# Chapter 2: Literature Review

## 2.1 Rhodophyta – the Red Algae

Algae are the major primary producers of oceans and other aquatic ecosystems forming the base of food chains for these systems. There are seven major divisions of algae differentiated by their dominant pigmentation, amongst which are Chlorophyta (green algae), Phaeophyta (brown algae) and Rhodophyta (red algae) (Wynne and Kraft, 1981). In comparison to the green and brown algae, the red algae are the most diverse in the tropics. Red algae owe their colour to water-soluble pigments called phycobilins, mainly γ-phycoerythrin. Rhodophyta can be unicellular, filamentous, parenchymatous or pseudoparenchymatous. Some are calcified or calcareous with calcium or magnesium carbonate (aragonite) (Calumpong and Menez, 1997).

## 2.2 *Gracilaria changii* Abbott, Zhang and Xia

*Gracilaria changii*, a red algae belonging to the Rhodophyta, is currently cultivated on experimental scale at Ban Merbok, Perak, Peninsular Malaysia, using an integrated polyculture system with shrimps in earthponds at cleared mangroves area (Phang, 1998). *G. changii* is widely distributed in Malaysia and the southern part of Thailand and grows abundantly on terrestrial mudflats and as attached epiphytes to mangrove roots, shells, pebbles and fish cages found throughout the years (Phang *et al.*,

1996). *G. changii*, being relatively bushy, is distinguished by abrupt constrictions at the base of the lateral branches, forming a slender stipe. Branching is of two to four orders with tetrasporangial and gametophytic plants similar in size and morphology (Phang, 1998).

As with other red algae, *G. changii* has the most complicated method of reproduction and phases in their life histories. *G. changii* exhibits three phases in their life history. They are, the one gamete producing phase (sexual phase) and two spore-producing phases (asexual phases). The asexual phases can be further divided into the caprospore and tetraspore-producing phases. Vegetative reproduction through fragmentation of the thallus also occurs (Trono, 1997).

## 2.3 Conventional Culture Techniques for *Gracilaria* species

Conventional culture techniques for seaweed culture are based on 'macro-vegetative' plant reproduction, whereby 'cuttings' from vegetative thalli are used as planting stocks for culture in ponds or attached to various contraptions including monofilaments, floating rafts, nets or suspended ropes in the open sea (Dawes and Kain, 1995).

Initial experimental cultivation of *Gracilaria* involved the use of unattached suspended culture (LaPointe and Ryther, 1978). One such example of this technique involved cultivating *Gracilaria* in narrow tanks or raceways which receive a rapid

exchange of seawater, with the seaweed kept in suspension by vigorous aeration (DeBusk and Ryther, 1984). Today, cultivation technique has been largely replaced by outdoor tanks and ponds (Friedlander *et al.*, 1995). Daily growth rates in the range of 10.76% (*G. firma*) to as high as 24.35% (*G. verrucosa*) have been achieved using these techniques (Chirapart and Ohno, 1993). Conditions required for optimal growth in ponds and tanks include suitable sources of carbon and nitrogen, culture density, temperature and pH (Santelices *et al.*, 1993; DeBusk and Ryther, 1984 ).

## 2.4 Important Factors Controlling Tissue Culture

Tissue culture provides relatively uniform plant materials at different organisational levels, which can be maintained under defined chemical and physical conditions, free from contaminating microorganisms. The ability to routinely obtain callus tissue, suspension of cells and protoplasts from seaweeds such as *Gracilaria,* among others, would open up new possibilities for increased genetic manipulation and provide systems for research into poorly understood areas of phycological research such as growth and differentiation at all levels, biochemistry, metabolism and molecular genetics (Evans, 1986).

### 2.4.1 Surface Sterilisation of Tissue

Seaweed surfaces are often densely covered with microorganisms (*e.g.*, protozoa, bacteria and fungi) and the production of axenic tissue requires either the sterilisation of

non-axenic plant parts, or the aseptic isolation of axenic internal tissues or reproductive stages (Butler and Evans, 1990). Sterilisation procedure must be optimised to produce the highest number of axenic plants at low incidence of tissue death. Table 2 summarises the sterilisation techniques applied to obtain axenic tissues in some seaweeds.

