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COMPARISON OF ENZYME SYSTEMS FOR THE RELEASE OF LARGE NUMBERS OF VIABLE PROTOPLASTS FROM GRACILARIA VERRUCOSA (RHODOPHYTA)

Stephanie Susan Dawes

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg in partial fulfilment of the requirements for Master of Science in the field of Biotechnology.

Johannesburg, February 1991

ABSTRACT

The response of Gracilaria verrucosa tissue to antibiotic treatment by polymyxin B sulphate and erythromycin, four different media (seawater, Provasoli Enriched Seawater, half strength Provasoli Enriched Seawater, and Provasoli Enriched Seawater supplemented with glycerol as a carbon source) and digestion with five different enzyme systems was investigated.

Gracilaria tissue was found to be unaffected by concentrations of polymyxin B sulphate up to 400 units/ml and concentrations of erythromycin below 60 µg/ml. These concentrations were found to be effective against the majority of the bacterial flora present on the algal tissue.

Gracilaria tissue survived the best on Provasoli Enriched Seawater, but produced the highest numbers of new lateral buds on seawater solidified with agar. Callus production was also optimal on seawater solidified with agar. Gracilaria explants survived equally well on osmolalities of 0.7 $Os.kg^{-1}$ and 1.0 $Os.kg^{-1}$ but viability of tissue explants improved on plates solidified with 0.3% agar as compared to those solidified with 0.75% agar.

Limpet Acetone Powder released the largest numbers of protoplasts from Gracilaria verrucosa, but only slightly fewer numbers were released with a crude enzyme extract from the gut of a local species of sea urchin, Stomponeustes variolis, and also with the acetone powder prepared from the digestive organs

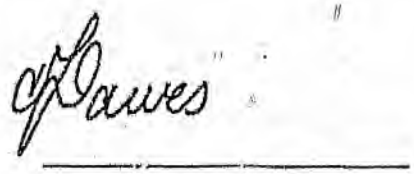
of this sea urchin. Release of protoplasts was also obtained from digestions with a crude enzyme extract from a bacterium that forms part of the natural flora of Gracilaria verrucosa. The latter enzyme systems are cost effective and efficient A low number of protoplasts was released after digestion with a cocktail of commercially available enzymes.

DECLARATION

I declare that this dissertation is my own unaided work.

It is being submitted for the degree of Master of Science in the field of Biotechnology at the University of the Witwatersrand, Johannesburg.

It has not been submitted for any degree or examination at any other university.



Stephanie Susan Dawes

Dated in Johannesburg on the 28th day of February 1991

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TABLE OF CONTENTS

	PAGE
Title page	1
Abstract	2
Declaration	4
Acknowledgements	5
Table of Contents	6
List of Figures	9
List of Plates	12
List of Tables	14
1 INTRODUCTION	15
2 LITERATURE REVIEW	21
Chapter 1: Algae as an economic resource	21
a) Food	21
b) Algal Polysaccharides	21
i) Agar	22
ii) Carrageenan	22
iii) Alginic acid	23
Chapter 2: Seaweed strain improvement	23
a) Plant breeding and selection of strains	23
b) Strain improvement by sexual hybridisation	25
c) Protoplast fusion	26
i) Interspecific	26
ii) Intergeneric	27

Chapter 3: Protoplast isolation 28

a) Isolation in the red, green and brown algae 28

b) Axenic culture 29

c) Enzymes 30

d) Media components 32

e) Staining dyes 35

f) Callus and single cell cultures 36

3 MATERIALS AND METHODS 38

3.1 Collection 38

a) Gracilaria verrucosa 38

b) Sea urchins 38

3.2 Media 39

3.3 Manipulation of Gracilaria explants 41

a) Betadine treatment of Gracilaria explants 41

b) Plating of Gracilaria disks 41

c) Plating of Gracilaria axes 42

d) Incubation of Gracilaria explants 42

e) Determination of tissue viability 42

f) Transfer of callus 42

3.4 Protoplast release 43

a) Crude sea urchin gut fluid extraction 43

b) Acetone purification 43

c) Protoplast release 43

i) Acetone Powder (Sea urchin and Limpet) 43

ii) Crude sea urchin extract 44

iii) 'Cheney cocktail' 45

v) Crude bacterial extract 47

3.5 Staining 47

a) Evans Blue dye 47

4 RESULTS AND DISCUSSION 48

4.1 Antibiotic treatment of disks 48

a) Response of algal flora to antibiotics 48

b) Response of Gracilaria tissue to antibiotics .. 50

4.2 Response of Gracilaria tissue to different media .. 58

a) Enrichment of media 58

i) Addition of nutrients 58

ii) Addition of an inorganic carbon source .. 63

b) Osmolality and solidity 67

4.3 Production of hydrolytic enzymes 67

4.4 Isolation of protoplasts from Gracilaria 68

a) Cheney cocktail 69

b) Limpet Acetone Powder 75

c) Sea Urchin Acetone Powder 75

d) Crude sea urchin gut extracts 78

e) Crude bacterial enzyme extract 82

5 SUMMARY AND CONCLUSIONS 85

6 APPENDIX 91

7 REFERENCES 95

LIST OF FIGURES PAGE

Figure 1. Flow diagram of protocols for the preparation of Gracilaria tissue for digestions, antibiotic treatments and media testing 41a

Figure 2. Percent mortality of tissue disks treated with erythromycin concentrations from 60 ug/ml to 250 ug/ml in a) SWA, b) ½PESA and c) PESA. 52

Figure 3 (a). Viability of tissue disks exposed to concentrations of polymyxin B from 100 units/ml to 400 units/ml on SWA. 53

Figure 3 (b). Viability of tissue disks exposed to concentrations of polymyxin B from 100 units/ml to 400 units/ml on ½PESA. 54

Figure 3 (c). Viability of tissue disks exposed to 100 units/ml, 200 units/ml, 300 units/ml and 400 units/ml polymyxin B on PESA. 55

Figure 4. Mortality rates of tissue disks exposed to concentrations of erythromycin on SWA. 56

Figure 5. Percent viability and percentage of normally pigmented tissue axes plated on SWA, ½PESA and PESA plates. 59

PAGE

Figure 6. The average number of buds formed per axis on SWA, ½PESA and PESA compared to the average length of the buds. 62

Figure 7. Frequency of points of callus production per healthy axis on SWA, ½PESA and PESA compared to the numbers of normally pigmented tissue pieces on each plate. 62

Figure 8. Mortality rates of tissue plated on various combinations of 100/70 PES, 0.75%/0.3% agar and ± 0.3 M glycerol. 66

Figure 9. Protoplast release during enzymatic treatment with the 'Cheney cocktail' enzyme mix. 69

Figure 10. Protoplast release (total and viable) from tissue treated with 5% Limpet Acetone Powder and 10% Limpet Acetone Powder. 73

Figure 11 (a). Viable protoplast release from 5% sea urchin acetone powder prepared from starved Stomponeustes, fed Stomponeustes and starved Pinocladus. 76

Figure 11 (b). Total protoplast release from 5% sea urchin acetone powder prepared from starved Stomponeustes, fed Stomponeustes and starved Pinocladus. 77

PAGE

Figure 12 (a). Protoplast release from the crude enzyme extract alone and supplemented with cellulase and macerozyme from the coelomic extract. 79

Figure 12 (b). Protoplast release from the crude enzyme extract alone and supplemented with cellulase and macerozyme from the digestive tract extract. 79

Figure 13. Protoplast release from Gracilaria tissue treated with a crude bacterial enzyme extract supplemented with cellulase and macerozyme and without additional enzymes. 83

LIST OF PLATES

PAGE

PLATE 1 93

Figure 1 Endophytic fungal contamination on Gracilaria verrucosa tissue disks.

Figure 2. Aerial hyphae of fungus (arrowed) from Gracilaria tissue that appears free of surface fungal contamination.

Figure 3. Cross section through Gracilaria axis showing large inner cortical cells and smaller outer cortical cells.

Figure 4. Squash of Gracilaria bud and callus (arrowed).

Figure 5. Callus production (one month old) on aerial lateral bud of Gracilaria axis plated on ½PESA medium.

Figure 6. Callus production (one month old) on aerial lateral bud of Gracilaria axis plated on PESA medium.

Figure 7. Callus production (one month old) on aerial lateral bud of Gracilaria axis plated on SWA medium.

PAGE

PLATE 2 94

Figure 1. Callus production and lateral bud formation at the wound site of a Gracilaria axis plated on SWA medium.

Figure 2. Callus production (three months old) at base of aerial shoot from a Gracilaria disk plated on PESA.

Figure 3. Lateral bud formation at the wound site of a Gracilaria axis plated on SWA.

Figure 4. Lateral bud formation at the wound site of a Gracilaria axis plated on SWA.

Figure 5. Agar 'pit' in PESA medium. The plastic bottom of the Petri dish is exposed (arrow).

Figure 6. Agar 'pits' in PESA medium. The pits appear to form independently of the presence of Gracilaria tissue.

LIST OF [illegible]ES

PAGE

Table 1. The maximum yield of protoplasts from *Gracilaria verrucosa* after digestion with seven enzyme systems 87

1 INTRODUCTION

Commercial interest in seaweeds and seaweed products has generated research into the cultivation and selection of seaweed strains. In the Far East, cultivation of Porphyra (Nori), Laminaria (Kombu) and Undaria (Wakame) is a major industry. Seaweeds are also used as a source of gel forming polysaccharides (phycocolloids) - notably agar and carageenan from red seaweeds and alginic acid from brown seaweeds (Percival and McDowell 1990).

Seaweed phycocolloids are widely used in the food industry as emulsifying agents, gelling agents, stabilisers, thickeners and suspension agents and have a combined annual market value in excess of US$ 250 million (Evans and Butler 1988). Seaweeds are also a source of unique and pharmacologically useful compounds (Polne-Fuller and Gibor 1987, Glicksman 1987). Additional applications of seaweeds include use as animal food, agricultural aids and in waste water treatments. Extensive research is also being carried out aimed at a commercially viable, large scale cultivation of seaweeds as a digestion feedstock for methane production (Polne-Fuller and Gibor 1987, Evans and Butler 1988).

Agar and agar-derived agarose are the highest priced of the seaweed phycocolloids due to their limited supply and an increasing demand for agar in many biotechnological areas (Percival and McDowell 1990). Top quality bacterial grade agar sells at US $ 38.60/kg (Sigma Chemical Co. 1986). Agarose is used in immunodiffusion and electrophoretic separations and some

specialised forms cost US $ 3300/kg (Evans and Butler 1988). Agar is extracted from a number of red algal species, the most important of which are Gelidium and Gracilaria.

Of the Gracilaria species, Gracilaria verrucosa (Hudson) Papenfuss is of major commercial importance in southern Africa. G. verrucosa is a red alga found off the coast of Namibia and harvested for its agar. The agar extracted from this strain is superior both in yield and gel strength to that of other seaweeds and is imported by Japan to improve inferior grades of agar. Japan imported 895 tons of Gracilaria and 100 tons of Gelidium from southern Africa in 1984 (Armisen and Galatas 1987).

G. verrucosa is harvested as a 'beach wash'- that is, seaweed that has separated from its bed and has been cast upon the beach by seasonal storms. This is an irregular method of harvest, but a large workforce is employed to manage the harvest during the stormy season (Rotmann 1987). In order to lessen the dependence of the harvest on the weather, it would be desirable to have some Gracilaria under cultivation. This would also increase the harvest and allow permanent employment for the workforce.

Attempts have been made to cultivate Gracilaria, but these have met with limited success (Armisen and Galatas 1987). However, cultivation for industrial purposes is undertaken in the People's Republic of China and in Taiwan by means of low rafts (Ren et al. 1984), and is also being initiated in Chile by seeding beds with thalli attached to sand filled polyethylene tubes (Martinez et al. 1990).

The most economical method of seaweed cultivation utilizes ropes strung in the ocean with clumps of seaweed suspended from the ropes. Gracilaria verrucosa is morphologically unsuited for this form of cultivation. It has spindly axes and its branches (which can grow up to 3 m in length) are easily fragmented.

Development of a strain of Gracilaria suitable for rope cultivation would be desirable. Characteristics of the desired strain would include high agar production coupled with suitable morphology for rope cultivation.

There has been no intensive strain selection procedure reported with Gracilaria although Levy and Friedlander (1990) have investigated the relationships between growth, morphology and agar quality in five strains of Gracilaria.

Traditional plant breeding methods such as simple strain selection have met with success with Eucheuma and Chondrus where highly productive strains have been selected (Doty 1985, Cheney et al. 1981), but simple selection is time consuming and limited to the genetic variability present in the population.

Sexual hybridisation techniques have been used extensively for the production of new cultivars in land plants. Gracilaria aculeata (Hering) Papenfuss is a species found off the east coast of South Africa that possesses desirable morphological attributes for rope cultivation (thick axes, short branches). The agar content of this species is inferior. However, a hybrid between these two species may exhibit the desired

morphology in conjunction with the high agar yield.

Unfortunately the contribution of sexual hybridisation to seaweed strain improvement has been limited, especially in the red algae (Cheney 1990, van der Meer 1986). The major stumbling block is the lack of interfertility between different species of red algae. Several interspecific crosses have been attempted in Gracilaria but in all cases there has been no evidence of interfertility between species (Plastino and De Oliviera 1988, 1989). Another stumbling block is the difficulty of obtaining both sexes of the strains desired (Cheney 1990). A cystocarpic Gracilaria plant was recovered from Namibia in the 1950's (R. Simons pers. comm.) but no sexual stages have been recovered from subsequent field samples of the Namibian G. verrucosa. It is likely that this population exists as a vegetative assemblage (Critchley et al. in press).

Protoplast fusion of somatic cells has been shown to break through species barriers and is a valuable tool for the genetic engineering of seaweeds. Protoplast research in seaweeds is a relatively new field and lags far behind that of land plants and unicellular algae (Cheney 1986). Most protoplast isolation has occurred in the Chlorophyta (7 species, 3 genera) and the Phaeophyta (6 species, 4 genera). Viable protoplasts have only been produced in two genera (6 species) of Rhodophyta; Gracilaria (Cheney et al. 1986) and Porphyra (Tang 1982, Saga and Sakai 1984, Polne-Fuller and Gibor 1984, Chen 1986).

Protoplast isolation in red and brown algae is relatively difficult because their cell wall components are different to those of land plants (Kloareg and Quatrano 1988, Polne-Fuller

and Gibor 1987). Alginic acid in the brown algae and agar and carageenans in the red algae are additional structural components of the cell wall (Kloareg and Quatrano 1988). Hence different enzymes are needed to break down the cell walls. The most promising enzyme sources are the gut extracts of some marine herbivorous invertebrates or culture filtrates from marine epiphytic bacteria which can digest the thalli of red or brown algae (Bradley et al. 1988, Polne-Fuller and Gibor 1987, Polne-Fuller 1987).

A prerequisite for many biotechnological manipulations of seaweeds is the successful isolation of large numbers of viable protoplasts from seaweed tissue under axenic conditions (Polne-Fuller and Gibor 1987). Axenic cultures have been difficult to obtain for multicellular species of marine algae. Methods used with success on land plants, such as surface sterilisation of tissue by treatment with a hypochlorite solution are not suitable for algae. Sodium hypochlorite and alcohols kill surface dwellers and also cells of the outer cortical layer. In most seaweeds it is this outer layer of cells that is the meristematic layer and harsh cleaning techniques may damage the cells (Polne-Fuller 1990).

Other surface sterilants have been more successful with algae, for example, iodine (Fries 1973), Betadine (Polne-Fuller and Gibor 1984) and detergents (Bradley and Pesano 1980). Antibiotics have been widely used with a variety of algae as well as combinations of antibiotics with Betadine treatment and sonication (Polne-Fuller and Gibor 1984). However, it appears that each alga collected from the field has its own unique

bacterial flora associated with it and no single method can be applied in all cases with consistent success (Bradley et al. 1988).

Optimisation of basic tissue culture techniques for Gracilaria verrucosa is necessary before strain manipulations can occur.

This study reports on investigations into :

i) The production of large numbers of viable protoplasts from Gracilaria verrucosa from low cost, alternative enzyme sources to the commercially available enzyme preparations

ii) The response of Gracilaria verrucosa explants to different culture media

iii) Isolation of axenic material from G. verrucosa

2 LITERATURE REVIEW

Chapter 1: Algae as an economic resource

Seaweeds are an important marine resource. During the last decade, they have been regarded as a promising biomass for fine chemicals and alternative energy production. Recently, cultivation of economically valuable seaweeds has become a large industry in Japan, primarily as a food source, but also in the extraction of phycocolloids.

a) Food

The most important use of seaweeds is as food (Evans and Butler 1988). Nori (Porphyra), Wakame (Undaria) and Kombu (Laminaria) are three major marine food crops and their annual yields are estimated to be in the region of 300 000, 120 000 and 30 000 tons dry weight respectively. The seaweed cultivation industry is estimated to be worth over US $ 1 billion per year (Evans and Butler 1988).

b) Algal polysaccharides

The second most important use of seaweeds is as a source of gel forming polysaccharides (phycocolloids), notably agar and carageenan from red algae and alginic acid from brown algae (Evans and Butler 1988). Seaweed phycocolloids are widely used in industry as emulsifying agents, gelling agents, stabilisers, thickeners and suspension agents (Glicksman 1987) and have a combined annual market value in excess of US $250 million (Evans

and Butler 1988).

i) Agar

Agar is extracted commercially from a number of red algal species, the most important of which are Gelidium and Gracilaria. Agar and agar-derived agarose are the highest priced of the seaweed phycocolloids due to limited supply and an increasing demand from many biotechnological fields. Top quality bacteriological agar sells at US $38.60/kg (Sigma Chemical Co. 1986). Agarose is also utilised in immunodiffusion and electrophoretic separations and some specialised forms cost US $ 3300/kg (Sandford and Baird 1983).

ii) Carageenans

Carageenans are mainly extracted from species of the red algae such asChondrus, Gigartina and Eucheuma. Commercial carageenan produces high viscosity gel solutions and gels, and reacts with proteins such as casein in milk and is thus widely used in the food industry as a thickening and stabilising agent. Kappa-carageenan is also used in biotechnological fields for cell immobilisation. Carageenan sells for US $ 49.50/kg (Sigma Chemical Co. 1986).

iii) Alginic acid

Alginic acid is extracted from brown algae, for example Laminaria and Macrocystis. Alginate has extremely wide ranging food and industrial uses. Calcium alginate is also used for cell encapsulation in bioconversion and immobilisation systems (Fujimura and Kajiwara 1990). Alginic acid sells for US $53.45/kg (Sigma Chemical Co. 1986).

