent, the organism perforated a hole in the intercellular cell wall, through which it migrated to the next cell. Apart from the unicellular organism, agglomeration of thread-like material surrounding the unicellular organism was observed in some instances (arrow-Fig.29). This thread-like structure was also found near the destroyed cell wall (Fig. 31) and along with the unicellular organism in the intracellular matrix of the cell.

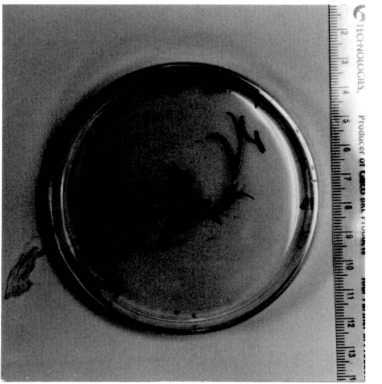

Fig. 28. Thalli with whitening and fragmentation where unicellular organism with assumed cell-wall degrading ability was identified.

Fig. 29

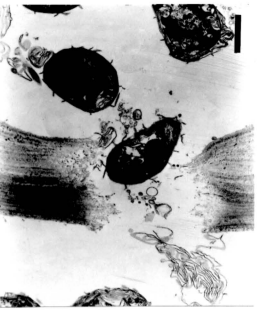

Fig. 30.

Fig. 31

Fig. 32

Fig. 29-32. Cross-section of 'sick' *G. changii* thalli showing the unicellular organism appearing to digest the cell wall (bar = 3 μm).

4.2 Re-infection of *G. changii* with Isolated Microorganisms.

Healthy thalli of *G. changii* were incubated with microorganisms isolated earlier from diseased thalli. The results when healthy thalli were grown with bacteria, fungi and virus are shown below.

Table 9 : Results of the Infection of Non-axenic Thallus with Bacteria (Seawater Medium).

	Number of Replicates		
Strain	1	2	3
K1B1	+	++	+++
K2B18	-	++	-
M1B25	-	+	+
M1B26	+	++	+
M1B27	-	-	+
M1B28	+	-	+
M1B29	++	+++	++
M2B31	+++	+++	+

+, generally healthy but with small pale or white patches;
++, 50% or less of whitening at injected area;
+++, 90%-100% of branches white or deteriorate;
-, no apparent change or any discoloration.

Of the 43 bacterial isolates individually tested for pathogenicity on non-axenic *G. changii*, 8 bacterial strains elicited disease symptoms in inoculated thalli after 10 days incubation, while the rest of the bacterial strains failed to

affect the appearance of the host alga over the same period of time. All eight bacterial strains eliciting disease symptoms were recovered from the infected thalli. Strain K1B1, M1B26, M1B29 and M2B31 elicited the strongest disease symptoms.

Table 10 : Results of the Infection of Axenic Thallus with Bacteria (Marine Broth Medium).

Strain	No. of Replicates					
	1	2	3	1	2	3
	Disease symptoms			Re-isolation of inoculant		
K1B1	-	+	++	i	i	i
K2B18	-	+*	+*	i	c	i
M1B25	+*	+*	+*	i	i	i
M1B26	-	+	++	i	i	i
M1B27	-	-	-	i	i	i
M1B28	-	-	+	i	i	i
M1B29	+++	+++	+	i	i	i
M2B31	-	-	+	i	c	i
Control	-	-	-	n	n	n

*, whitening of tips
+, generally healthy but with small pale or white patches;
++, 50% or less of whitening at injected area;
+++, 90%-100% of branches white or deteriorate;
-, no apparent change or any discoloration.
i, inoculant re-isolated; c, contaminant (s); n, no bacterial growth