Table 2. Sterilisation procedures used in obtaining seaweed tissues

| Seaweed species | Sterilisation procedure | Reference |
|---|---|---|
| *Laminaria digitata, L. hyperborea and Fucus spiralis* | Calcium hypochlorite solution as surface sterilant. Proportion of axenic explants was low as hypochlorite readily penetrates seaweed pieces leading to rapid bleaching and death of the tissues. | Fries, 1984. |
| *Chondrus crispus* | Axenicity obtained through 'dip and drag' on antibiotic containing agar followed by incubation in antibiotic mixture. A wide range of antibiotics that were screened showed that they provide broad-spectrum antibacterial activity but with minimal toxicity to the tissues. | Chen and Taylor, 1978. |
| *Pylaiella littoralis* | Ultrasonic cleaning in 0.1% Tween 80 coupled with antibiotic treatment resulted in axenicity of explants.<br><br>Ultrasonic cleaning (2-10 min) followed by exposure to antibiotics and/or mild surface sterilants such 1-2% polyvinylpyrrolidone-iodine complex (PVP-1) or chlohexidine digluconate have been shown to be effective . | Loiseaux and Rozier, 1978.<br><br>Butler and Evans, 1990. |
| *Ecklonia radiata* | Aseptic coring of the tissues e.g. stipes. Suitable only for larger algae. | Lawlor *et al.*, 1989. |
| *Undaria pinnatifida* | Tissues submerged in sterile seawater and sonicated for 20 sec for three times followed by antibiotic treatment for several days. Antibiotic solution contained 100 mg penicillin G potassium, 100 mg phosphomycin, 40 mg gentamycinsulfate and 8 mg nystatin per 100 ml enriched seawater. | Tokuda and Kawashima, 1988. |
| *Laminaria japonica* | The tissues were cleaned with sterilised water, steeped in a 1.5% KI solution (10 min) and treated with 70% alcohol. | Zuo-mei, 1984. |
| *Laminaria and Dictyosiphon* | Gentle surface sterilisation of the thallus and aseptic release and culture of the microscopic, axenic, reproductive stages such as the spores. | Saga *et al.*, 1982. |

### 2.4.2 Callus Formation and Frond Regeneration

In higher plants, callus formation is seen as a deviation from normal growth and differentiation patterns that occur when the explant is separated from the rest of the thalli. This occurs as a result of removal of the endogeneous controls, which normally maintain growth and differentiation in the intact plant. Active mitosis and cytokinesis would subsequently resume in the excised tissue resulting in the proliferation of an undifferentiated callus (Yeoman and Forsche, 1980).

Many simple seaweeds which exhibit diffuse growth have cells which are capable of division and regeneration in simple media and even the more complex red algae are composed of filaments which may continue to grow independently of the thallus in the presence of adequate light and nutrients. However, callus formation in most seaweeds was shown to be somewhat different to that observed in higher plants, as the explants may remain photoautotrophic, slow-growing and difficult to maintain indefinitely (Butler and Evans, 1990).

### 2.4.3 Media and Growth Substances

Success in plant tissue culture is largely determined by the quality of nutrient media and optimisation of growth and plant regeneration from cells and tissues may require modifications rather than novel formulations of nutrient media (Constable and Shyluk, 1994). Most of the media used in seaweed tissue and cell cultures are derived or modified from those used in higher plants.

Culture media provide a source of growth substances (growth hormones), amino acids and vitamins in addition to the macronutrients and micronutrients that are essential for callus formation or plant regeneration. Macronutrients are required in large amounts by cultured plant cells while micronutrients are required only in trace amounts (Burbidge *et al.*, 1993). The various components of culture media and their respective functions are summarised in Table 3.

Table 3. Major components of culture media and their functions.

**Macronutrients**

| Elements | Description of Functions |
|---|---|
| Carbon | Usually supplied in the form of carbohydrate. Required by explants that do not photosynthesise. Sucrose is usually used. |
| Nitrogen | Provided either as nitrate ions or ammonium. Usually supplied in form of various salts. Needed for cell growth. |
| Phosphorous | Usually supplied as the phosphate ion from salts such as ammonium, sodium or potassium di-hydrogenorthohosphate. Play key role in the transfer of energy within the cell, regulates the activity of many enzymes and is a component of biomolecules such as DNA and RNA. |
| Potassium | Supplied as chloride, nitrate or orthophosphate salt. Generally the most important cation and has major roles in cellular homeostasis, such as pH and osmotic regulation. Potassium also regulates the activity of certain enzymes. |
| Magnesium | Usually supplied as magnesium sulfate. It is a component of chlorophyll and is important biochemically as enzyme co-factor. |
| Calcium | Can be provided in form of calcium chloride and calcium nitrate. It functions as second messenger and is an important enzyme co-factor and enzyme regulator. Important in the maintenance of cell walls and membrane integrity. |
| Sulphur | Generally supplied as magnesium sulphate or ammonium sulphate. Important as part of amino acids and also play key role as enzyme co-factor. |
| Chloride ions | Play important role in osmotic regulation by balancing cations such as magnesium, potassium and sodium. |