Seaweed phycocolloids have played an important role in biotechnology, since most of the major advances in technology would not have been possible without the availability of phycocolloids from seaweeds. No commercial applications have emerged for polysaccharides from green seaweeds.

Chapter 2: Seaweed strain improvement

Polysaccharides from seaweeds have allowed scientists to develop modern biotechnological tools and techniques (Renn 1990). These tools and techniques can be applied in turn to the manipulation of seaweed strains, either through conventional cross breeding, production of mutants or by somatic hybridisation and somaclonal variation (Renn 1990).

a) Plant breeding and selection of strains

For some of the seaweeds with known or potential commercial value, there have been attempts to improve the performance of cultivated or experimental lines. The Chinese have been the

leaders in this area with improvements in their cultivated strains of Laminaria (Lipkin 1985, van der Meer 1987). Gametophytes of Laminaria japonica Aresch. were exposed to high temperatures and surviving plants were inbred for four generations. In the field, selected sporophytes performed better during late spring than the controls.

Selection of Porphyra in Japan has also progressed to the point where commercial cultivars differ greatly from the wild populations (Yoshida and Akiyama 1980). The advent of cultured Conchocelis and artificial seeding has facilitated the process of selecting superior strains of Porphyra (Miura 1975, van der Maer 1987). The cumulative effect of the selection process has been impressive with frond lengths raised from 10 - 15 cm to up to 60 cm in length (van der Meer 1986).

A disease resistant strain of Porphyra yezoensis Ueda was obtained by selection of single cells from islands of living cells in mostly dead blades of plants attacked by the fungus. These cells were regenerated into plantlets which exhibited resistance to the fungus, Pythium (Polne-Fuller 1987).

A genetic selection regime was incorporated into the design of local seaweed farming operations for the red alga Eucheuma (Doty and Alvarez 1975, Doty 1978) but no quantitative genetic analyses of growth performance have been reported.

For red algae other than Porphyra and Eucheuma, vigorous clones have been obtained by simple selections of fast growing plants from wild populations. This approach has been reported for Chondrus crispus Stackh. (Cheney et al. 1981), Gigartina

exasperata (auth. unavail.) (Waaland 1979), Gracilaria tikvahiae McLachlan (Ryther et al. 1979) and Gracilaria sjoestedtii (auth. unavail.) (Hansen 1984). There has however been no commercial cultivation of any of these strains as new clones are still being isolated (van der Meer 1986).

There has been no intensive strain selection effort with Gracilaria spp. although most of the world commercial agar is extracted from this genus (Hurtado-Ponce and Umezaki 1988) although Gracilaria has been cultivated in land based ponds for agar production in Taiwan for many years (Shang 1976)

b) Strain improvement by sexual hybridisation.

Sexual hybridisation has proved enormously successful in land plants for obtaining experimental cultivars for potential commercial exploitation. However, hybridisation tests in seaweeds have often been incomplete - if a hybrid plant is produced, it is not often tested for fertility.

The first attempt to cross red algae was made by Sundene (1959) with Antithamnion. Other genera of red algae have been studied for cross hybridisation capabilities and these include Gigartina and Petrocelis (Guiry 1984), Callithamnion (O'Kelly and Baca 1984), Ceramium (Rueness 1978), Champia (Steele et al. 1986), Chondrus (Chen and Taylor 1980), Gigartina (Guiry et al. 1987), Laurencia (Howard et al. 1980), Palmaria (van der Meer 1987a) and Polysiphonia (Kapraun 1977). Both intra and interspecific incompatibility have been reported in several red algae

including Chondrus (Chen and Taylor 1980), Gigartina (Guiry 1984) and Gracilaria (Plastino and de Oliviera 1989)

Because of such interfertility contraints, it would appear unlikely that interspecific hybridisations can be utilized as effectively in commercially valuable red algae as it has in land plants to develop new strains (Cheney 1990).

c) Protoplast fusion

In the past, seaweed strain improvement techniques have generally been restricted to classical plant breeding approaches such as simple selection of wild plants or variants produced by mutagenesis and colchicine treatment, or to a lesser extent by polyploid construction and sexual hybridisation (Cheney 1984, van der Meer 1986, Lewis et al. 1986). Although there have been some notable successes (Fang et al. 1983, Cheney et al. 1981) these remain few in number and generally require many years of research effort to develop.

Protoplast fusion techniques provide the opportunity to produce unique genomic combinations which are impossible or impractical by classical breeding techniques, such as the production of hybrids between sexually incompatible species.

i) Interspecific protoplast fusion

Protoplast fusion has been reported between Gracilaria tikvahiae and G. chilensis (auth. unavail.) (Cheney 1990) using the chemical fusogen polyethylene glycol (PEG). More

than 20 of these fusion products have been regenerated to whole plants that exhibit unusual morphological features that suggest they may be putative hybrids.

Fujita and Migita (1987) attempted to fuse protoplasts from two different colour morphs of Porphyra yezoensis. Although heterochromatic thalli were produced, these authors were unable to demonstrate that true somatic hybrids had actually been produced and that the thalli were not chimeras .

Kapraun (1990) fused asexual zoospores from Enteromorpha linza (L.) J. Agardh., and also fused asexual zoospores from Ulvaria oxysperma (Kuetz.) Bliding. In both cases, isolated germlings regenerated from the fusion products developed into thalli and hybridisation was confirmed by DNA examination.

ii) Intergeneric protoplast fusion

Saga et al. 1986 reported an attempt to fuse protoplasts from the green alga Enteromorpha with protoplasts from Porphyra, a red alga. The authors were unable to regenerate whole plants.

The development of protoplast fusion - somatic hybridisation procedures usually involves five key steps (Cheney 1990). All five steps are essential for success and should be developed more or less sequentially. One of the first steps is to develop techniques for isolation and culture of viable protoplasts. A

second step is to achieve regeneration of protoplasts into whole plants. Fusion of protoplasts follows with appropriate selection procedures for the selection of hybrid plants and finally confirmation of the hybridity of putative hybrids is necessary (Cheney 1990). This review will only deal with the first objective.

Chapter 3: Protoplast isolation

a) Isolation in the red, green and brown algae.

The production of protoplasts from algae has been accomplished numerous times using single celled species (Berliner and Wenc 1976, Schlosser et al. 1976). Only in the last ten years have protoplasts been isolated from macroscopic algae. Currently protoplasts have been obtained from 19 species of multicellular marine algae. The majority of these have been from three genera of Chlorophyta, Monostroma (three species), Enteromorpha (two species) and Ulva (three species - Reddy et al. 1989, Saga 1984). Viable protoplasts have been produced from four genera of Phaeophyta; three species of Laminaria (Butler et al. 1989, Saga and Sakai 1984), Undaria (Fujita and Migita 1985), Sargassum (Fisher and Gibor 1987) and Macrocystis (Saga et al. 1986). Protoplasts have also been isolated from three genera of Rhodophyta; two species of Gracilaria (Cheney et al. 1986) viz. G. tikvahiae and G. lemaneiformis, four species of Porphyra viz P. miniata, P. perforata, P. yezoensis and P. suborbiculata (Chen 1986, Polne-Fuller and Gibor 1984, Saga and Sakai 1984, Tang 1982), and from Chondrus crispus (Le Gall et al. 1990).

b) Axenic culture

Axenic cultures of algae are needed for studies of cell nutrition, plant metabolism and cell and tissue culture, but have been difficult to obtain for multicellular species. Surfaces of seaweeds are heavily infested by various epiphytes, both macroscopic and microscopic. Some of these organisms are embedded in cell walls and between the living cells. This presents a unique obstacle for the isolation of axenic tissue from seaweeds since the meristematic cells are frequently located on the surface and will be damaged by the application of chemical cleaning (Polne-Fuller and Gibor 1987).

Algal material has been semi-cleaned in the past by mechanical manipulations such as vigorous shaking and brushing, or by dragging the tissues through semi-solid agar medium (Fries 1967). Other researchers used ultraviolet irradiation, various antibiotic mixtures (Polne-Fuller 1987) and other antiseptic solutions such as Argyrol (Gibor and Izawa 1963) and commercial bleach (Fries 1980) for cleaning algal tissues.

The most effective germicidal agent was an iodinated polyvinyl-pyrolidone preparation, commercially marketed under the trade name of Betadine. Exposure of algal tissue to a 1 % Betadine solution for up to 10 minutes was tolerated by most of the seaweeds. This mild germicidal treatment does not kill all infecting microbes and subsequent treatment of tissue with antibiotics is necessary (Polne-Fuller and Gibor 1987).

Bradley et al. (1988) devised a method to test the sensitivity of algal tissue and contaminating flora to different antibiotics

simultaneously. Plant material was chopped finely and spread evenly across the surface of agarose solidified culture medium. Antibiotic-impregnated paper disks were placed on top of the tissue pieces and the tissue was incubated. Axenic plant pieces can be recovered from areas free of bacterial growth. Polymyxin B and erythromycin impregnated disks yielded axenic tissue from Gracilaria lemaneiformis (Bory) Weber van Bosse when tested by these authors.

A unique contamination problem may be encountered with endophytic organisms. Some tropical Sargassum species carried systemic infections of fungi (Polne-Fuller 1987). Surface cleaning does not eliminate these organisms and some systemic infections of fungus are resistant to treatment by antibiotics.

c) Enzymes

Seaweed cells are set in a matrix consisting of a complex carbohydrate network and a variety of proteins. The chemical nature of this matrix is quite variable. This variability is found not only between different species of algae, but also between different regions in one plant (Polne-Fuller and Gibor 1984).

Only a few seaweeds can be dissociated by the enzyme systems utilised for protoplast production in higher plants. Green algae such as Enteromorpha and Ulva are among those while red and brown algal cell walls resist degradation by these enzymes.

Mixtures of some commercial enzymes such as agarase, pectinase and pectolyase soften some red algal tissue (Polne-Fuller 1987). An enzyme mix consisting of agarase, cellulase, macerozyme and pectolyase was used by Cheney et al. (1986) to release protoplasts from Gracilaria spp.. The 'cuticle' on the outside of many of the more complex alga prevents the penetration of the enzymes which otherwise might be able to digest the cell walls.

New sources of enzymes for the digestion of red and brown algal tissue are being sought (Polne-Fuller and Gibor 1987). Sources investigated are the digestive tracts of algal grazers and enzyme production from pathogenic bacteria, fungi and amoebae (Polne-Fuller and Gibor 1987).

The crude enzyme preparations which are presently used, with partial success, are extracted from the guts of abalone or limpet molluscs. As the animals are collected from the field, the gut enzyme composition may vary depending on the season, the physiological state of the organism and its diet. The gut enzyme composition of the invertebrate can be standardised, to a certain extent, by feeding the animal a diet of the algal tissue to be dissociated for a period of one month. Boyen et al. (1990) fed abalones and sea hares with Macrocystis tissue for one month but found no significant difference in the average yield of enzyme although enzyme concentrations from individual animals were more consistent.

Reproducible protocols for the isolation of protoplasts using crude gut extracts are difficult to achieve because both the

composition and the activities of the constituent enzymes are undefined. Crude extracts also contain low molecular weight substances and enzymes such as proteases, lipases or ribonucleases. There is evidence that these substances can reduce protoplast yield and viability (Evans and Bravo 1984, Butler et al. 1989). Boyen et al. (1990) found that proteolytic activity in abalone gut extracts purified by ion exchange chromatography was negligible.

Enzyme extracts from abalone and limpets that have been partially purified by acetone treatment are available commercially (Sigma Chemical Co.). An acetone purification procedure for crude enzyme extracts is given in Polne-Fuller and Gibor (1987). Enzyme extracts can also be purified by ammonium sulphate fractionation (Kloareg and Quatrano 1987).

j) Media components

Although tissue culture techniques have been well established for higher plant orders, studies using these techniques for algal physiology and morphogenesis studies have been limited (Fries 1980). Progress in these fields depends largely on the use of existing culture methods and their successful modification. There have been attempts to establish axenic cultures of macroalgae in defined media. In most cases the growth of the alga concerned was relatively poor. This has been attributed to a necessary mutualistic relationship between the alga and its associated microorganisms (Provasoli and Pintner 1980, Tatewaki et al. 1983).

However, poor algal growth could also be attributed to a number of factors related to culture conditions, especially the media used. Although multicellular species have been cultured successfully in some media, most of the media used were developed for maintenance of unicellular algae. Thus a deficiency in the media might be directly responsible for the poor algal growth frequently reported (Mooney and van Staden 1985).

Plant cells in culture require similar nutrients to those needed by whole plants. In addition, isolated tissues often require substances that would be normally synthesised by the whole plant, eg vitamins. Higher plant tissue culture media contain inorganic nutrients, vitamins, plant growth substances and a carbon source. Complex organic mixtures such as casein hydrolysate may also be used to supplement the medium (Evans and Butler 1988).

Existing seawater media are either enriched natural seawater or synthetic media. Natural seawater is complex and is the standard medium for the growth of marine plants. When seawater is enriched with additional nutrients it supports the growth of many seaweed species in culture. Synthetic media are fully defined and consist of the salts of the major ions found in seawater and mixtures of chelated metals and vitamins. The use of fully defined seawater media is preferable, as these are more suitable for nutritional experiments and are less variable than natural seawater. However, defined media due to intrinsic simplicity may not support good growth in some species of algae and enriched seawater media are most commonly used for the

culture of macroalgae.

The inorganic requirements of seaweeds are diverse and are the subject of extensive reviews by O'Kelley (1974) and McLachlan (1982). Seaweed tissues have been cultured successfully on several media, but the inorganic requirements have not been optimised for any species (Evans and Butler 1988).

A carbon supply which can be metabolised is a necessary prerequisite for the rapid growth of heterotrophic tissues, but the inclusion of a carbon source is not reported in many protocols. Carbon sources effective for the culture of seaweeds and tissue cultures seem to be those that are accumulated by seaweed cells (Saga et al. 1982, Fries 1984). Fries (1973) reported that glycerol is an effective carbon source for the vegetative propagation of several red seaweeds. Robaina et al. (1990) verified this for the red alga Grateloupia but observed that the effects of glycerol were only observed in explants cultivated under light. Organic carbon has a central role in supporting the growth of tissue in culture and the carbon nutrition of seaweeds requires clarification.

Vitamin requirements in seaweeds have been reviewed by Provasoli and Carlucci (1974). Seawater media already contains mixtures of vitamins thought to be required by algae. Axenic tissues may need additional vitamins supplied by the epiflora or available in the whole plant. Organic-nutritional experiments can only be carried out in the absence of microorganisms and axenic tissue culture may be useful in establishing the vitamin requirements of macroalgae.

Plant growth substances are usually present in tissue cultures of higher plants and are sometimes necessary to maintain active cell division and induce callus formation. There is very little understanding of the role of plant growth substances in the growth and differentiation of marine plants. Bradley and Cheney (1990) found that several mixtures of auxins and cytokinins stimulated cell division and growth in tissue cultures of the red alga Agardhiella. Other work with red algae includes evidence for the stimulation of growth in Goniotrichum (Fries 1974) and Porphyra (Fries and Iwasaki 1976) when treated with phenolics and auxins, and in Gracilaria (Jennings 1971) treated with gibberellin. The development of specialised nutrient media is crucial to the advancement of tissue culture techniques in seaweeds (Evans and Butler 1988).

e) Staining dyes

The accurate evaluation of cell viability is an important aspect of tissue culture and the genetic engineering of cells. Determining cell viability is essential to the development of efficient methods for mutagenesis, transformation and/or cryopreservation of cultured algal cells. Saga (1989) found that the most simple and reliable method for determining cell viability in the red and brown algae utilised neutral red for the detection of live cells and Evans Blue for the detection of dead cells. Appearance (spherical), the retention of pigments and organelles and observation of cytoplasmic streaming are also considered useful indicators of the viability of the protoplasts (Butler et al. 1989).

Evaluation of the complete removal of the cell wall can be performed in a number of ways. Sensitivity to reduced osmolarity (protoplasts burst on exposure to fresh water), a spherical appearance and observation of the protoplasts under an electron microscope have been criteria used to evaluate the removal of the cell wall (Fisher and Gibor 1987, Butler et al. 1989, Cheney et al. 1986). Calcofluor White stains cellulose, hence the absence of this stain indicates the absence of a cell wall (Berliner 1981). However, Cheney et al. (1986) found that putative protoplasts that indicated the absence of a cell wall by staining negatively for Calcofluor White, stained positively with Toluidine Blue O (a stain for sulphated polysaccharides) indicating the partial presence of a cell wall.

f) Callus and single cell cultures

Callus cultures are used as a source of vegetative cells for regeneration experiments or protoplast isolation, or as a source of secondary products. The formation of callus tissue has been reported in at least 27 species of macroalgae, eight of which were reported in red seaweeds. Of these eight reports, the callus tissue has been successfully regenerated into plants for Aghardiella (Cheney 1986), Gelidium (Polne-Fuller unpublished), and Porphyra (Polne-Fuller and Gibor 1984, Saga et al. 1986, Zhao and Zhan 1981). The formation of callus has also been reported in Gelidium (Misawa 1977), Grateloupia (Robaina et al. 1990) and Gracilaria (Misawa 1977, Collantes et al. 1989).

There are reports that agar can be successfully produced by callus culture of algae such as Gelidium and Gracilaria; 100g of

dried callus cells was reported to produce 75 g of agar (Misawa 1977).

The development of techniques for protoplast isolation have allowed researchers to establish single cell cultures for marine multicellular algae in much the same way that single cell cultures have been established for higher plants. Tait et al. (1990) have established a single cell culture of Porphyra perforata J. Agardh. tissue and assayed the cells in the culture for the production of polysaccharides. The polysaccharide produced was structurally similar to that extracted from the naturally grown alga. Carageenan has been produced by tissue cultures of Agardhiella (Cheney et al. 1987).

Reports of protoplast isolation and the culture of members of the Chlorophyta and their intentional uses in cell engineering have been more common compared to other seaweed groups. Fujimura and Kajiwara (1990) have prepared immobilised cell cultures of Ulva for the production of bioflavour.