The symptoms observed were whitening of the injected area. The experimental control remained symptomless for at least a month, after which whitening and fragmentation of thallus followed. Potential physical damage to the thallus as a consequence of injection was tested by injecting sterile seawater into thallus segments. In all 3 replicates of the experiment, the thallus pieces remained in the same condition as that of the uninjected control. Disease symptoms began to show by the third day of incubation. When the eight bacterial isolates showing positive disease symptoms in non-axenic thalli were injected in axenic thalli and grown in marine broth, only strain M1B29 showed strong disease symptoms in two replicates and slight whitening in the third, followed by strain K1B1 and M1B26 with slight whitening observed in two replicates. Whitening of the thalli tips (not injected sites) were observed when injected with strain M1B25 and K2B18 (Fig. 33). Strain M2B31 showed slight disease symptoms in one replicate. For the repeat experiment, using marine broth and seawater medium, axenic thalli were injected with strain K1B1, M1B26 and M1B29.

Fig. 33 . Axenic thalli injected with strain K2B18 exhibiting whitening of tips (arrow).

Table 11: Comparison of the Infection of Axenic Thallus with Bacteria in Marine Broth and Seawater Medium.

Strain No.	No. of Replicates					
	Marine Broth			Seawater Medium		
	1	2	3	1	2	3
	Disease symptoms			Disease symptoms		
K1B1	+	-	-	+++	+++	+
M1B26	+	+*	+*	+	+	+
M1B29	+++	+	++	++	++	+

*, whitening of tips;
+, generally healthy but with small pale or white patches;
++, 50% or less of whitening at injected area;
+++, 90%-100% of branches white or deteriorate;
-, no apparent change or any discoloration.

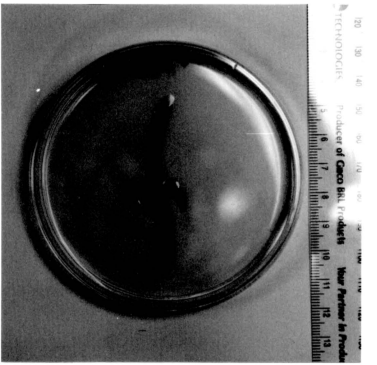

Fig. 34. Axenic thalli (in marine broth) injected with bacterial strain M1B29 showing whitening at injected site.

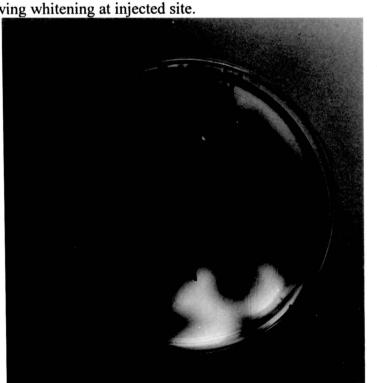

Fig. 35. Axenic thalli (in seawater medium) injected with bacterial strain K1B1 showing whitening at injected site.

Bacterial growth was not observed on Marine Agar 2216E over 1 month when axenic *Gracilaria changii* was tested for sterility. M1B29 showed strong disease symptoms in both media (Fig. 34). Strain K1B1 showed strong disease symptoms in seawater medium (Fig. 35). Strain M1B26 showed slight whitening of tips in replicate 2 and 3 (marine broth) and slight whitening in all three replicates of seawater medium. All bacteria were re-isolated.

4.2.1 Identification of the Three Bacterial Isolates Showing Disease Symptoms in Two Growth Media.

Based on the Biolog Microstation with the Database Microlog GN, the bacteria were identified as below:

Table 12 : Identification of Two Disease Causing Bacteria.

Strain No:	"SIM" value	Identified species
K1B1	0.798[a]	*Deleya aesta*
M1B26	0.874[b]	*Deleya aesta*
M1B29	0.975[c]	*Deleya marina*

[a, b, c] - refer Appendix 2, 3 and 4 respectively.

Both K1B1 and M1B26 are the same species which is *Deleya aesta,* formerly *Alcaligenes* and strain M1B29 is *Deleya marina,* formerly *Pseudomonas marina* in Bergey's Manual of Systematic Bacteriology, Vol. 1. (Holt *et al.*, 1994).