**Micronutrients**

| | |
|---|---|
| Iron | Required for the formation of several chlorophyll precursors and an important component of ferredoxin. It is the most important micronutrients. |
| Manganese | Required to maintain chloroplast ultrastructure and for photosynthesis. |
| Copper and Zinc | Important components of some enzymes such as oxidases and superoxide dismutase. |
| Molybdenum | Important for nitrogen metabolism. |
| Cobalt | A component of vitamin B12. |
| Iodine | Not essential but nevertheless generally incorporated into media. |

**Vitamins**

| | |
|---|---|
| Myo-inositol | Considered to be a B-vitamin, which is important in cellular metabolism and physiology. |
| Thiamine | Also called vitamin B1. Essential for carbohydrate metabolism and biosynthesis. Also found to influence organogenesis in some plant cell cultures. |

Table 3. Major components of culture media and their functions (*continued*).

| **Miscellaneous** | |
|---|---|
| Arginine, glutamic acid and glutamine. | Generally supplied to provide a reduced source of nitrogen which can be easily assimilated. |
| Glycine | Although often considered to be a vitamin in plant cell, its continued use seems to be based on historical precedents as no definite proof of it essentiality is available. |
| EDTA (diaminetetraac-etic acid) | Usually supplied in form of $Na_2EDTA$. Require for slow and continuos release of iron into the medium, which it achieves by complexing with the iron. |

Burbidge *et al.* (1993), Constable and Shyluk, (1994) and Holdgate, (1977)

The most widely used growth substances are auxin and cytokinin. Others include gibberellins and abscisic acid (ABA). They are usually employed together in different ratios. Indole-3-acetic acid (IAA) is a natural auxin and is weaker than naphthalene acetic acid (NAA). Media containing IAA deteriorate faster than those containing NAA. The most potent auxin is 2,4-dichlorophenoxy acetic acid (2,4-D). The most active cytokinin is N6-isopentenyladine (2iP), though kinetin and N6-benzylagenine are more commonly use (Constable and Shyluk, 1994).

Table 4. Growth substances and their functions.

| Growth Substances | Functions |
|---|---|
| Auxins | Auxins such as IAA are important for cellular elongation, phototropism, geotropism, apical dominance, root initiation and elongation and ethylene production. |
| Cytokinins | Promote cell division and organ formation; seed germination, cell and organ enlargement; root initiation and growth; and bud and shoot development. |
| Gibberellins | Belong to a large group of terpenoids. Responsible for growth of intact plants, genetic dwarfism and mobilization of storage compounds. |
| ABA | Sesquiterpine composed of three isoprene units. Responsible for stomatal closure, defense against environmental stresses, dormancy, abscission of leaves and flowers; and seed germination. |

Artheca, (1995) and Davies, (1988)

The common commercially available media used in seaweed tissue culture are MS and PES. MS medium was formulated by Murashige and Skoog, (1962) and revised by Linsmaier and Skoog (1965), Gamborg *et al.* (1968) and Schenk and Hildebrant (1972). PES or Provasoli Enriched Seawater Medium was formulated by Provasoli, (1968). Other media that have been employed in seaweed tissue culture are ASP-12-NTA (Provasoli, 1963), SWMD-1 (Chen and Taylor, 1978) and TC-11 (Chen, 1987). All of these media contain the major components described above. Diluted sterile seawater is usually use as base for media preparation. Silicon may be optionally supplied as sodium salt to prevent the growth of diatoms as in the case for SWMD-1 medium.

### 2.4.4 Growth and Maintenance of Culture

In contrast to calluses of higher plants, which grow under heterotrophic conditions due to loss of photosynthetic ability, seaweed calluses generally grow when there is adequate light and nutrients (Butler and Evans, 1990). The morphological plasticity of seaweeds can be induced by a variety of factors including pH, temperature, light intensity and photoperiod (Moss, 1974).