Culture conditions still need to be optimised before the economic viability of the production of polysaccharides from plant cell cultures as opposed to the extraction of the polysaccharides from cultivated plants can be determined.

3 MATERIALS AND METHODS

3.1) Collection of material

a) Gracilaria verrucosa

Gracilaria verrucosa was collected from Lüderitz, Namibia from August - November 1990. The seaweed was transported in cooled plastic bags to Johannesburg by refrigerated lorry and air freight. The plants were checked and visible epiphytes removed. The plants were transferred to aquaria containing seawater. The experimental material was incubated at 70 $\mu E.m^{-2}.s^{-1}$ with a light/dark (L/D) cycle of 16:8.

b) Sea urchins

Stomponeustes variolis Lam. and Pinocladus sp. were collected at Reunion Rocks, Isipingo, Natal during September - October 1990 from low shore rock pools. The sea urchins were transported in seawater to Johannesburg in cooled plastic jars and transferred on arrival to aerated aquaria. The experimental animals were divided between two treatments: the sea urchins were either starved for three days or fed for three days with Gracilaria verrucosa prior to sacrifice and extraction of the gut fluids.

3.2 Media

Seawater (SW) was obtained from the Oceanographic Research Institute, Durban. It was filtered twice through Whatman No. 1 filter paper and autoclaved. Autoclaved seawater (ASW) was used to prepare all media unless otherwise indicated.

A stock solution of Provasoli Enriched Seawater (PES) was prepared (Provasoli 1968) (see Appendix).

The stock solution was diluted with ASW to produce PES and half-strength PES (½PES) solutions for Gracilaria tissue culture. The solutions were solidified with 0.75 % agar (Saarchem) or 0.3% agar (Lawlor et al. 1989, Robaina et al. 1990). PES and ½PES media solidified with agar were designated PESA and ½PESA respectively. Seawater solidified with agar was designated SWA.

Glycerol was incorporated (prior to autoclaving) at a concentration of 0.3 mol/l (Robaina et al. 1990) in plates poured to test the response of Gracilaria to a carbon source (see section 4.2 (a)).

Osmolality of the media was adjusted by replacing a percentage of the seawater used to prepare the media with distilled water (Robaina et al. 1990). Plates were poured on a laminar flow bench immediately after the media had been autoclaved (121°C at 1.5 bar for 20 minutes) to minimise contamination. The plates were left to harden overnight at room temperature before use. Three replicates were poured for each treatment.

Plates incorporating antibiotics were poured when the agar had cooled to ~ 65 ° C after autoclaving to avoid heat inactivation of the antibiotics.

Antibiotic stock solutions were prepared from erythromycin (Boehringer Mannheim GmbH) and polymyxin B sulphate (Sigma Chemical Co.). 10 mg/ml erythromycin was dissolved in 99% ethanol and 8000 units/ml (u/ml) polymyxin B sulphate was dissolved in distilled water and filter sterilised with a 0.22µm sterile filter (Millipore).

Antibiotics were incorporated into media in the following manner. Suitable aliquots (depending on the final concentration required) of the stock solutions of antibiotics were dispensed into autoclaved test tubes. The test tubes were all of the same volume (20 ml). Another series of autoclaved test tubes were prepared. Liquid agar was poured into the test tube containing the aliquot of antibiotic. The agar was then transferred back and forth between the original test tube and another empty autoclaved test tube to mix the contents thoroughly. The contents were then decanted into a sterile Petri dish and the surface of the media was briefly flamed with a Bunsen burner flame to eliminate air bubbles.

A stock solution (50 u/ml) of agarase (Sigma Chemical Co.) was prepared in seawater and filter sterilized with a 0.22 µm filter (Millipore).

Enzyme solutions specific for each digestion protocol are described under the relevant digestion protocols.

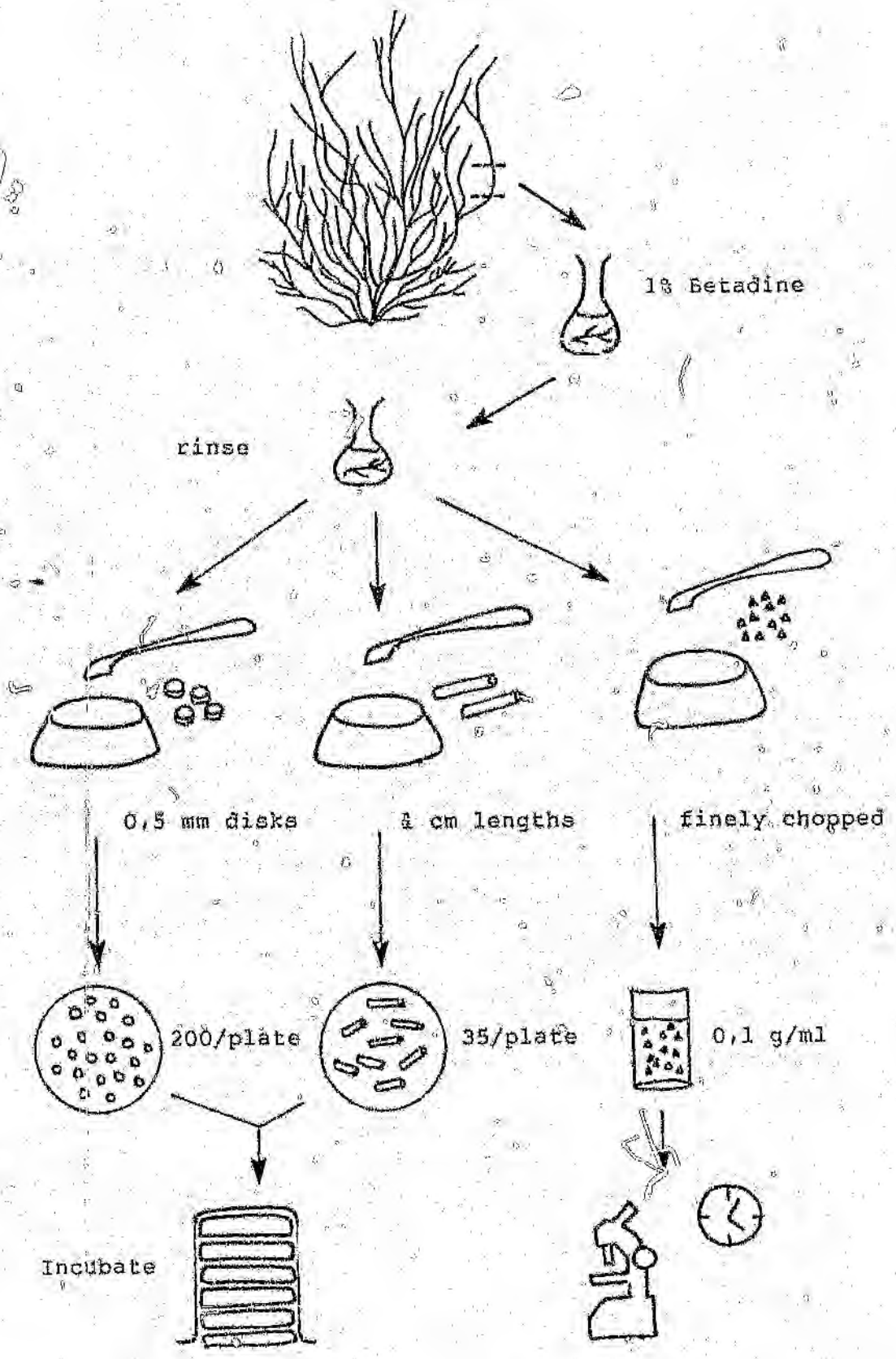


Figure 1. Flow diagram of protocols for the preparation of Gracilaria tissue for digestions, antibiotic treatments and media testing

3.3 Manipulation of Gracilaria explants (See Fig. 1)

a) Betadine treatment of Gracilaria tissue

Samples of the experimental material were removed from the aquaria as necessary. The Gracilaria tissue was washed briefly in distilled water (Robaina et al, 1990) and rinsed several times in ASW. The algal tissue was then immersed in a 1% Betadine (Adcock Ingram Lab. Inc.) solution in seawater for 5 minutes (Liu and Gordon 1987). The tissue was rinsed five times in ASW.

b) Plating of Gracilaria tissue disks

The tissue was sliced into fine sections (0.5mm in width) with commercially pre-sterilised scalpel blades under aseptic conditions. The sections of tissue were kept in ASW until sufficient tissue disks had been obtained for the number of plates to be plated. The disks were transferred to the agar plates using a shortened pre-sterilised plastic Liquipette (Elkay Products Inc.) and spread over the surface of the plate with an alcohol sterilised glass spreader.

c) Plating of Gracilaria axes

The algal tissue was cut into centimeter lengths with pre-sterilised scalpel blades and placed onto the agar medium with sterile forceps.

d) Incubation of Gracilaria explants

The plates spread with Gracilaria tissue disks or axes were incubated stacked on top of one another and loosely enclosed in a plastic bag. The culture conditions were identical to those for the maintenance of whole Gracilaria plants (see section 3.1 (a)).

e) Determination of tissue viability

The tissue was observed to lose pigmentation as the health of the tissue decreased so death of the tissue disks or axes was correlated with a lack of pigmentation although loss of colour does not necessarily indicate death since recovery and resynthesis of pigments might take place. Counts of dead tissue disks were taken at regular intervals (every three days or every four days) and used as an indication of tissue response to the various treatments.

f) Transfer of callus

Without antibiotic treatment : Callus tissue was excised with sterile scalpels along with pieces of the surrounding tissue and transferred to PESA plates made with 70% seawater and 30% distilled water (see section 3.2). The agar concentration was either 0.75% or 0.3% and glycerol at 0.3 M was included in some of the plates.

With antibiotic treatment : Callus tissue was either transferred immediately to PESA 70 plates (0.75%/0.3% agar ± 0.3 M glycerol) supplemented with 30 µg/ml erythromycin and 200 u/ml polymyxin B, or pretreated with Betadine and then transferred.

3.4) Protoplast Release

a) Crude sea urchin gut fluid extraction

The sea urchins were sacrificed by removal of the mouth followed by harvest of the coelomic fluid. The gonads were removed and discarded. Fluid from inside the digestive tract was harvested and stored at -8°C. The remaining digestive tract tissue and tract contents were partially purified by acetone treatment (see 3.4 (b)).

b) Acetone purification (Polne-Fuller and Gibor 1987)

100 g of sea urchin guts were immersed in 500 ml of ice-cooled acetone and homogenised in a blender. The homogenate was filtered through Whatman No. 1 filter paper and the filtrate washed twice with ice-cooled acetone. A final wash was performed with ice cooled ether to remove the acetone. The powder was scraped off the filter paper and stored at -8°C in airtight glass vials.

c) Protoplast release

i) Acetone Powder (Sea Urchin and Limpet)

The method followed was that used by Polne-Fuller and Gibor (1984). 10% acetone powder was suspended in filtered seawater. The mixture was stirred with magnetic stirrer bars for 1 h at

20°C and then centrifuged at 500xg for 20 minutes. The mixture was placed in wide dialysis tubing and dialysed against seawater for 16 hr at 4°C. Sorbitol was added to the dialysed solution to a final concentration of 0.6 M and the pH was adjusted to 6.0. The solution was filter sterilized through a 0.45 µm Millipore filter and used immediately or stored at -8 °C.

Gracilaria tissue was finely chopped (Cheney et al. 1986, Nelson 1990) and 0.5g fresh weight tissue was allocated per 5 ml of enzyme solution (Cheney et al. 1986). Small magnetic stirrers (Chen 1986) were used to agitate the enzyme mix. The tissue was incubated in the dark (Cheney et al. 1986, Nelson 1990) for 20 hours with protoplast release and viability (see section 3.5 (a)) evaluated by haemocytometer counts every five hours.

ii) Crude sea urchin gut extract

An aliquot of gut extract taken either from the coelomic cavity or from inside the digestive tract was thawed and made 0.6 M with respect to sorbitol (Nelson 1990) and 5 mM CaCl was added to stabilise the cell membrane of the protoplasts when released (Cheney et al. 1986). 2% cellulase 'Onozuka' R-10 (Yakult Honsha Co. Ltd) and 1% macerozyme R-10 (Yakult Honsha Co. Ltd.) were added to some digestions (for assay of increased protoplast release.) The mixture was stirred for 20 minutes and centrifuged at 200xg for 20 minutes. The solutions were then filter sterilized with 0.45 µm Millipore filters.

0,5g finely chopped Betadine treated (see section 3.3 (a)) tissue was allocated per 5 ml gut filtrate in a glass vial. Small magnetic stirrers were used to agitate the mixture and the tissue was incubated in the dark for 48 hours (Cheney et al. 1986, Nelson 1990). Evaluation of protoplast release and viability occurred every 12 hours.

iii) Cheney cocktail (Cheney et al. 1986)

After Betadine treatment (see section 3.3 (a)) the tissue was incubated for two days in an antibiotic medium comprising :

3.3 mg/ml streptomycin sulphate (Boehringer Mannheim GmbH)

1.67 mg/ml penicillin G (Boehringer Mannheim GmbH)

0.33 mg/ml neomycin (Boehringer Mannheim GmbH)

0.17 mg/ml mycostatin (Squibb Lab. Pty. Ltd.)

1.67 mg/ml kanamycin (Highveld Biological Pty Ltd)

Plants were incubated in the dark for 24 hrs prior to enzyme treatment. Tissue was then chopped finely and rinsed three times in protoplast isolation medium (SW, 0.3 M mannitol, 5 mM CaCl at pH 5.8. 5 ml enzyme solution :

3% cellulase 'Onuzuka' R-10

3% macerozyme R-10

0.5% pectolyase Y-23 (Seishin Pharm. Co. Ltd.)

1 u/ml agarase

1 M mannitol

5 mM CaCl

60% seawater

40% deionised water

pH 5.8, centrifuged at 200xg for 20 minutes and filter sterilised through a 0.22 um filter was aliquotted per 0.5g of fresh weight tissue. Small magnetic stirrers were introduced into the vials and enzyme treatment proceeded in the dark with protoplast release monitored every hour for four hours by haemocytometer counts.

iv) Crude bacterial enzyme extract

A sample of bacteria found in an agar pit (see Results and Discussion, section 4.3) was transferred to 10 ml PES supplemented with 0.15% agar in a 100 ml Ehrlenmeyer flask. The culture was grown until turbid and centrifuged at 200xg for 5 minutes and the supernatant filter sterilised with a 0.45 µm filter. 0.5 g fresh weight finely chopped tissue was allocated per 5 ml enzyme solution in a glass vial. Small magnetic stirrers agitated the mixture which was incubated in the dark. Protoplast release was monitored on a regular basis.

3.5) Staining

a) Viability - Evans Blue dye

Tissue and cells were stained with a 0.01% solution of Evan's Blue dye in seawater at pH 8.0 (Saga et al. 1989). Viable cells do not take up the dye whereas dead cells stain blue. Stained cells and tissue were viewed under an Olympus light microscope.

4 RESULTS AND DISCUSSION

4.1) ANTIBIOTIC TREATMENT OF DISKS

(a) Response of algal flora to antibiotics

Gracilaria disks that were spread onto agar plates (section 3.3 (b)) after treatment with Betadine (section 3.3 (a)) exhibited a limited variety of bacterial growth. The bacterial growth was confluent and wax-coloured and visual separation of different species (by noting differing colony formations, for example) was not possible. A sample of the bacterial contamination was Gram stained and observed at a 1000 x magnification with an oil immersion lens. The majority of flora that survived the Betadine treatment were Gram negative rods and cocci, although a small number (<5%) of Gram positive cocci were present. The uncontrolled growth of these bacteria does not seem to affect the health of the algal tissue even when the tissue is covered with confluent growth, since the majority of the Gracilaria disks retained their normal, healthy pigmentation.

On the agar plates that were treated with erythromycin and polymyxin B, no growth of the wax-coloured bacteria occurred but the plates were contaminated by fungi. Some isolated areas of fluorescent green and orange bacterial contamination also arose but these were not noted to be closely associated with Gracilaria tissue.

On the antibiotic treated plates, the fungal contamination was mostly limited to two species. One dark green/black fungus was noted to appear not only on the antibiotic-treated plates, but also on non-treated plates that also did not contain Gracilaria disks. This implies that this particular fungal contamination was not Gracilaria-borne, but that spores of this fungus were present in the laboratory.

The second fungal contaminant (rust-coloured) appeared only on the antibiotically-treated plates spread with Gracilaria tissue (Plate 1 Fig. 1). The control plates did not exhibit growth of this fungus. The fungus was also observed to occur primarily on the algal tissue and rarely on the agar although bacterial contaminants were observed on both the algal tissue and on the agar. On closer observation (stereo microscope), aerial hyphae were noted to arise from tissue that appeared free of surface contamination (Plate 1 Fig. 2). A cross section of this tissue showed filamentous (fungal) growth within the cortex. It seems likely that the Gracilaria tissue used for the antibiotic testing was systemically infected by an endophytic fungus. This fungal growth was not controlled by the antibiotics present in the plate, but the absence of any fungal growth on the control plates spread with Gracilaria disks of the same origin suggested that the fungal growth may in some way be controlled by the growth of the wax-coloured bacteria.

Bradley et al. (1988) found that treatment of Gracilaria lemaneiformis tissue with polymyxin B and erythromycin yielded axenic tissue. However, these authors also noted that each alga

collected from the field has its own unique bacterial flora associated with it. This study on Gracilaria verrucosa flora indicates that erythromycin and polymyxin B are efficient at controlling the growth of the majority of the bacteria present on the algal tissue, but are ineffective against endophytic fungi. Tissue disks may however be recovered from antibiotically-treated plates that are not infected with a fungus and appear (under stereo microscope) to be free of bacterial contamination.

(b) Response of Gracilaria tissue to antibiotics

A range of antibiotic concentrations was tested for the dual purpose of ascertaining the optimum concentration of antibiotic that was bacteriocidal without deleteriously affecting the algal tissue. Three different media were also tested for their effect on the growth and maintenance of Gracilaria tissue disks. Mortality rates of the disks were monitored by visual counts of dead tissue disks every three days. A complete loss of pigment indicated death of the tissue. The mortality rates for the tissue treated with the erythromycin range of antibiotic concentrations in SWA, ½PESA and PESA are shown in Figure 2(a), 2(b) and 2(c) respectively. The mortality rates for the tissue treated with concentrations of polymyxin B in SWA, ½PESA and PESA are shown in Figures 3(a), 3(b) and 3(c) respectively. No growth of the tissue (new lateral bud production) was observed.