4.3 Re-infection of Axenic Thalli with Fungi Isolated Earlier from Diseased *G. changii* Thalli.

All eleven fungal isolates were grown together with axenic thalli. Two showed mycelial growth on the thalli. All other isolates failed to affect the appearance of the host alga.

Table 13: Results of the Infection of Thallus with Fungi Isolates.

Strain	No. of Replicates					
	1	2	3	1	2	3
	Disease symptoms			Re-isolation of inoculant		
AF3	-	+++	-	-	i	-
AF10	+++	++	++	i	i	i

++, 50% or less of mycelial growth on thalli;
+++, 90%-100% of branches with mycelial growth along thalli;
-, no mycelial growth observed;
i, inoculant re-isolated

Fungal isolate AF3 (*Aspergillus* sp.) had mycelia growing along the entire length in one of the replicate by the seventh day. Mycelial growth of isolate AF10 (*Pestalotia* sp.) was apparent by the tenth day on all three replicates (Fig . 36). All 3 replicates of the control showed slight bleaching on some areas of the thalli by the 20th day. Free-hand cross-sectioning of the thalli grown with mycelia of isolate AF10 showed cyst-like growth within the cell. Non-stained sections showed a cyst-like growth to be light green in colour

whereas sections stained with iodine shows a brownish-red colour which can be seen in Figure. 37.

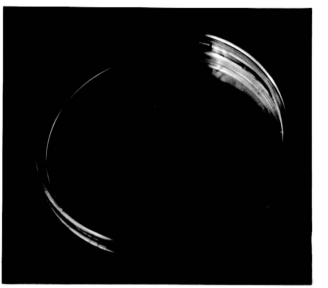

Fig. 36. Mycelial growth of *Pestalotia* sp. along the thalli of *G. changii*.

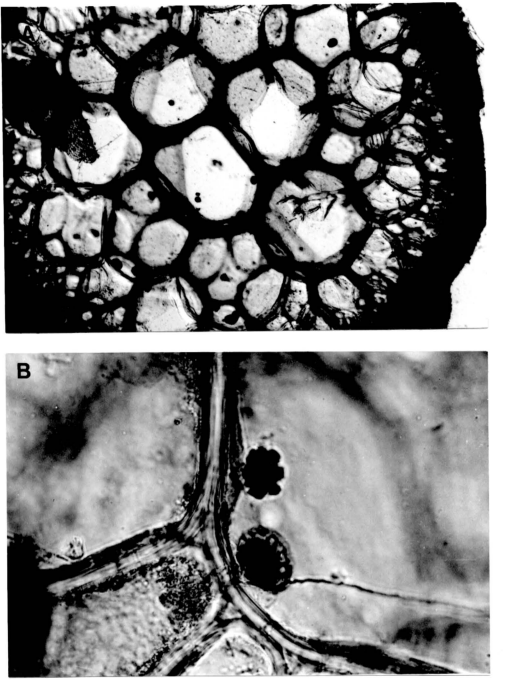

Fig. 37. A, cross-section of thalli with fungal isolate AF10 (*Pestalotia* sp.) showing cyst-like growth within the cell, X100. B, a larger magnification, X200.

4.4 Re-infection of Axenic Thalli with Virus-like Particles Obtained from Diseased Thalli of *G. changii.*

Dried thalli specimens believed to contain VLP as observed earlier, failed to cause disease symptoms in all three replicates.

4.5 Influence of *G. changii* Extract on Bacterial Growth

Seaweed extract appeared not to encourage increased bacterial growth around the disc neither creating inhibition of bacterial growth.

4.6 Influence of *G. changii* Extract on Fungal Isolates

Fungi grew irregularly and extensively on the plate regardless of the concentration of seaweed extract. There does not appear to be any correlation between fungal growth and seaweed extract in this experiment.