## 2.5 Tissue Culture of Seaweeds

The history of seaweed tissue culture dates back to 1950 when Aharon Gibor started experimenting with seaweed tissues as a possible system for developing mariculture in desert countries (Polne-Fuller, 1988). Chen and Taylor in 1978, presented the first report on seaweeds tissue culture which involved tissue of the red alga, *Chondrus crispus*. Their study showed that nonpigmented medullary cubes regenerated into complete and normal plants. Since then, few reports of regeneration of tissue in whole plants have been reported.

To date, tissue culture of many species of seaweeds have been investigated as summarised in Table 5.

Table 5. Tissue culture of selected seaweed species.

| Seaweed species | Methodology | Results | Reference |
|---|---|---|---|
| *Laminaria digitata* and *L. hyperborea* | Meristematic explants cultured in ASP6 F2 medium with 0.6% agar. | Outgrowth of callus and filaments were obtained. | Fries 1980. |
| *Undaria pinnatifida* | Medullary-stipe explants cultured in PESI medium. | Callus growth and sporophyte regeneration. | Zhang, 1982 |
| *Dictyosiphon foeniculaceus* | Microthalli were cultured in ASP 12-NTA medium supplemented with 3% mannitol, 0.1% yeast extract and 1.5% agar. | Callus was induced for the first time in this species. | Saga *et al.*, 1982 |
| *L. japonica and U. pinnatifida* | Sporophyte explants cultured in MS (Murashige and Skoog) medium with added Vitamin B2 and Sodium Napthenate. | Callus and filaments sporophyte regeneration from cells of filaments. | Fang *et al.*, 1983; Yan, 1984 |
| *Agardhiella subuculata* | Cross-sections of the thalli were cultured in F5 and ASP-12-NTA media with added nitrate | Callus and filaments plantlets regeneration were observed. | Cheney *et al.*, 1986 |
| *Euchema* spp. | Cross section of tahlli were cultured in solid ASP-12-NTA, PES, ASP F2 media. | Callus regeneration observed for explants cultured in all the media. | Polne-Fuller and Gibor, 1986 |
| *Pterocladia capillacea* | One mm of axenic segments were grown in PES medium at 22°C, 135 $\mu E\ m^{-2}\ s^{-1}$, 16:8 h L:D. | Segments developed buds and uniseriate hairs both on the new buds and on the cur surface. Some buds produced normal rootlets (haptera) after four days. | Xue-wu and Gordon, 1987 |
| *Ecklonia radiata* | Stipes explants inoculated in PES medium with added 1 mg $l^{-1}$ biotin and cyanocobalamine, 0.5 mg $l^{-1}$ nicotinic acid and pyridoxine HCl and 0.1 mg $l^{-1}$ Thiamine HCl. | Development of unpigmented cells from newly cut explants and pigmented clumps of cell after 10 weeks. | Lawlor *et al.*, 1989 |
| *Eucheuma denticulatum and Kapaphycus alvarezii* | Seed stocks of both species cultured in ESS, SWMD-1 and soil extract. | Clonal propagation and callus induction from axenic explants within 4 to 8 weeks. | Dawes and Koch, 1991 |
| *Laminaria digitata* | Investigate the effect of $Ca(ClO)_2$ and growth regulators such as NAA, IAA and 2,4-D on callus formation. The effect of white, red, blue and green lights were also investigated. | Sterilization using $Ca(ClO)_2$ produced 87.5% sterile explants and 87.5% callus induction. Blue light inhibited callus formation but promoted callus growth. White, red and green light favoured callus induction. | Folefack and Cosson, 1995 |
| *Grateloupia dichotoma* | Intercallary segments were cultured in ASP 12-NTA medium supplemented with combination of IAA, 2,4-D and BA. | Callus-like-structure (CLS) was induced in ASP 12-NTA medium supplemented with IAA and BA in 1 + 5 and 5 + 1 mg $l^{-1}$ and IAA, 2,4-D and BA at 0.5 and 5.0 mg $l^{-1}$. | Yokoya *et al.*, 1996 |

### 2.5.1 Tissue culture of *Gracilaria*

Although *Gracilaria* is of great economic importance, there have been very few reports on tissue culture of this important seaweed because current farming techniques based on artificial beds established by anchoring thalli to sand-filled polyethylene tubes are able to produce this seaweed in large quantities (Martinez *et al.*,1990). However, tissue culture is indispensable in maintaining species diversity in view of the low natural populations of *Gracilaria*, especially *Gracilaria changii*, due to over-harvesting for agar extraction and seasonal variation in their reproduction cycle (Chirapart *et al.*, 1992). Moreover, micropropagation provides fundamentals for biological studies, production of high valued biochemical using cell culture and production of plants with novel characteristics using protoplast fusion (Burbidge *et al.*, 1994).