The mortality rates of the disks plated on all three sets of media (SWA, ½PESA and PESA) were noted to increase sharply with

increasing concentrations of erythromycin. 100% mortality was noticed almost immediately on plates with 250 µg/ml while the control plates showed a low mortality rate. Concentrations of erythromycin below 60 µg/ml exhibited similar mortality rates to the control value (Fig. 4).

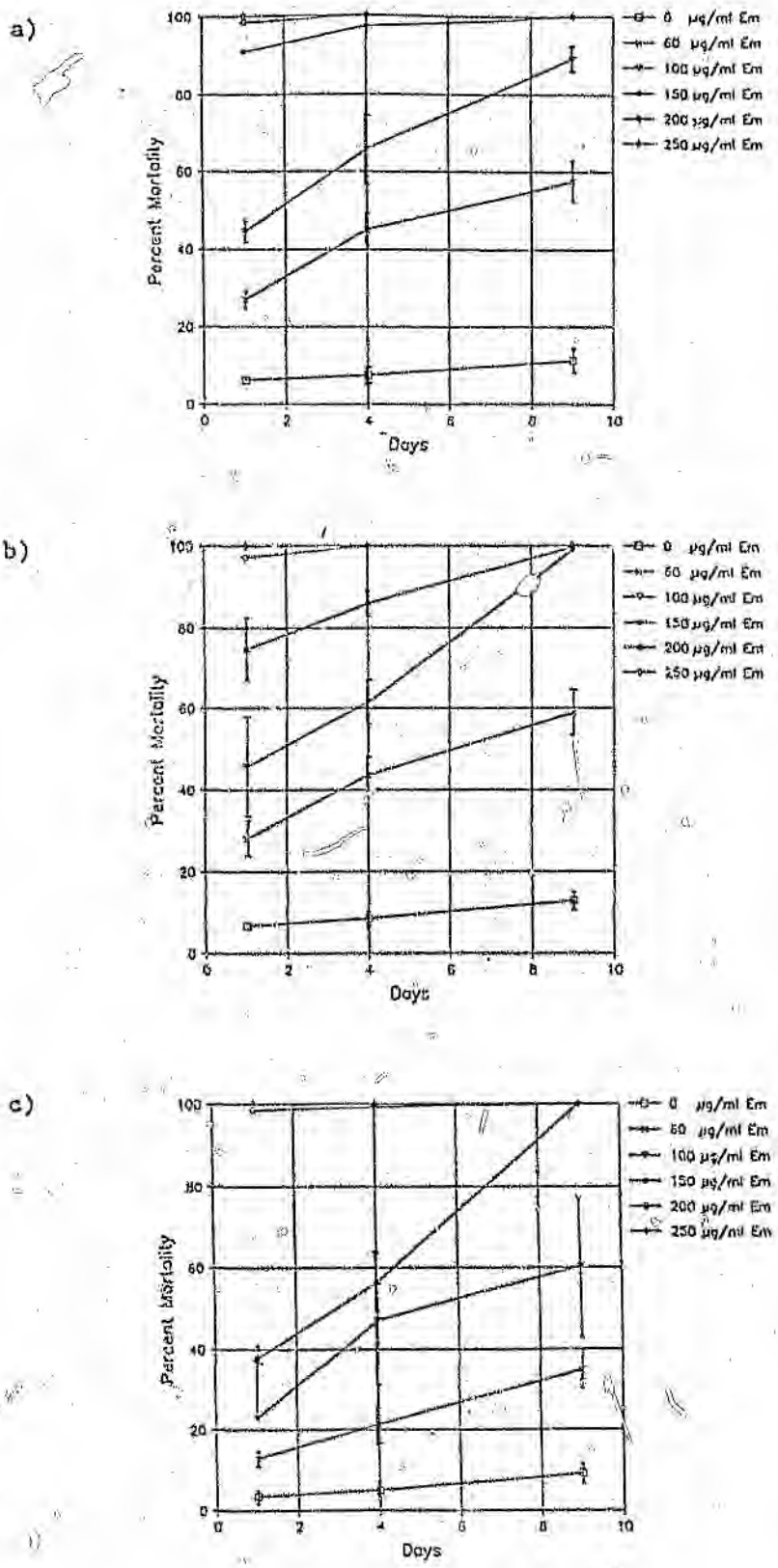


Figure 2. Percent mortality (± SE) of tissue disks treated with erythromycin concentrations from 60 µg/ml to 250 µg/ml in a) SWA, b) hPESA and c) PESA.

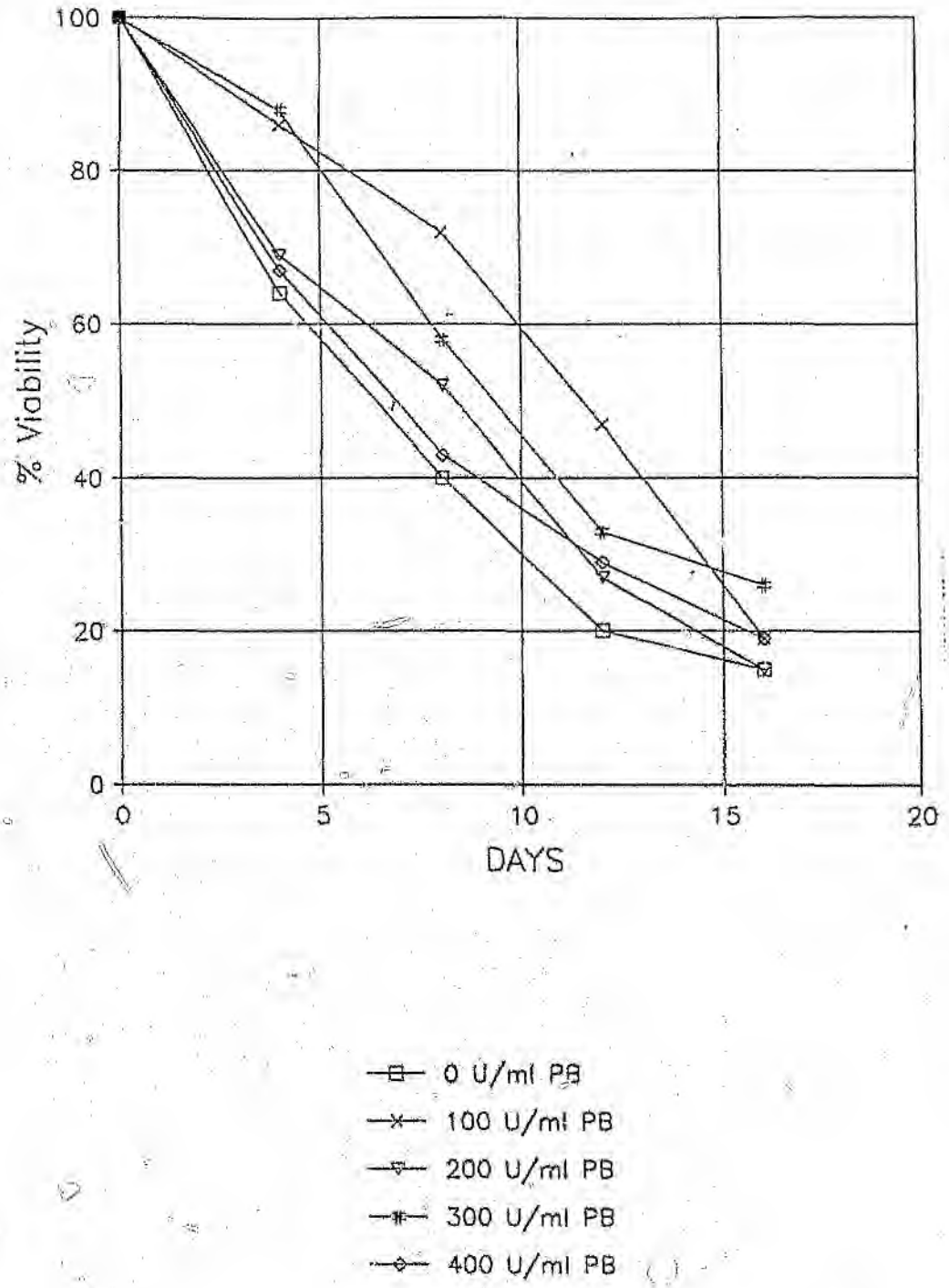


Figure 3 (a). Viability of tissue disks exposed to 100 units/ml, 200 units/ml, 300 units/ml and 400 units/ml polymyxin B on SWA.

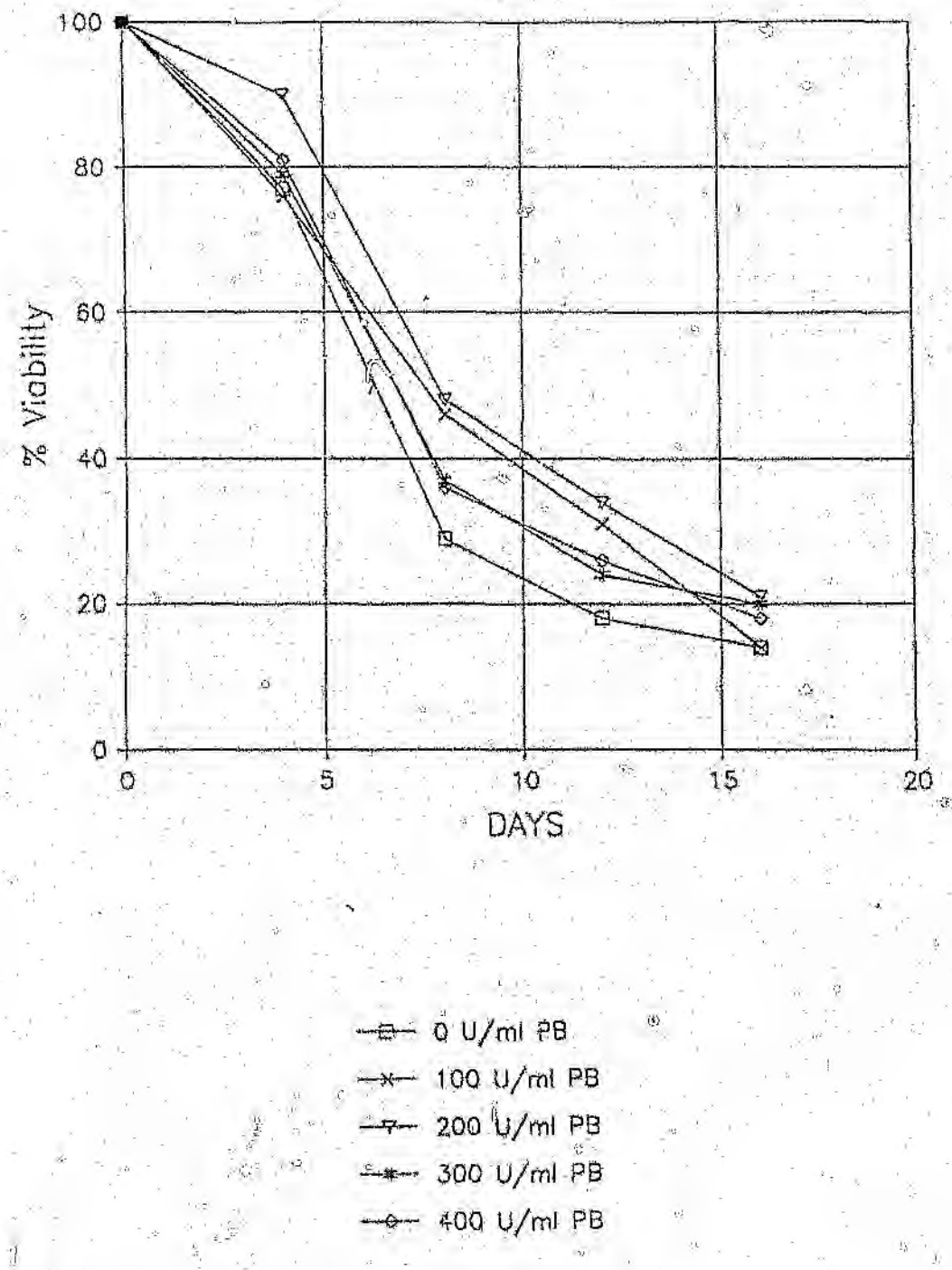


Figure 3 (b). Viability of tissue disks exposed to 100 units/ml, 200 units/ml, 300 units/ml and 400 units/ml polymyxin B on ^{4}PESA.

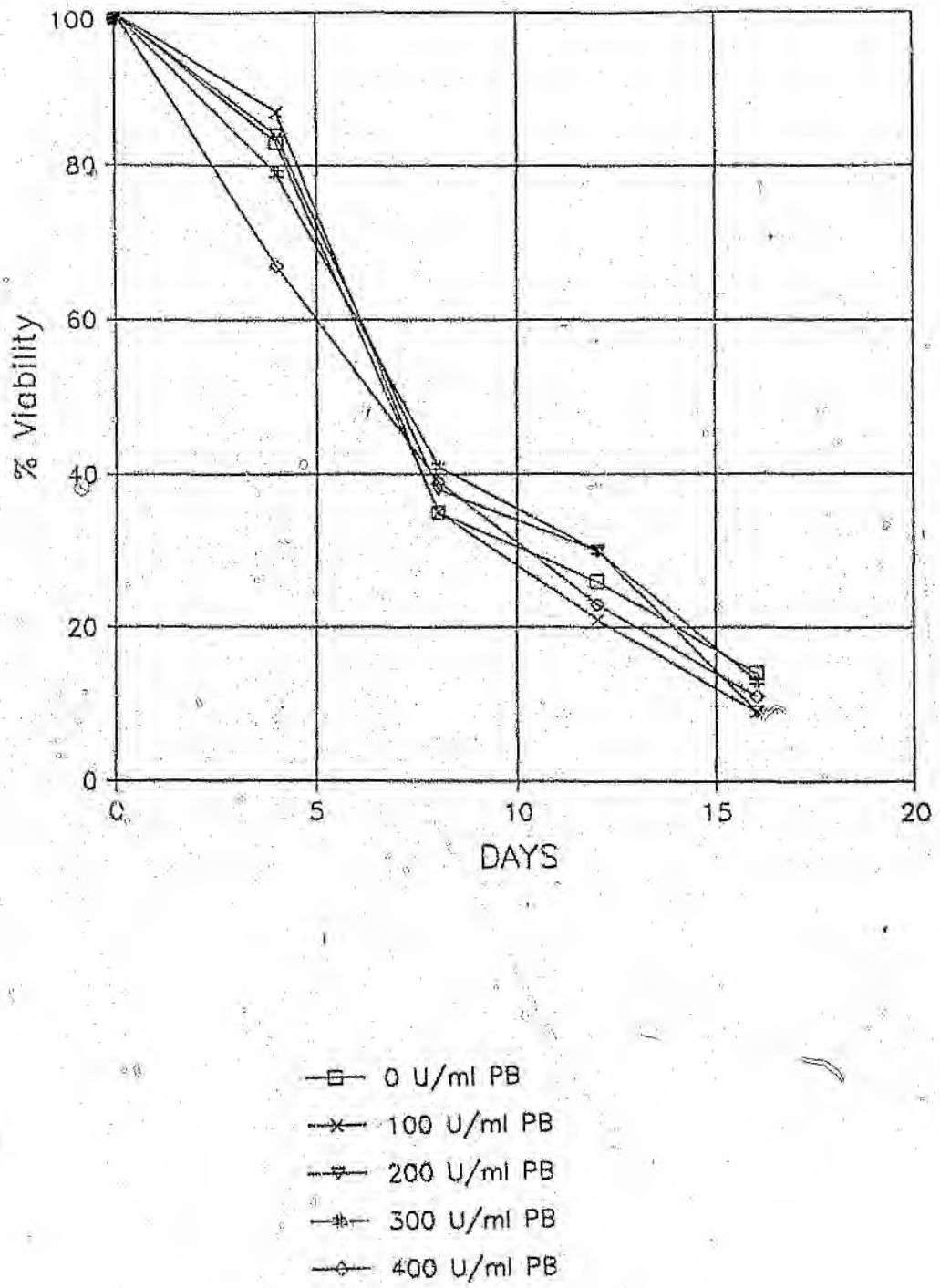


Figure 3 (c). Viability of tissue disks exposed to 100 units/ml, 200 units/ml, 300 units/ml and 400 units/ml polymyxin B on PESA.

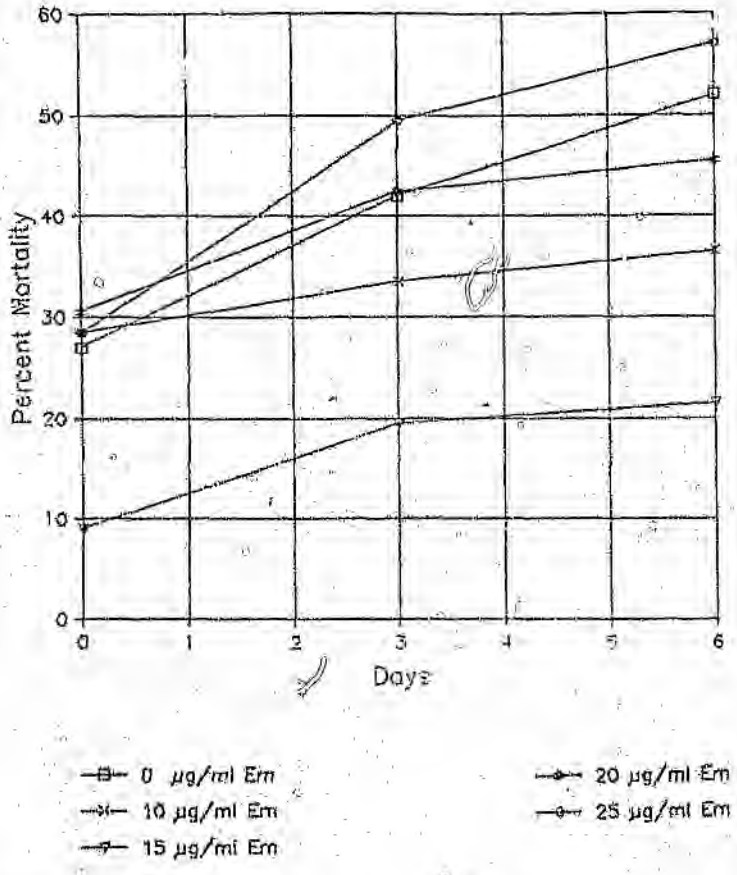


Figure 4. Mortality rates of tissue disks exposed to 10 µg/ml, 15 µg/ml, 20 µg/ ml, 25 µg/ml and 30 µg/ml erythromycin on SWA.