## 2.6 Important Factors Controlling Protoplast Culture

### 2.6.1 Structure and Organisation of Marine Algal Cell Wall

Seaweeds exhibit a variety and complexity of cell wall organisation in contrast to the relatively homogeneous characteristics of higher plants. The algal cell wall consists of a crystalline phase (skeleton) embedded in a more amorphous phase (matrix) (Butler *et al.*, 1990). The 'immediate' cell wall enveloping the cells contains both skeletal and matrix polysaccharides. The 'intercellular matrix' between cells however contains little or no skeletal components (Cronshaw *et al.*, 1958).

The skeletal polymers of seaweeds are natural linear polysaccharides most common being cellulose. Brown algae contain very little cellulose. Caulerpaceae and Dichotomosiphonaceae contain xylans while Codiaceae and Dasyladales synthesise mannans. In the red algae such as *Gracilaria*, the cell wall contains small amounts of cellulose and much of gelatinous or amorphous sulphated galactan polymers such as agar, carageenan, furonan, furcellarin, etc. (Butler *et al.*, 1990; Trono, 1997).

### 2.6.2 Cell Wall-degrading Enzymes

Protoplasts were initially isolated mechanically in higher plants as reported by Klerckel (1982). Research on protoplast isolation using enzymatic removal of cell walls was initiated by Cocking (1960). Since then cell wall-degrading enzymes have been produced commercially and routinely used in preference to the mechanical means in almost all protoplast studies (Takabe *et al.*, 1968). Complete cell wall degradation requires the use of a combination of enzymes that digest the matrix components.

Enzymes employed in isolating protoplasts from seaweeds can be derived from seaweed pathogens such as fungi, bacteria or amoebae, and from grazers such as snails, fish, copepods, etc., (Polne-Fuller, 1988). Extracts of the digestive systems of such organisms, or culture filtrates in the case of microbes, contain potent cell wall-degrading enzymes (Butler *et al.*, 1990). Cellulases, xylanases and alginases have been detected in extracts of the gastropod *Turbo* (Liu *et al.*, 1984). Alginate-lyase that cleaves the alginate by β-elimination at the glycosidic bond is, by far, the most common (Doubet and Quatrano, 1984). Extracts from *Haliotis tuberculata* (abalone) and *Aplysia depilans* (sea hare) contained significant amounts of alginate lyase (Boyen *et al.*, 1990). Table 6 summarises the types of cell wall-degrading enzyme derived from various marine organisms.

Table 6. Natural cell wall degrading enzymes and their organism sources

| Organisms | Enzymes | References |
|---|---|---|
| Marine pseudomonads | Fucanase | Yaphe and Morgan, (1959). |
| Calm | α-L-fucosidase | Presper *et al.*, (1986) |
| *Aplysia, Patella, Haliotis, Littorina* and *Dolabella* | Alginases | Franssen and Jeuniaux, (1965); Nakada and Sweeny, (1967); Nisizawa *et al.*, (1968). |
| *Haliotis rufescens, Dolabella auricula, Littorina* sp., and *Turbo cornatus* | Alginate lyase | Nakada and Sweeny, (1967) and Muramatsu and Egawa, (1980). |
| *Haliotis, Aplysia, Turbo and Patella* | Combinations of endo-polymannuronate lyases | Fisher and Gibor, (1987). |

Problems associated with enzymes present in crude extract is that its composition and activities are not precisely known and thus detailed investigation of the effects of individual enzymes is impossible which also impairs the reproducibility of isolation techniques. Moreover, the presence of proteases, lipases and ribonucleases may also result in low yield and viability of the protoplasts. These problems, however, can be partially solved by the use of purified enzymes with known activity towards the cell wall component (Butler *et al.*, 1990; Polne-Fuller, 1988).