The mortality rates exhibited by the Gracilaria tissue disks on the polymyxin B-treated SWA, ½PESA and PESA plates were similar. The SWA plates in general exhibited higher viability than PESA or ½PESA. ½PESA exhibited less viability than PESA. This could be due to the contamination levels of other bacteria present on the places. SWA plates had fewer varieties of contaminants while PESA had the most variety and highest growth. However, the Gracilaria disks on PESA showed higher viability than those on ½PESA which could be due to the higher availability of nutrients on the PESA plate.

The mortality rates of the disks treated with polymyxin B on SWA were found to differ. The mortality rate of tissue treated with 100 u/ml polymyxin B was found to be significantly different to that of the control ($p=0.99$) as were the mortality rates of 400 u/ml polymyxin B treated tissue and control tissue ($p=0.995$). The control plate differed only slightly from the plate treated with 300 u/ml polymyxin B and no significant difference ($p=0.975$) was found between the control and the 200 u/ml polymyxin B treatment. The 100 u/ml and 200 u/ml polymyxin B plates were found to differ slightly in their mortality rates while the 200 and 300 u/ml polymyxin B treated tissue showed no significant difference ($p=0.975$) in their mortality rates. The 300 u/ml and 400 u/ml polymyxin B plates were found to have significantly different mortality rates ($p=0.995$). However, no direct trend could be inferred from these results.

%PBSA plates treated with polymyxin B showed fewer differences in mortality rates between the different concentrations of polymyxin B. The mortality rates of the tissue on 100 u/ml, 200 u/ml and 300 u/ml polymyxin B plates were not significantly different to the control ($p=0.975$). However, the plates treated with 400 u/ml exhibited a significantly different mortality rate to the control ($p=0.975$). Slight differences in mortality were exhibited between 100 u/ml and 300 u/ml polymyxin B plates, and between 200 u/ml and 400 u/ml polymyxin B plates, but no significant differences in mortality rates ($p=0.975$) were observed between 100 and 200, or 200 and 300, or 300 and 400 u/ml polymyxin B treated plates.

PESA plates containing polymyxin B in varying concentrations showed no significant difference ($p=0.975$) between any of the treatments.

Although no obvious trend could be inferred from the response of the Gracilaria tissue to polymyxin B, at various concentrations on the SWA plates, it seems that polymyxin B affects Gracilaria tissue more if the tissue is plated on SWA than ½PESA, and similarly if the tissue is plated on ½PESA and PESA. However, the final percentage mortality of the tissue disks was not found to be significantly different between the three plates and it can be assumed that concentrations of polymyxin B up to 400 u/ml do not deleteriously affect Gracilaria tissue.

4.2) RESPONSE OF GRACILARIA TISSUE TO DIFFERENT MEDIA

a) Enrichment of media

i) Addition of nutrients

To quantify the response of Gracilaria tissue to different media, centimetre lengths of axis tissue were Betadine-treated and placed on SWA, ½PESA and PESA plates (section 3.3 (a)). Mortality rates were monitored as before. As a further indication of tissue response, the numbers of normally pigmented pieces of tissue were also recorded. A graphic representation of these results is shown in Figure 5.

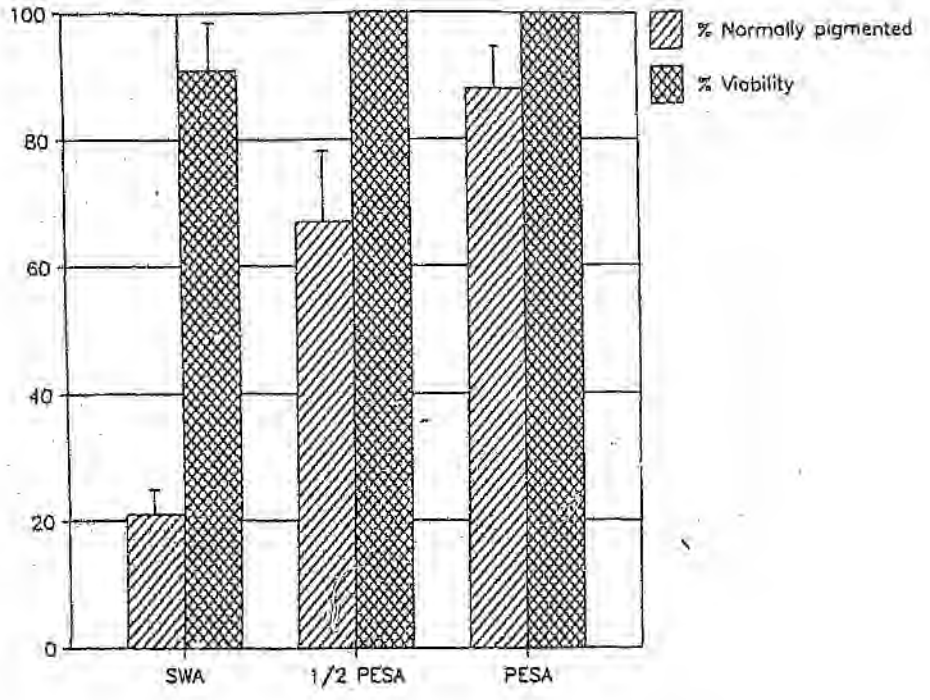


Figure 5. Percent viability and percentage of normally pigmented tissue axes plated on SWA, ½PESA and PESA plates.

A significant difference (p=0.975) in viability was noted between the SWA and PESA plates with PESA exhibiting the greater viability. There was no difference in viability between PESA and ½PESA.

There was however a significant increase in the numbers of normally pigmented tissue between SWA and ½PESA (p=0.995), and between ½PESA and PESA (p=0.975).

The effect of adding vitamins in tissue culture media for red algae has not been investigated in depth. Under natural conditions, the algae are bathed in seawater containing vitamins derived from algal and bacterial metabolism (Lawlor et al.

1989). Bacterial growth was present on all plates, but showed the greatest variety on the PESA plates. Vitamins may have been supplied to the algal tissue in this manner, as well as by increasing concentrations of vitamins in ½PES and PES media. Either way, improved vitality correlated with the increasing concentrations of vitamins present in the media.

Other workers have not found algal tissue to be responsive to addition of various vitamins (Lawlor et al. 1989) but no experiments on algal tissue response to vitamins under light conditions have been reported.

The improved growth from SWA to PES plates may also be due to the differing concentrations of trace metals present in the media although no reference to experiments with different trace metals could be traced in the literature.

Mooney and van Staden (1985) quantified the response of Sargassum heterophyllum (Turn.) J. Agardh. to different media by counting the number of proliferations produced by explant segments in the different media. The response of Gracilaria tissue to SWA, ½PESA and PESA was further investigated in this study by counting the numbers of lateral buds present on the wounded ends of the axes. This was also compared to the average length of the buds. These findings are graphically represented in Figure 6.

The numbers of buds produced by explants on SWA and ½PESA media were not found to be significantly different (p=0.975). In contrast, the number of buds produced on PESA plates differed significantly from the numbers produced on SWA plates (p=0.99) and ½PESA plates (p=0.975). The greatest number of buds per explant were produced on PESA plates. However, only healthy tissue produced new lateral buds and if the number of buds produced per healthy explant were compared for the three media, the number of buds produced by the surviving explants on the SWA agar plates was significantly higher (p=0.995) than those produced on the PESA plates while the number of buds produced on PESA and ½PESA plates were not significantly different (p=0.975).

The lengths of the buds produced on SWA, ½PESA and PESA were significantly different (p=0.9995). The new lateral buds grew better on SWA than on PESA and ½PESA, but buds grew better on PESA than on ½PESA medium.

The formation of callus tissue on axes plated on all three media (Plate 1 Figures 5, 6 and 7) was observed after three weeks. An attempt was made to quantify the relationship between media and callus production by counting the number of points of callus production on each axis and relating this to the media on which the axis was plated. The results are shown in Figure 7.

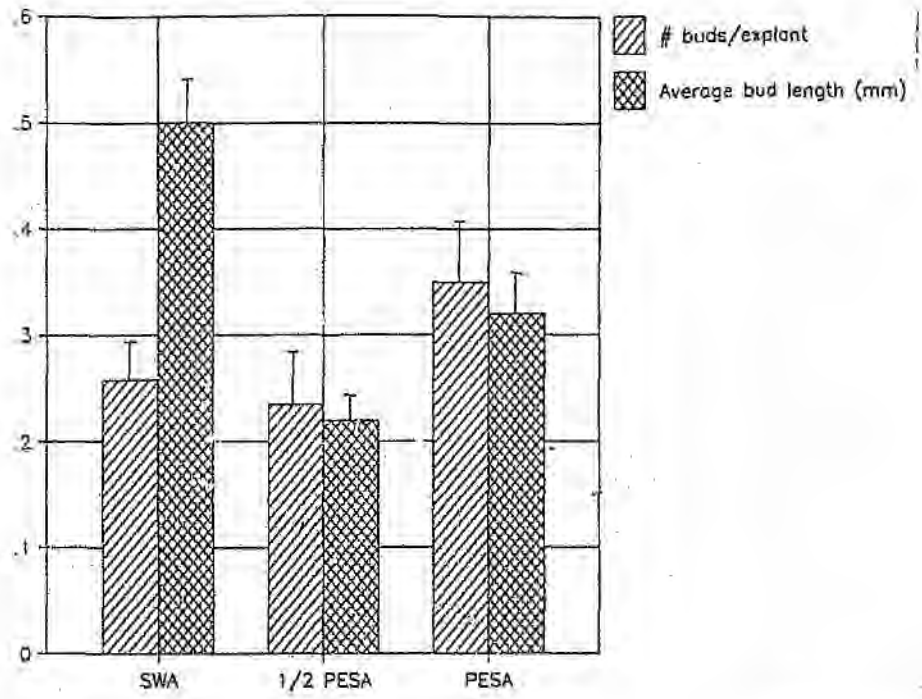


Figure 6. The average number of buds (±SE) formed per axis on SWA, ½PESA and PESA compared to the average length of the buds (±SE).

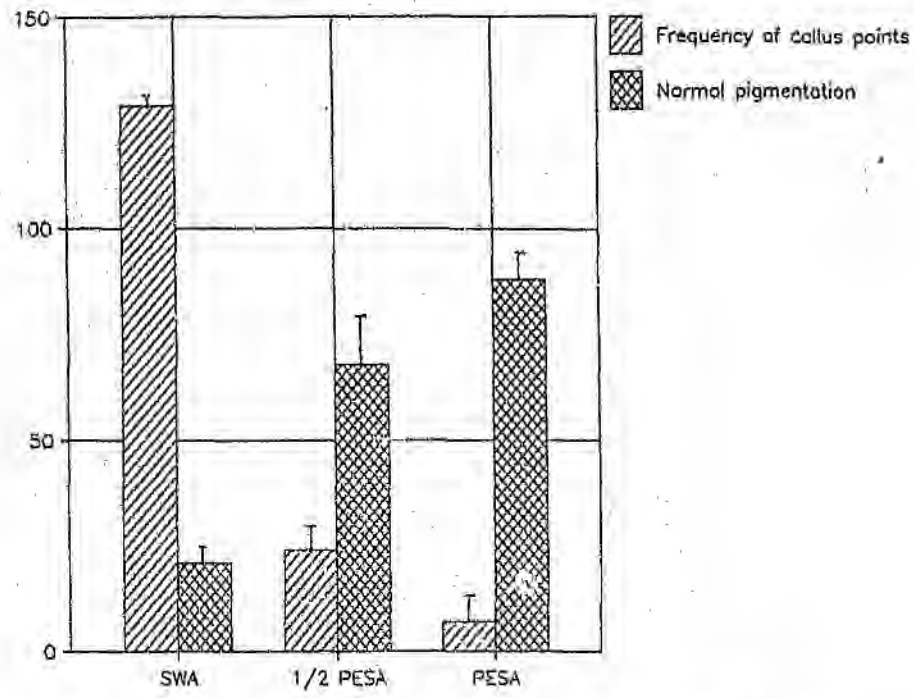


Figure 7. Frequency of points of callus production per healthy axis (±SE) on SWA, ½PESA and PESA compared to the numbers of normally pigmented tissue pieces on each plate (±SE).

It can be seen that the amount of callus produced on the SWA plates was at least three-fold the amount noted on the ½PESA plates and almost 20 x the amount on the PESA plates. The callus tissue was only produced on healthy tissue in each case. The callus tissue was non-friable, but dissociated easily when pressure was applied with a coverslip (Plate 1 Fig. 4). The volume of callus per callus point was noted to be visibly greater on the SWA axes than the PESA and ½PESA plates (compare Plate 1 Figs. 5, 6 and 7), but it was not possible to measure the volume of the callus produced as the callus had not grown sufficiently in the time period available for this experiment (Compare Plate 1 - Figs. 5 and 6 (month-old calli) to Plate 2 - Fig. 2 (three-months-old callus)

However, it was clear that SWA was more efficient at producing new bud and callus growth. The callus was mainly produced at the apices of aerial lateral shoots and sometimes on the cut ends of the axes (Plate 1 - Figs. 5, 6 and 7; Plate 2 - Fig 1). No callus was observed on tissue buried in the agar.

ii) Addition of an organic carbon source

Robaina et al. (1990) observed that the red alga Grateloupia doryphora (Montagne) Howe grew 400 % better in liquid media supplemented with glycerol than in media without glycerol. To test these observations with Gracilaria, a series of plates were poured (Section 3.2). In this study only solid media were tested. The addition of glycerol raised the osmolality of the medium so this was compensated for by dilution of seawater with

distilled water (70PES = PES made with 70% seawater and 30% distilled water). The appropriate controls (100PES and 70PES without glycerol solified with 0.3%/0.75% agar) were included. Eight combinations of media were tested. The mortality rates (with death determined by complete loss of pigmentation) of the tissue disks on these different media are depicted in Figure 8.

No antibiotics were included in the media as it was thought that their action might mask the reponse of the Gracilaria tissue to the glycerol. Previous experiments in this study were successful in indicating varied tissue response to SWA, ½PESA and PESA despite bacterial contamination, but the addition of glycerol promoted confluent bacterial growth on the plates and all the tissue disks exhibited a greatly lowered mortality on these plates when compared to the control plates without glycerol.

To test the response of Gracilaria tissue to glycerol under conditions of controlled bacterial growth, tissue explants were transferred to plates supplemented with 200 u/ml polymyxin B and 30 µg/ml erythromycin as well as 0.3 M glycerol.

The tissue explants were obtained from the SWA plates used in the previous experiment and each explant exhibited callus production and new lateral bud formation. Half of the tissue explants were treated with Betadine prior to placement on the antibiotic-supplemented plates and the other half of the tissue explants were transferred without Betadine treatment.

The callus tissue was incubated at 22°C in a L:D cycle of 16:8 at 70 $\mu E.m^{-2}.s^{-1}$. irradiance. After three weeks, stereo microscopic examination of the callus and bud pieces showed no contamination of the tissue. Not all of the tissue was still alive, but the pieces that were alive all showed normal pigmentation. The tissue pieces on 70 PES, 0.75% agar appeared to be performing the best. No further callus growth or differentiation of the callus was observed after three weeks and the study was terminated due to time constraints.

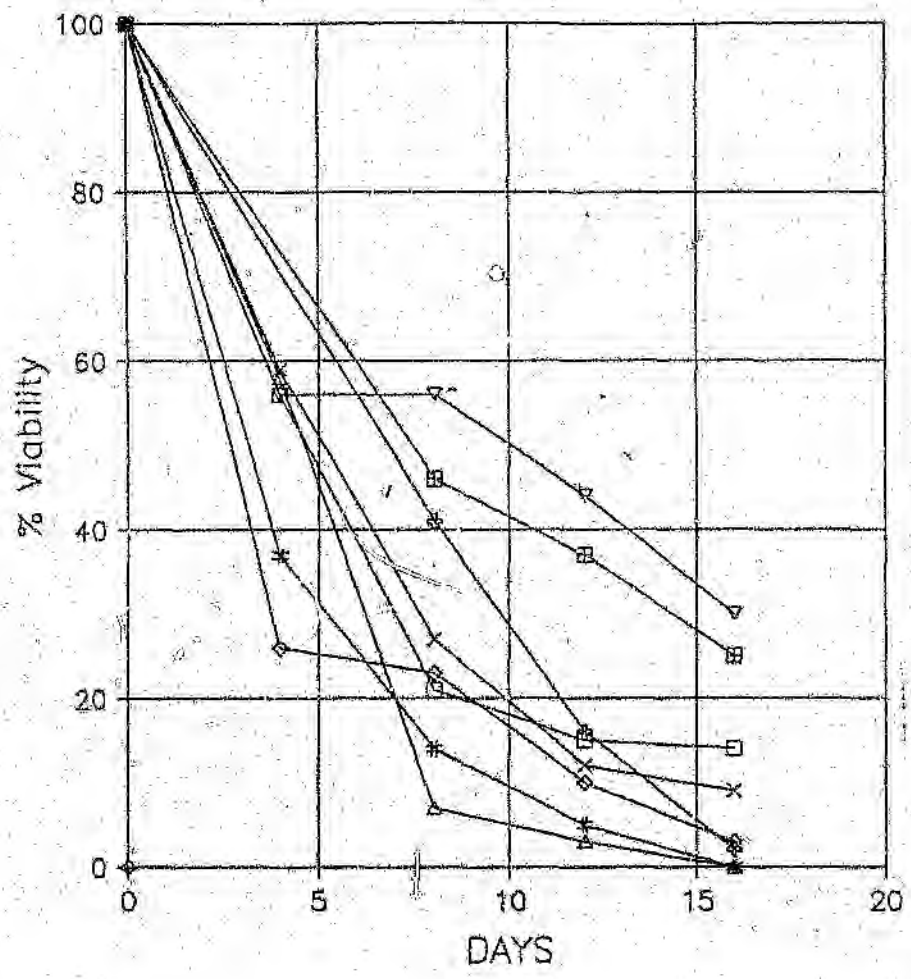


Figure 8. Mortality rates of tissue plated on various combinations of 100/70 PES (P), 0.75%/0.3% agar (A) and ± 0.3 M glycerol (G).

b) Osmolality and solidity of media

Robaina et al. (1990) noted that osmolality and solidity of the media affected bud and callus formation in the red algae Grateloupia doryphora, Gelidium versicolor (S. G. Gmelin) Lamouroux and Laurencia sp.. This was not tested for Gracilaria in this study, but the previous experiment included two different osmolalities (100PES and 70PES) and two different agar concentrations among the control plates without glycerol (See Fig. 8). Analysis of the mortality rates exhibited by the tissues disks on these plates showed a significant difference in the mortality rates of tissue on 100 PES plates containing 0.75% and 0.3% agar ($p=0.99$), and on 70 PES plates containing 0.75% and 0.3% agar ($p=0.975$). Tissue disks on plates with 0.3% agar exhibited higher viability than those on 0.75% agar plates.

No significant difference ($p=0.975$) between the mortality rates of Gracilaria tissue was noticed on the plates containing 100 PES and 70 PES.

4.3 PRODUCTION OF HYDROLYTIC ENZYMES

Macroscopic observation of SWA, ½PESA and PESA plates after four weeks suggested that a bacterium present on the plate might secrete hydrolytic enzymes specific for agar. Agar pits, or depressions in the agar, formed on SWA, ½PESA and PESA plates (See Plate 2 - Figs. 5 and 6). Not all of the pits were formed around the tissue which suggested that pit formation was not due to the hydrolytic enzymes produced by the Gracilaria tissue. The formation of the pits on the plates where the wax-coloured

bacterium was predominant (ie SWA plates) suggested that this bacterium might be secreting the enzyme(s) into the medium. A sample of the bacteria in the pit was taken and spread onto PESA plates to separate out single bacteria and note whether more than one sort of bacterium was present. Only one type of colony was formed, as determined by visual observation. The bacterium was inoculated into SW, ½PES and PES. No growth was observed. A sample of this bacterium was inoculated into PES supplemented with 0.15% agar and incubated. Growth (turbidity) was difficult to assess due to the presence of agar in the medium, but a Gram stain indicated growth of a Gram negative coccus. The supernatant of this culture was then analysed for enzyme activity (Section 3 (e)).

4.4 ISOLATION OF PROTOPLASTS FROM GRACILARIA

Initial enzymatic digestion experiments were performed with the gentle agitation (25 - 50 rpm on an orbital shaker) recommended in many of the published protocols (Saga and Kudo 1989, Le Gall et al. 1990). No protoplast release was observed. Greater agitation in the form of magnetic stirrers was introduced (Chen 1986, Chen 1989) and protoplast release was obtained. Five different enzyme systems were tested for protoplast release (Section 3.4) and viability (Section 3.5 (a)).

a) 'Cheney cocktail' (Cheney et al. 1986)

Protoplast release was monitored every hour by counting protoplasts with a haemocytometer. The number of protoplasts released per gram of fresh weight, as a function of time, is represented in Figure 9.

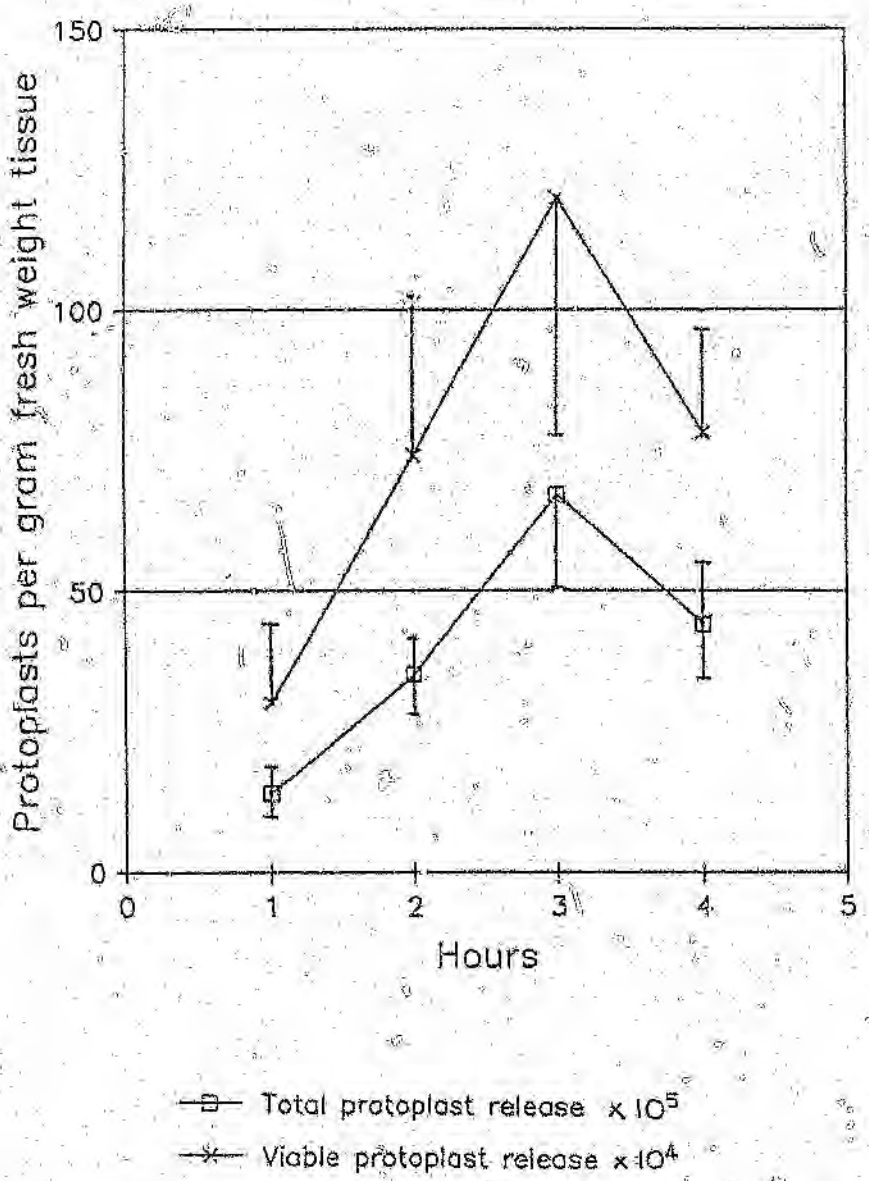


Figure 9. Protoplast release during enzymatic treatment with the 'Cheney cocktail' enzyme mix (±SE).

Previous work by Nelson (1990) indicated that 2 - 3 hours was the optimum time for digestion with the 'Cheney cocktail'. Peak release and viabilty was obtained after three hours in this study. Maximum protoplast release was in the order of 6.7×10^5. protoplasts per gram of fresh weight tissue (p/g fwt). Maximum viability of the released protoplasts was lower and in the order of 1.2×10^4 p/g fwt. Cheney et al. (1986) reported protoplast release from Gracilaria tikvahiae in the region of $3x\ 10^5$ to $1x\ 10^6$ protoplasts per gram of fresh weight tissue after digestion with the 'Cheney cocktail' enzyme mix. This release appears on a par with the release obtained from Gracilaria verrucosa in this study, but it must be borne in mind however, that the number of protoplasts released was estimated prior to ary washing steps in this study and loss of protoplasts in the washing steps must be included to enable comparisons. Thus the release of protoplasts from Gracilaria verrucosa is less than the release obtained by Cheney et al. for G. tikvahiae. Nelson (1990) obtained 2.99×10^4 protoplasts per gram of fresh weight tissue from Gracilaria verrucosa with the 'Cheney cocktail' enzyme mix. This is slightly lower than the release obtained in this study. The enzyme mix used differed slightly from the enzyme mix used by Cheney et al. (1986) in that the agarase used in this study was obtained from Sigma Chemical Co. and not from Calbiochem-Behring Corp. and concentrations of this agarase were calculated from units of activity and not by percentage weight/volume as is the agarase supplied by Calbiochem Behring Corp.

Cheney et al. (1986) found that substitution of Cellulysin for Onozuka R-10 Cellulase in digestion experiments with their

enzyme mix produced lower yields of protoplasts from G. tikvahiae. These authors also found that lower concentrations of the enzymes in their enzyme mix produced substantially lowered yields without improving viability. It is possible that the agarase used in this study was either not as effective at releasing protoplasts from Gracilaria verrucosa as the agarase used by Cheney et al. (1986) for G. tikvahiae, or that the optimal concentration of agarase was not used.

However, the release of viable protoplasts from Gracilaria verrucosa in this study is at least 20-fold lower than the number of viable protoplasts obtained by Cheney et al. (1986) with G. tikvahiae. These authors found that protoplast viability was highly variable and dependent on the strain and age of the donor plant material and on the osmolality of the enzyme mixture. 1.0 M mannitol produced the greatest percentage of viable protoplasts (70%) from G. tikvahiae, but 0.6 M sorbitol was used in this study as this concentration had been previously determined optimal for the maintenance of protoplasts from G. verrucosa (Nelson 1990). Approximately 2% of the protoplasts recovered from G. verrucosa treated with the Cheney cocktail enzyme mix were viable.

Saga and Kudo (1989) found that increased concentrations of cellulase (>5%) were toxic to Monostroma protoplasts and a similar effect might exist with agarase if a non-optimal concentration was used in this study.

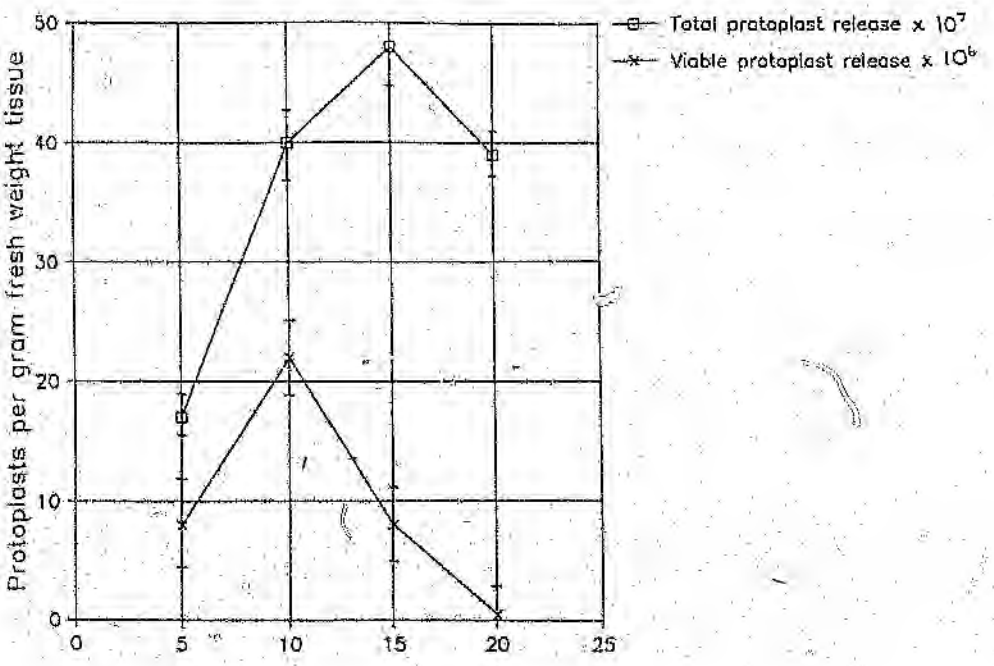
It is also possible that the agitation of the enzyme mixture by the magnetic stirrers instead of rotary shaking (although the

stirring speed setting was the lowest possible) may have caused a decrease in protoplast viability. Chen (1989) utilised magnetic stirrers for agitation of Porphyra enzymatic digestions. The protoplasts released from Porphyra (~35 µm) were larger than the protoplasts released from Gracilaria (~15 µm) and so shear forces should affect Porphyra protoplasts to a greater extent, but Chen (1986) does not report on the percentage viability of the protoplasts released using magnetic stirrer bars.

b) Limpet Acetone Powder digestions

Gracilaria tissue was Betadine-treated and chopped finely. Two sets of experiments were performed with different concentrations of the Limpet Acetone Powder. The generally utilised concentration of Limpet Acetone Powder for digestions is 10% (w/v) (Polne-Fuller and Gibor 1984). However, this experiment needed to be compared to the release obtained from the sea urchin acetone powder and insufficient sea urchin acetone powder was available to perform the number of digestions (with sea urchin acetone powder concentrations of 10%) necessary for the results of the digestions to be statistically significant. A 5% Limpet Acetone Powder digestion was therefore included. Protoplast release was monitored every five hours by haemocytometer counts and the number of protoplasts released per gram of fresh weight tissue was plotted as a function of time. Results are shown in Figure 10.

a)



b)

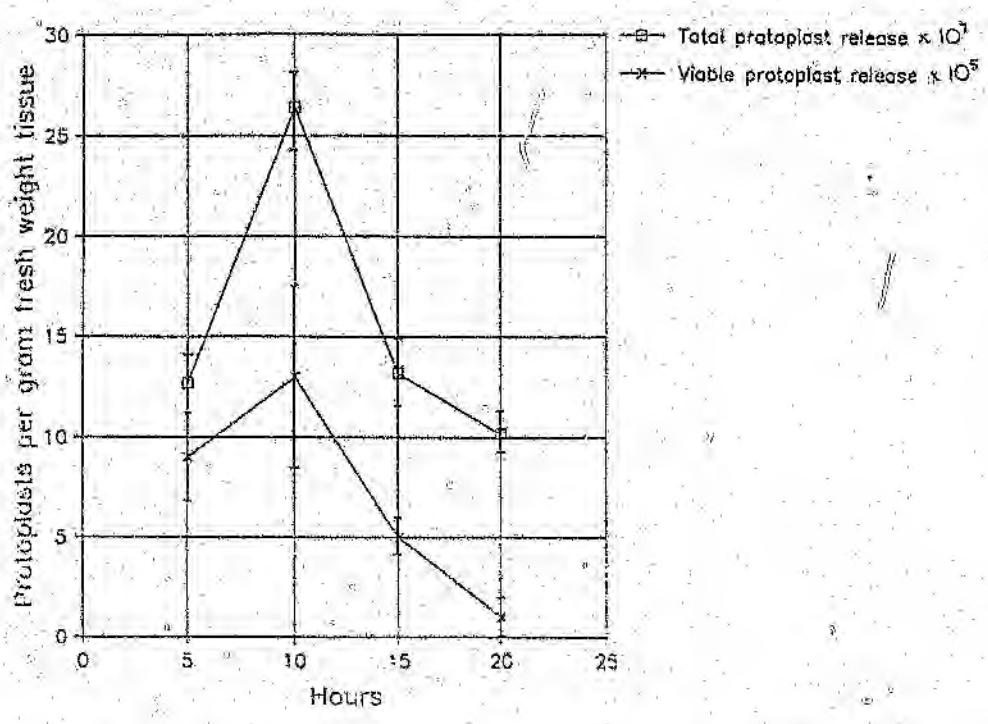


Figure 10. Protoplast release (total and viable) (±SE) from tissue treated with a) 5% Limpet Acetone Powder and b) 10% Limpet Acetone Powder.

The optimum time for digestion was confirmed at 10 hours for both 5% and 10% Limpet Acetone Powder as the optimum viable protoplast production occurred at 10 hours. Optimum total protoplast production occurred after 15 hours of digestion for 10% LAP and after 10 hours of digestion for 5% LAP. A decreased production (~50%) of protoplasts was observed for 5% as compared to 10% LAP, but maximum protoplast release was in the region of 4.8×10^7 p/g fwt for 10% LAP and 2.6×10^7 p/g fwt for 5% LAP. Again, it must be borne in mind that the number of protoplasts released was estimated prior to any washing steps in this study and loss of protoplasts in the washing steps must be included to enable comparisons. However, this compares favourably with the release of 3.55×10^4 p/g fwt from Gracilaria verrucosa reported by Nelson (1990). The number of protoplasts released from Gracilaria tissue after digestion with the Cheney enzyme cocktail in this study is ~ 14% of the number obtained from digestion with 10% LAP. However, Nelson (1990) found that the release of protoplasts from Gracilaria verrucosa by digestion with 10 % LAP and the 'Cheney cocktail' was similar and Cheney et al. (1986) found that treatment of G. tikvahiae with 10% LAP resulted in yields that were considerably lower than those obtained with the Cheney cocktail. Cheney et al. (1986) ascribed this difference in efficiency to the fact that the Limpet Acetone Powder is effectively utilised for protoplast release from Porphyra whose cell wall composition is sufficiently different to that of Gracilaria to warrant a different enzyme mix for optimal protoplast release. It is also possible that the cell wall composition of Gracilaria verrucosa is sufficiently different to that of G. tikvahiae such that higher protoplast release is obtained with the enzymes present in LAP

than with those present in the 'Cheney cocktail'.

Although a high release of protoplasts was obtained from Gracilaria verrucosa with 10% LAP in this study, the highest percentage of viable protoplasts obtained with the 10% LAP digestion was 4.7%, and 0.5% for the 5% LAP digestion. Maximum viable protoplast release is still 18-fold higher than the maximum viable protoplast release obtained with the Cheney cocktail in this study.

c) Sea urchin acetone powder digestions

Protoplast release was monitored by haemocytometer counts of subsamples taken every five hours. The data are depicted in Figure 11 below. Since two genera of sea urchins were compared, the numbers of viable protoplasts released for the two treatments were plotted on one graph (Fig. 11 (a)) and the total protoplast release for the two treatments on another graph (Fig. 11 (b)) instead of release and viability for each treatment on the same graph as appears elsewhere.