To date, many commercially available enzymes are available for isolating protoplasts from seaweeds. Commercially available cellulases appear to effectively degrade algal cellulose. Cellulase preparations differ in their relative proportion of cellulytic enzymes and may also contain a mixture of other enzymes such as xylanases, mannanases and β-1,3-glucanases, which may be important for complete cell wall digestion (Saga, 1984). Agarase (Sigma Ltd.) is already available commercially and has been used to isolate protoplasts from species of *Gracilaria* (Cheney *et al.*, 1986). Table 6

also gives details of some enzyme mixtures successfully employed for protoplast isolation.

Besides optimising the combination of enzymes, the success of protoplast isolation also depends on factors such as sources of protoplasts and incubation condition during protoplast isolation.

### 2.6.3 Source of Protoplasts

Cellular composition, age and previous growth conditions of the parent tissue influence the effectiveness of enzyme treatment and protoplast development (Burbidge *et al.*, 1994). Some of the cell wall characteristics such as the mannuronate and guluronate composition of alginate in *Laminaria*, may influence protoplast isolation (Butler *et al.*, 1990). Position on the thallus in *Porphyra perforata* determines susceptibility to digestion and success in regeneration of plantlets (Polne-Fuller and Gibor, 1984). Species strain influenced protoplast yields in species of *Gracilaria* (Cheney *et al.*, 1986) and *Sphacelaria* (Ducreux and Kloareg, 1988). The particular tissue selected depends upon the nature of investigation.

### 2.6.4 Incubation Conditions During Protoplast Isolation

Activity of cell wall-degrading enzymes are influenced by pH, presence of cations and osmoticum. Protoplasts have been isolated from seaweeds at pH values between 6.0 and 7.0 as compared to the range employed in higher plants (5.4 to 6.2) (Evans and Bravo, 1984). The pH value chosen is often a compromise between the optimal value for enzymatic activity and at the pH offering the least stress to the protoplasts, (Burbidge *et al.*, 1994).

The presence of divalent cations such calcium may increase the rigidity of the cell wall of some species of gluconate-rich, alginate containing seaweeds through the

formation of alginate gel. The use of chelating agents such as EGTA to remove $Ca^{2+}$ will result in dissolution of the alginate gel and at the same time contribute to the destabilisation of the wall through the release of fucan components (Butler *et al.*, 1990; Quatrano *et al.*, 1985). However, the presence of minute concentration of calcium ions in the washing solution use for protoplast purification and harvesting is often necessary for membrane stability (Chen *et al.*, 1988).

Protoplast isolation is carried out in a hypertonic solution, created using osmoticum, in order to cause cell plasmolysis. Plasmolysed cells will round off and separate from the cell wall resulting in the release of protoplasts devoid of cell walls. The nature and concentration of osmoticum must be chosen so as to create the least possible pertubation to the physiological system of the protoplast. The most common choices are sugar-alcohol compounds such as mannitol and sorbitol (Burbidge *et al.*, 1994). During plasmolysis, protoplasts take up substances from the osmoticum. The osmotic potential of isolating media for seaweeds is usually in the range of 1000 to 1700 mOsm $kg^{-1}$, a range much higher than the levels used with terrestrial and freshwater plants (300 to 1000 mOsm $kg^{-1}$) (Butler *et al.*, 1990). Optimum osmotic potential for *Gracilaria* was shown to be as high as 1600 mOsm $kg^{-1}$ (Cheney *et al.*, 1986).

## 2.7 Protoplast Culture of Seaweeds

Techniques for the isolation and culture of protoplasts from seaweeds have only been developed recently and the amount of background research in this field is limited. The first report of protoplast isolation was from the green alga *Enteromorpha intestinalis* (Millner *et al.*, 1979). Controlled regeneration of protoplasts into new plants is essential if genetically improved plants are to be produced. Cell wall regeneration in the protoplast occurs without difficulty in all the genera studied. However, only few cases of plant regeneration have been reported and is limited to the less complex seaweeds like *Ulva*, *Monostroma*, *Enteromorpha*, *Sphacelaria* and *Porphyra* (Buter *et al.*, 1990). The various culture media used, protoplast isolation condition and the growth developments achieved are summarised in Table 7.

Table 7. Protoplast isolation and culture of selected seaweed species.

| Seaweed species | Methodology | Results | Reference |
|---|---|---|---|
| *Enteromorpha intestinalis* | Thallus treated with 4% driselase and 0.4% pectinase. Isolation medium contained 5 mM $MgCl_2$, 25 mM MES and 1.2 M sorbitol. The medium pH was 6.0. | Protoplast yield of 2-300µg chl. $g^{-1}$ fresh weight was obtained. Viability in the range of 85-90%. Oxygen evolution was similar to cells of the thallus, | Millner *et al.*, (1979) |
| *Porphyra suborbiculata* | Protoplasts isolated using ammonium sulphate purified extract of *Turbo coronatus* followed by 25 cellulase R10. 2% glucose used as osmoticum. | Cell wall regeneration and division up to the two cell-stage was observed. | Tang, (1982) |
| *Ulva linza and Monostroma angicava* | Protoplasts isolated using 4% cellulase R10 and 2% pectolyase. 2% glucose used as osmoticum. | Microcolony formation and plant regeneration in MPES medium observed. | Zhang, (1983) |
| *Enteromorpha linza, M. zostericola* and *Ulva pertusa* | Only 2% cellulase R10 was used for protoplast isolation. Isolating medium contained 1 M mannitol at pH 6.0. | Yield up to $10^6$ protoplast $g^{-1}$ fresh weight was obtained. Plant regenerated in PES medium. | Saga, 1984. |
| *Macrocystis pyrifera, Sargassum muticum, Porphyra perforata* and *Porphyra yezoensis* | Crude extract of *Haliotis* species (dialyzed) macerozyme (1%), pectinase (1%) and cellulase R10 (2%) were employed for protoplast isolation. 1 M sorbitol, 50 mM $MgCl_2$ and 10 mM $CaCl_2$ was added to the enzyme solution. pH was set at 6.0. | Up to 2 X $10^4$ protoplast $g^{-1}$ fresh weight was obtained. | Saga *et al.*, 1986. |
| *Fucus distichus* | Zygotes were treated with 1.25 to 2% cellulase (CELF), alginate lyase (6-10 UA $ml^{-1}$) in a two-step procedure. | More than 95% cell wall regeneration was observed. | Kloareg and Quatrano, (1987) |
| *Sphacelaria* sp. | Enzyme preparation containing 2% cellulysin, 0.5% pectolyase Y23 and 0.25 UA $ml^{-1}$ alginate lyase was used. Seawater, pH 5.8, used as base with added 0.8 M mannitol. | About 260 – 4600 protoplasts $g^{-1}$ fresh weight were obtained. Plant regenerated from apical cell protoplast. | Ducreux and Kloareg, (1988) |
| *Undaria pinnatifida* | Blade and midrib-stipe parts were treated with crude enzymes solution extracted from acetone powder of sea hare, *Aplysia kurodai*. Cultured in ICS medium. | Up to 4 x $10^5$ protoplasts $g^{-1}$ fresh weight of midrib-stipe parts and 1.5 x $10^5$ protoplasts $g^{-1}$ fresh weight of blade parts were isolated. Generated cell wall in ICS medium within 2 days. Cells with cell wall divided by 7 days but no further development. | Tokuda and Kawashima, (1988) |

Table 7. Protoplast isolation and culture of selected seaweed species (*continued*).