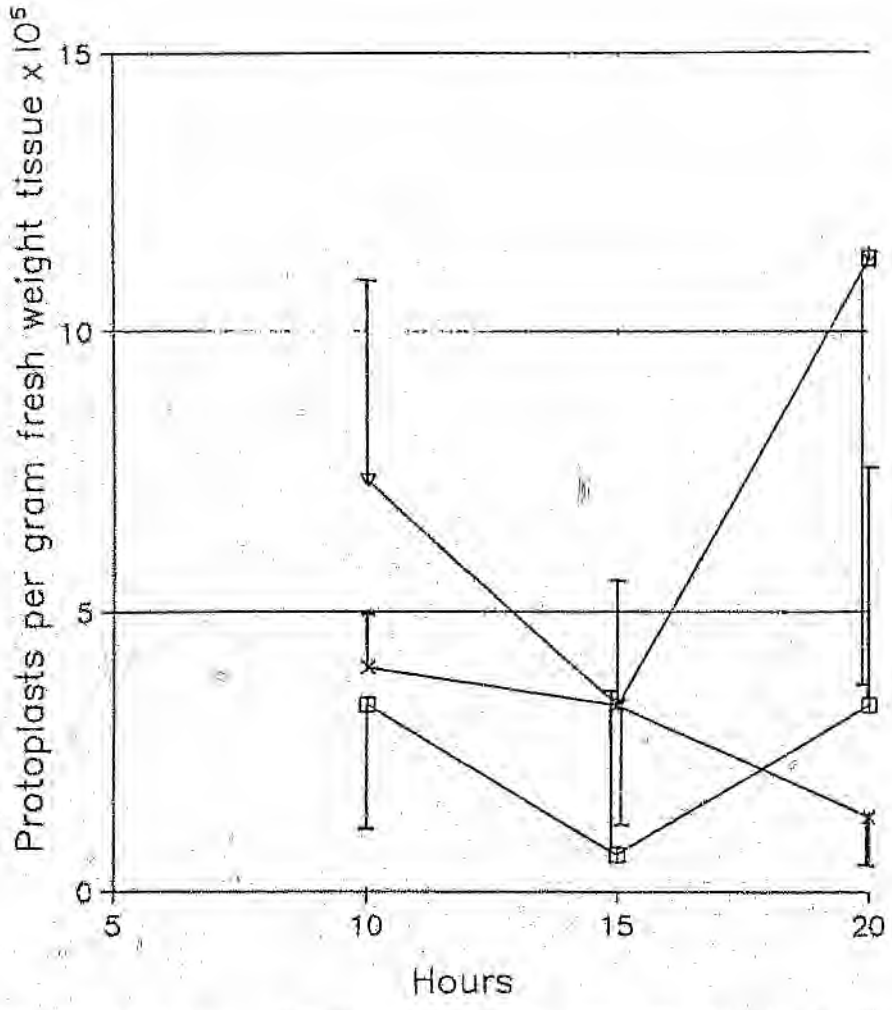


Figure 11 (a). Viable protoplast release (±SE) from 5% sea urchin acetone powder prepared from (—□—) starved Stomponeustes, (—×—) fed Stomponeustes and (—○—) starved Pinocladus.

A significant decrease ($p=0.975$) in protoplast production occurred after 10 hours of digestion with the sea urchin acetone powder prepared from starved Stomponeustes while no significant decrease ($p=0.975$) was observed in the protoplast release from digestions of Gracilaria with starved Pinocladus or fed Stomponeustes acetone powder.

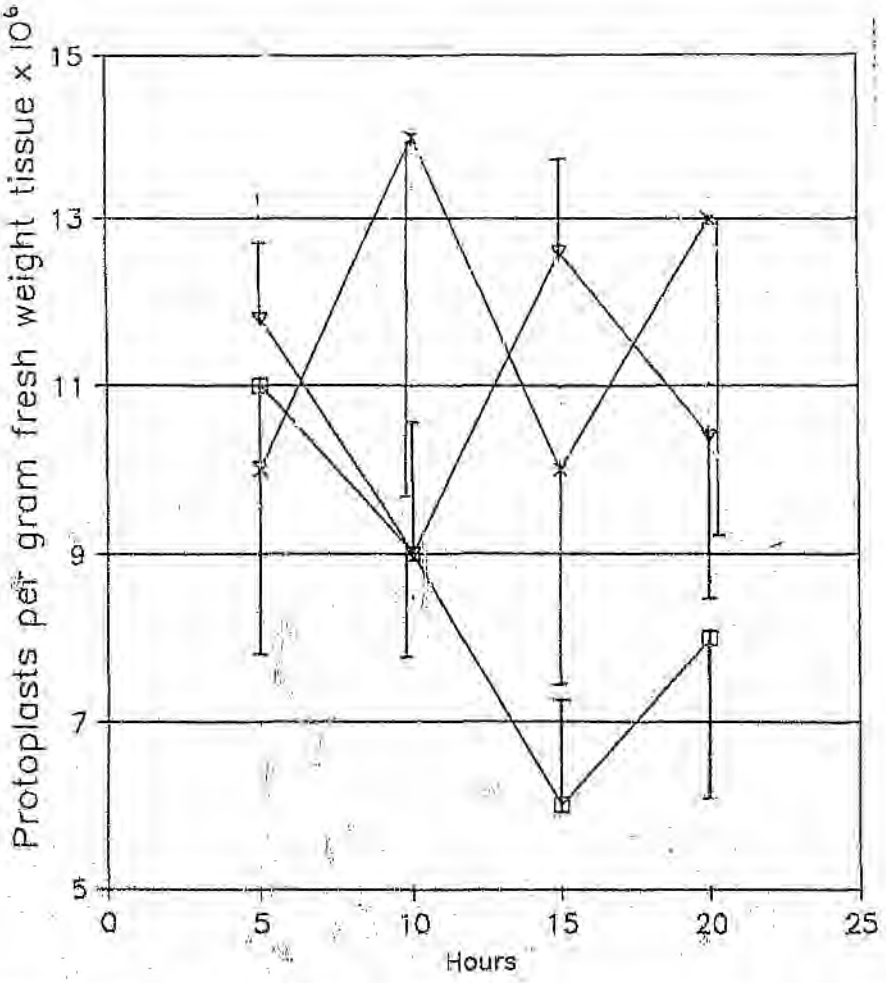


Figure 11 (b). Total protoplast release (±SE) from 5% sea urchin acetone powder prepared from (—▫—) starved Stomponeustes, (—×—) fed Stomponeustes and (—▽—) starved Pinocladus.

Protoplast release from starved and fed Stomponeustes was not found to be significantly different (p=0.975). Protoplast release from digestion with starved Pinocladus and starved Stomponeustes was found not to be significantly different (p=0.975) except for protoplast release obtained at 15 hours which was significantly lower (p=0.995) for the starved Stomponeustes digestion than for the starved Pinocladus digestion. There was no significant difference in the protoplast release from digestions with starved Pinocladus or

fed Stomponeustes (p=0.975).

No significant difference (p=0.975) in protoplast viability was noted between the three different digestions or at any of the sample times in each digestion.

Protoplast release was in the order of 1 x 10^7 p/g fwt for all digestions with protoplast viability in the order of 3.8 x 10^5 p/g fwt. Protoplast release for 5 % sea urchin acetone powder was 38 % lower than the release obtained with 5% Limpet Acetone Powder.

d) CRUDE SEA URCHIN GUT EXTRACT

The crude sea urchin gut was harvested from Stomponeustes in two sections. The coelomic fluid was withdrawn and then the fluid released by the rupture of the digestive tract (Section 3.4 (a)). Both fractions were tested for enzyme activity by monitoring protoplast release every 12 hours. Protoplast release was also monitored from crude extracts supplemented with cellulase and macerozyme. Figure 12 overleaf represents the protoplasts released as a function of time.

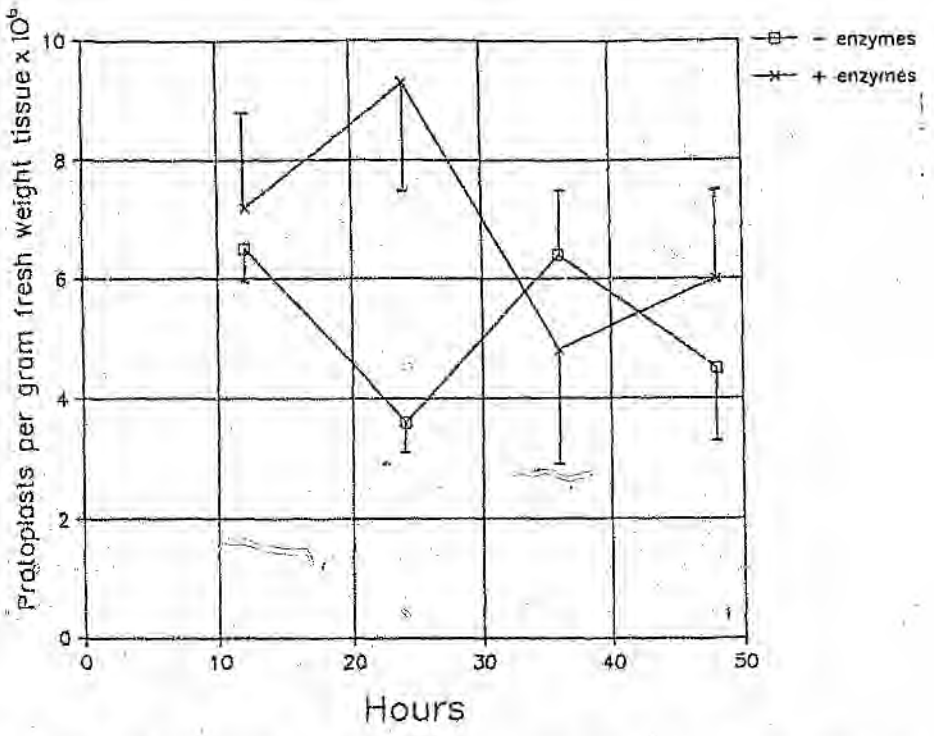


Figure 12 (a). Protoplast release (±SE) from the crude enzyme extract alone and supplemented with cellulase and macerozyme from the coelomic extract.

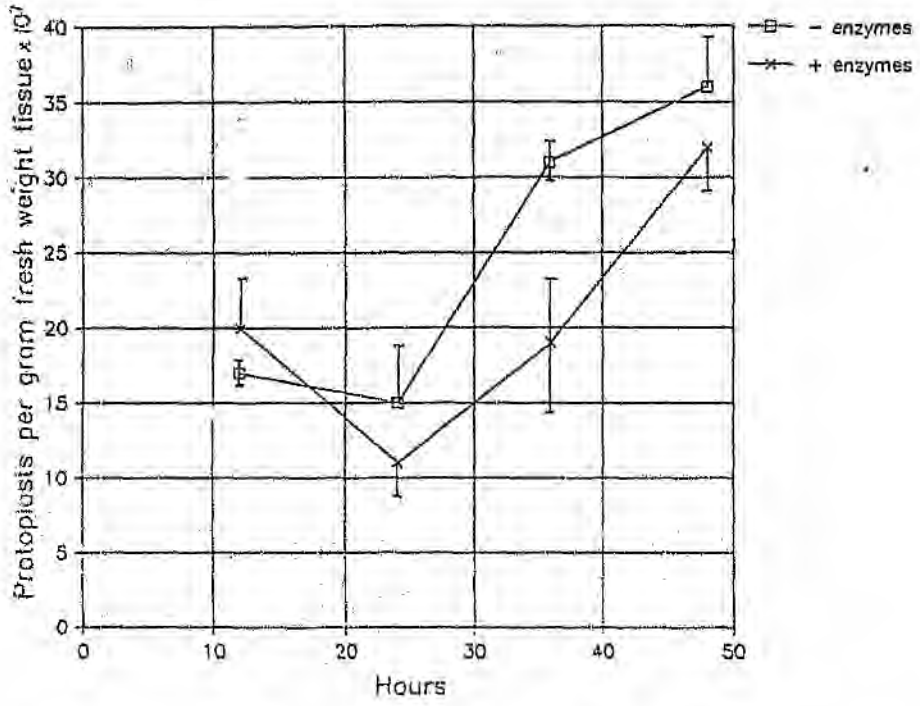


Figure 12 (b). Protoplast release (±SE) from the crude enzyme extract alone and supplemented with cellulase and macerozyme from the digestive tract extract.

The coelomic extract was found to have some enzymatic action as was evidenced by the release of protoplasts in the region of 9 x 10^6 p/g fwt. No significant difference (p=0.975) in protoplast release was noted between 12 and 24 hours, or between 36 and 48 hours, but the protoplast release decreased significantly (p=0.995) after 24 hours of incubation in the coelomic extract supplemented with cellulase and macerozyme. Two significant peaks (p=0.995) in protoplast release were noted at 12 and 36 hours in the digestion of Gracilaria tissue with the coelomic extract not supplemented with additional enzymes.

A slightly higher maximum release of protoplasts (8.25 x 10^6 p/g fwt) was obtained with the coelomic extract that was supplemented with cellulase and macerozyme than was obtained with the crude extract alone (6.4 x 10^6 p/g fwt).

Maximum protoplast release for digestion of Gracilaria tissue with the crude extract from the sea urchin digestive tract occurred at 48 hours for both the crude extract supplemented with macerozyme and cellulase and the unsupplemented crude extract. No significant difference (p=0.975) in protoplast release was observed between the extract supplemented with enzymes and the extract without additional enzymes until 36 hours of digestion, when the extract without additional enzymes showed a significantly higher (p=0.975) protoplast release than the extract supplemented with enzymes. However, no significant difference (p=0.975) between the two treatments was observed at 48 hours.

A significantly higher (p=0.9995) release of protoplasts (~3.4 x 10^7 p/g fwt) was obtained with the digestive tract extract as compared to the coelomic fluid (6.4 - 8.25 x 10^6 p/g fwt). It is interesting to note that the release of protoplasts from the crude sea urchin extract is very much higher than the release of protoplasts from the acetone purified gut extract. The same pool of sea urchins was used for both enzyme digestions, that is the gut fluid was harvested from certain sea urchins and pooled (to obtain an average enzyme composition), following which, the digestive tracts from the same sea urchins were removed, pooled, and treated with acetone (Section 3.4 (b)). When the acetone powder was reconstituted (Section 3.4 c (i)) the volume of the acetone powder mix was coincidentally the same as the volume of the corresponding crude extract obtained from the coelom. The volume of digestive tract fluid obtained from the sea urchins was approximately one-quarter of the coelomic fluid volume. It is therefore possible to compare, to some degree, the relative enzyme concentrations of the extracts by comparing the release of protoplasts obtained in these treatments.

Protoplast release from the digestive tract crude extract digestion was ~ 3.4-fold higher than the protoplast release obtained from the sea urchin acetone powder digestion (3.42 x 10^7 cf. 1 x 10^7 p/g fwt). If the volumes of the two enzyme concentrations are compared, the release of protoplasts with the digestive tract crude extract needs to be reduced by a factor of four, which brings the release of protoplasts from this digestion on a par with that of the coelomic extract (8.5 x 10^6 cf. 8.25 x 10^6 p/g fwt) and slightly less than the digestion with the acetone preparation of the digestive tract extract (8.5

$\times 10^6$ cf. 1×10^7 p/g fwt).

The release of viable protoplasts from both the acetone powder digestion and the crude gut extract digestion was approximately 3 % of the total protoplast release.

e) Bacterial crude enzyme extract

A sample of the bacteria from one of the agar pits present on the SWA plates was transferred to PES medium supplemented with 0.15% agar and grown for one week (Section 3.4 c (iv)). The supernatant was tested for enzyme activity by monitoring protoplast release by haemocytometer counts. The supernatant was also supplemented with cellulase and macerozyme and tested for protoplast release. Protoplast release for the two fractions was monitored every 12 hours and the results are shown in Figure 13.

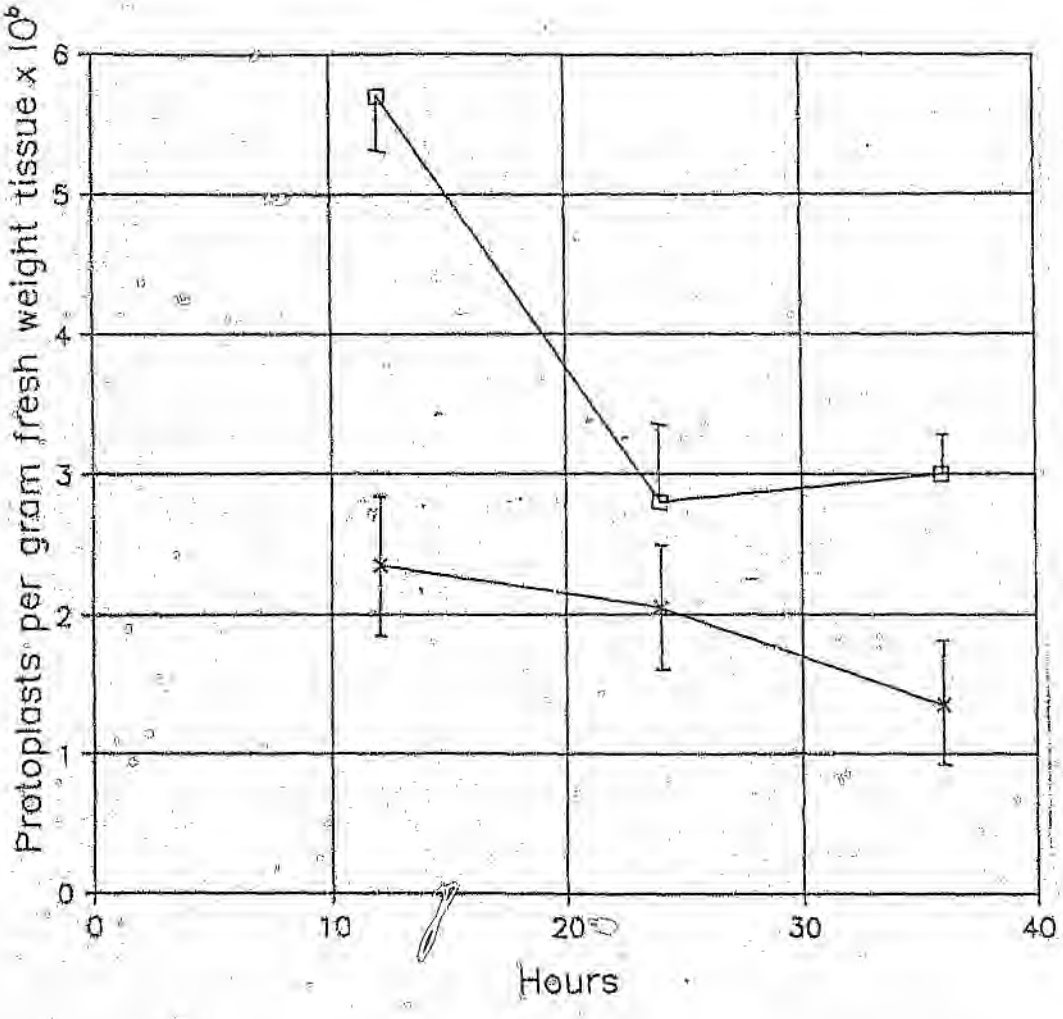


Figure 13. Protoplast release (±SE) from Gracilaria tissue treated with a crude bacterial enzyme extract (-×-) supplemented with cellulase and macerozyme and (-□-) without additional enzymes.