| | | | |
|---|---|---|---|
| *Macrocystis pyrifera* | Enzymes used are 2% cellulase (CELF) and 30 US $ml^{-1}$ alginate lyase at pH 6.5. | Protoplast yield in the range of $10^7$ –$10^8$ $g^{-1}$ fresh weight. Cell wall regeneration and microcallus formation obtained in 40% PES medium with 0.6 M sorbitol. | Kloareg *et al.*, (1989) |
| *Monostroma angicava* | Female gametophyte was used in protoplast isolation. Various concentrations of cellulase onozuka R-10 were tested. Isolated protoplasts cultured in PES medium. | Protoplast yield over $4 \times 10^6$ $g^{-1}$ fresh weight was obtained when 2-5% Cellulase was used. Yield of protoplast decrease with enzyme concentration over 5%. Cell wall regenerated after one day and within two days most of the protoplasts had generated cell walls. Cell division occurred after four days and by successive cell divisions and branching. Small discs were developed within one month. | Saga and Kudo, (1989) |
| *Ulva fasciata, U. pertusa* and *U. congloblata.* | Vegetative thalli were cleaned and treated with abalone powder, cellulase onozuka R-10, cellulase onuzuka RS, driselase, maceroenzyme R-10 and combinations of these enzymes. | None of the enzymes when tested alone produced any protoplasts. However, mixtures with abalone crude enzyme produced protoplasts ranging from $1.5 - 6.0 \times 10^5$ cells per 0.1g fresh weight of plant. | Reddy *et al.*, (1989) |
| *Chondrus crispus* | Female gametophytes were used for protoplast isolation using commercial cellulase and various carrageenases prepared from marine bacteria. | Protoplast yield ranging from $1.0\text{-}8.5 \times 10^8$ $g^{-1}$ fresh tissue were obtained depending on the nature of the donor tissue. A few protoplasts regenerated cell wall and divided. | Gall *et al.*, (1990) |
| *Porphyra linearis* | Explants were treated with 0.025% agarase in seawater without organic osmoticants. | Mean percentage of viable protoplasts obtained was more than 97%. Protoplast remained viable for at least 24 h in the digestion medium. | Chen *et al.*, (1994) |
| *Bangia atropurpurea* | One unit each of agarase, β-1,4-mannanase and β-1,3-xylanase was used for protoplast isolation from cleaned fronds. | A maximum of $5.7 \times 10^6$ protoplasts per 200mg of fronds were obtained. | Araki *et al.*, (1998) |

### 2.7.1 Protoplast Culture of *Gracilaria* species

Few reports on protoplast isolation and culture of *Gracilaria* sp. have been reported. The first protoplast isolation of *Gracilaria* sp. was reported by Cheney *et al.*, (1986) where protoplasts were isolated from young sporelings and matured plants. Complete cell wall removal was achieved using sporelings and high protoplast yield was obtained, although cell division was not observed. The most successful work on protoplast isolation and culture was reported by Yan and Wang (1993). The authors managed to produced 2-4 $\times$ $10^5$ protoplasts per 0.5 g fresh weight of tissues using 4% w/v cellulase and 1% w/v sea-snail enzymes. The isolated protoplasts underwent divisions and subsequently formed callus-like cell masses after five to seven days culture in MES medium. Finally, young buds were developed from the cell masses after three months in aerated culture. However, there was no further report from the said authors to prove that the young buds regenerated from protoplasts can indeed be matured into young plants.

Table 8 summarises the details of protoplast isolation and culture of several species of *Gracilaria*.

Table 8. Protoplast isolation and culture of *Gracilaria* spp.

| Seaweed species | Methodology | Results | References |
|---|---|---|---|
| *Gracilaria tikvahiae* and *G. lemaneiformis* | Young sporelings and matured plants were tested for protoplast isolation. Enzyme combination used: 1-3% onozuka R-10 (cellulase), 1-3% macerozyme R-10, 0.5% pectolyase Y-23. Three percent abalone gut enzyme mixture was tested alone. Length of enzyme treatment: 2-2.5 h at 26°C in the dark. | Optimal enzyme concentration that resulted in complete cell-wall removal was found to be 3% Onozuka R-10, 3% Macerozyme R-10, 1% agarase and 0.5% Pectolyase Y-23 in 60% seawater containing 1.0 M mannitol. Protoplast yield ranged from 1.5-5.0 per 0.5 g fresh weight of tissue. Young, healthy sporelings produced the most viable protoplasts compared to matured plants. Spontaneous protoplast fusion was observed in several occasions. | Cheney *et al.* (1986) |
| *Gracilaria verrucosa* | Protoplasts were isolated using only agarase prepared from marine bacteria and commercial cellulase. | Protoplast yield up to a maximum of $1.0 \times 10^7$ $g^{-1}$ fresh weight was achieved. Protoplasts isolated from young blades showed higher percentage of viability. Cell viability was better with NaCl as osmoticum than sorbitol. Cell wall regeneration was noted within seven days. | Mollet *et al.*, (1995) |
| *Gracilaria tenuispitata* and *G. lemaneiformis* | Enzymes used: 2% onozuka R-10 and macerozyme R-10 and 0.01% agarase. Enzymatic incubation: 4.5h. Protoplasts were characterized using Flow Cytometry. | Protoplast yield ranging from $10^6$ to $10^7$ $g^{-1}$ fresh weight was obtained. | Corzo *et al.*, (1995) |