Maximum protoplast release occurred after 12 hours of digestion with the unsupplemented crude extract with a significant decrease ($p=0.975$) in the release of protoplasts occurring after 24 to 36 hours of digestion. No significant difference ($p=0.975$) in the release of protoplasts by the supplemented crude extract was observed although a decrease in release was significant with $p=0.95$.

The protoplast release obtained by digestion of Gracilaria tissue with crude extract (unsupplemented by macerozyme and cellulase) was significantly higher ($p=0.995$) at 12 and 36 hours than the release obtained by the extract supplemented with enzymes.

Maximum protoplast release was in the region of 5.7×10^6 p/g fwt (unsupplemented crude extract after 12 hours), which is six-fold lower than the protoplast release obtained with the crude sea urchin digestive tract extract (3.42×10^7 p/g fwt). The percentage of viable protoplasts obtained from the bacterial crude extract digestions was in the region of 9% (average percentage as the protoplast viability for the two treatments was not significantly different ($p=0.975$)).

The maximum protoplast release obtained by digestion of Gracilaria verrucosa tissue with the crude bacterial extract is 30 % less than the maximum protoplast release obtained with the crude sea urchin coelomic extract and six-fold lower than the maximum protoplast release obtained with the digestive tract crude extract. However, the viability of the protoplasts obtained with the crude bacterial extract was higher than that obtained with the sea urchin crude extracts (9% cf 3%).

5 SUMMARY AND CONCLUSIONS

a) Isolation of axenic tissue

Gracilaria tissue was exposed to various concentrations of two antibiotics, polymyxin B and erythromycin, that had been found to generate axenic tissue pieces from Gracilaria lemaneiformis (Bradley et al. 1988).

The tissue pieces were observed to be unaffected by concentrations of polymyxin B up to 400 u/ml, and concentrations of erythromycin below 60 µg/ml. Low concentrations of these two antibiotics (200 u/ml polymyxin B and 20 µg/ml erythromycin) were noted to control bacterial growth to the extent that only isolated colonies of bacterial contaminants appeared on plates treated with the antibiotics.

Tissue explants transferred to plates containing suitable concentrations of these antibiotics (200 u/ml polymyxin B and 30 µg/ml erythromycin) appeared free of microbial contamination (microscopic examination after a period of time).

In this study, polymyxin B and erythromycin were found to be suitable antibiotics for obtaining axenic tissue from Gracilaria verrucosa.

b) Optimisation of media

The response of Gracilaria verrucosa tissue explants to three media (SWA, ½PESA and PESA) was quantified in terms of explant viability, callus production and new lateral bud formation.

Explants exhibited the greatest viability on PESA media and least viability on SWA media, but SWA stimulated callus and new lateral bud formation to a greater extent than ½PESA or PESA.

Explant viability was noted to increase with decreasing agar concentrations (explants on 0.3% agar performed better than those on 0.75% agar). A 30% difference in osmolality (70% seawater used compared to the 100% seawater usually utilised for media) did not affect Gracilaria explant viability.

The addition of an organic carbon source to media was attempted, but contamination of the experiment obscured any trends that may have been observed. A subsequent experiment with decreased contamination levels was terminated prematurely due to time constraints. However, the response of Gracilaria to the presence of glycerol in the growth medium should be investigated further.

c) Protoplast isolation

Protoplast isolation from Gracilaria verrucosa was attempted with seven different enzyme sources. Isolation with the 'Cheney cocktail' was found to be the least efficient, while protoplast isolation with 10% Limpet Acetone Powder was the most efficient. The following table lists the enzyme treatments in the order of decreasing efficiency in the release of protoplasts from G. verrucosa:

Table 1. The maximum yield of protoplasts from Gracilaria verrucosa after digestion with seven enzyme cocktails.

Enzyme treatment	Protoplasts released per gram fresh weight tissue
10% Limpet Acetone Powder	4.8×10^7
Crude sea urchin extract (digestive tract)	3.42×10^7
5% Limpet Acetone Powder	2.6×10^7
Sea Urchin Acetone Powder	1×10^7
Crude sea urchin extract (coelomic cavity)	8.25×10^6
Crude bacterial extract	5.7×10^6
Cheney cocktail	6.7×10^5

Although the most efficient release was obtained with Limpet Acetone Powder, only slightly decreased yields can be obtained with the crude gut extract and also with the acetone purified gut extract of a local species of sea urchin. Digestion of Gracilaria verrucosa tissue with enzymes obtained from the gut extracts of either Stomponeustes variolis or Pinocladus is a viable proposition. To standardize the enzyme content of the gut extracts to obtain reproducible protocols, the sea urchins should be fed for at least a month on Gracilaria verrucosa.

The production of hydrolytic enzymes from the bacteria isolated from the natural flora of Gracilaria verrucosa should be investigated further. The doubling time of a bacterium (time taken for a given population to double in number) is measured in hours and is many times faster than the doubling time of a population of sea urchins. It should be borne in mind that a large number of sea urchins (~ 10) needed to be culled to produce 0.5 g of acetone powder. Bacterial cultures are low on maintenance (seed cultures can be stored at 4°C until needed) and can produce unlimited amounts of enzyme. A homogeneous population of bacteria can be obtained by the selection and propagation of one bacterial colony. This would favour consistent enzyme production (under controlled growth conditions) which in turn would lead to consistent digestion yields.

The bacterium isolated in this study has not been identified, and identification of a bacterium is expensive and time consuming. Protocols for growth and enzyme production need to be optimised. However, once this has been accomplished, a consistent and ready supply of enzymes at low cost will have

been obtained.

Alternatively, another bacterium known to produce hydrolytic enzymes specific for agar can be isolated from seawater samples. In particular, Bacillus cereus occurs naturally in seawater and many protocols for the specific isolation of this bacterium exist. Growth media have also been optimised for this bacterium.

FUTURE WORK

a) Enzyme sources

Microorganisms such as bacteria and fungi are increasingly being used as enzyme sources for the digestion of algal cell walls (Polne-Fuller and Gibor 1987, Butler et al, 1989, Boyen et al. 1990) due to the ease with which these organisms are propagated and maintained, and the unlimited and consistent production of hydrolytic enzymes obtained from cultures of these microorganisms. In this study, a bacterium isolated from the natural flora of Gracilaria verrucosa was found to digest G. verrucosa tissue. Bacillus cereus is also known to produce hydrolytic enzymes and is a common contaminant of Gracilaria tissue (Armisen and Galatas 1987). Agarase is commercially prepared from Pseudomonas atlantica (Sigma Chemical Co, 1986).

Polne-Fuller (1987) mentions that some tropical Sargassum species were found to carry systemic infections of fungi. The presence of an endophytic fungus in some samples of

Gracilaria verrucosa suggests the possibility that this fungus may possess hydrolytic enzymes that can degrade G. verrucosa cell walls. No investigation into this fungus was made in this study, but future research on enzyme production as well as the nature of the symbiosis should be directed in this area.

Research in the area of enzyme production from microorganisms for the release of protoplasts from Gracilaria verrucosa seems promising.

ii) Callus Potential

To the best of this author's knowledge, this study represents the first report of callus production in Gracilaria verrucosa. Callus tissue is actively dividing, undifferentiated tissue that can be readily dissociated into single cells and utilised for regeneration studies, as well as a source of cells for protoplast production. Protoplasts released from callus tissue should be less stressed than protoplasts from normal somatic tissue (shorter digestion time -Cheney et al. (1986), less agitation necessary) which in turn would result in higher viability as shorter digestion times have been correlated with increased viability (Saga 1984). Research into the production of protoplasts from callus tissue of Gracilaria verrucosa for protoplast fusion studies, and research into the regeneration of G. verrucosa plants from single cells could assist in the regeneration of putative hybrid plants obtained from protoplast fusion.

6 APPENDIX

1. Nutrient media

Provasoli Enriched Seawater (stock solution)

100 ml distilled water

350 mg $NaNO_3$

50 mg Na-glyceroPO_4

2.5 mg Fe (as EDTA; 1:1 mol)

25 ml PII metals (see below)

10 ug vitamin B_{12}

0.5 mg thiamine

5 ug biotin

500 mg Tris

pH 7.8

Add 2 ml of this stock solution to 100 ml autoclaved seawater.

PII metals

100 ml distilled water

0.1 g Na_2-EDTA

1 g Fe (as Cl^-)

20 g B (as H_3BO_3)

4 g Mn (as Cl^-)

0.5 g Zn (as Cl^-)

0.1 g Co (as Cl^-)

Add 25 ml PII metals to 100 ml stock solution of PES.

2. Antibiotic media

Cheney cocktail antibiotic medium

100 ml filtered seawater

330 mg streptomycin sulphate

167 mg penicillin G

33 mg neomycin

17 mg mycostatin

167 mg kanamycin

Filter sterilise

PLATE 1

Figure 1. Endophytic fungal contamination on Gracilaria verrucosa tissue disks. Bar = 1 mm

Figure 2. Aerial hyphae of fungus (arrowed) from Gracilaria tissue that appears free of surface fungal contamination. Bar = 0.2 mm

Figure 3. Cross section through Gracilaria axis showing large inner cortical cells and smaller outer cortical cells. Bar = 1 mm

Figure 4. Squash of Gracilaria bud and callus (arrowed). Bar = 0.2 mm

Figure 5. Callus production (one month old) on aerial lateral bud of Gracilaria axis plated on ½PESA medium. Bar = 0.2 mm

Figure 6. Callus production (one month old) on aerial lateral bud of Gracilaria axis plated on PESA medium. Bar = 0.2 mm

Figure 7. Callus production (one month old) on aerial lateral bud of Gracilaria axis plated on SWA medium. Bar = 0.2 mm

Plate 1

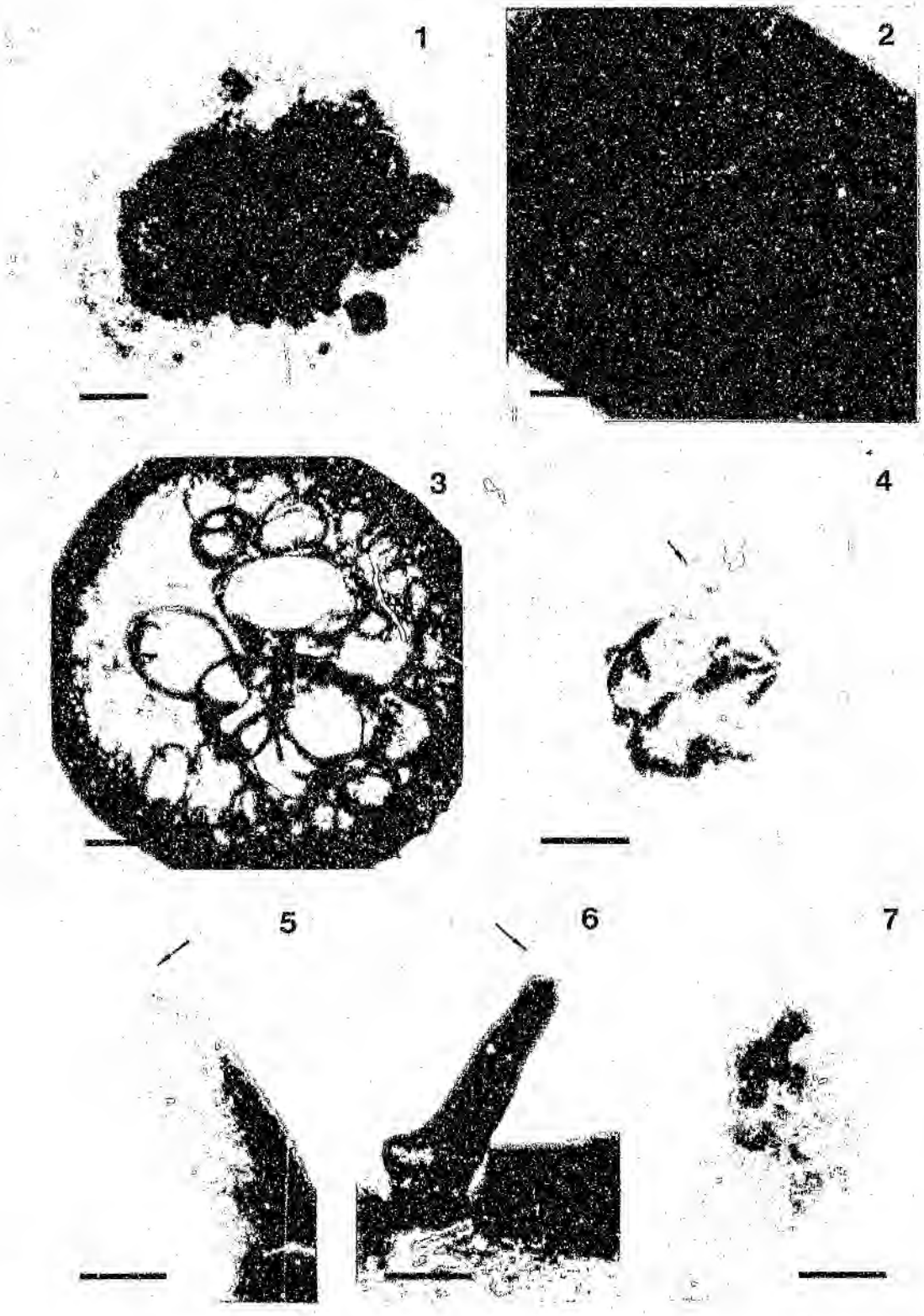


PLATE 2

Figure 1. Callus production and lateral bud formation at the wound site of a Gracilaria axis plated on SWA medium. Bar = 0.5 mm

Figure 2. Callus production (three months old) at base of aerial shoot from a Gracilaria disk plated on PESA. Bar = 0.2 mm

Figure 3. Lateral bud formation at the wound site of a Gracilaria axis plated on SWA.

Bar = 0.2 mm

Figure 4. Lateral bud formation at the wound site of a Gracilaria axis plated on SWA.

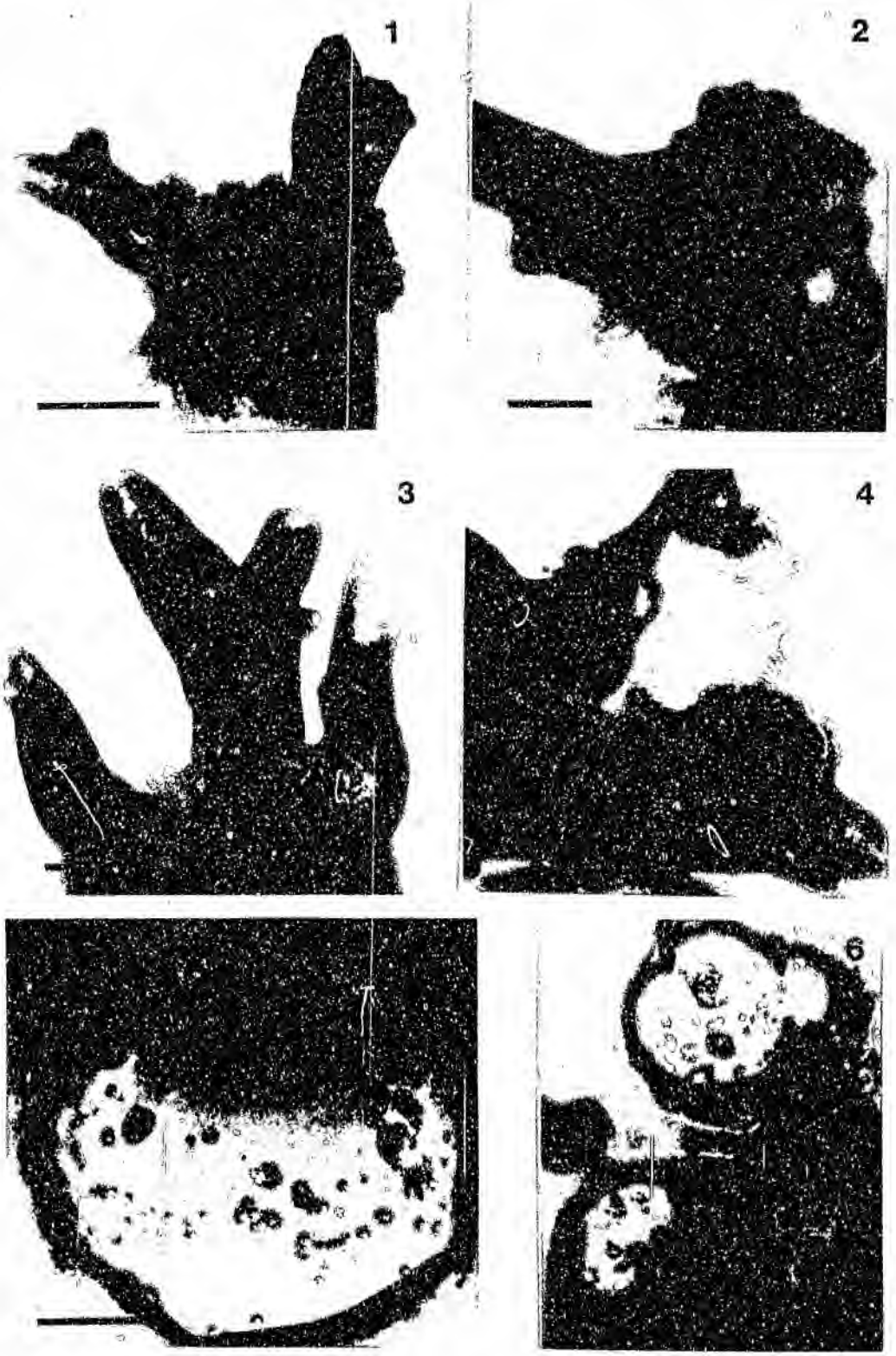
Bar = 1 mm

Figure 5. Agar 'pit' in PESA medium. The plastic bottom of the Petri dish is exposed (arrow).

Bar = 1 mm

Figure 6. Agar 'pits' in PESA medium. The pits appear to form independently of the presence of Gracilaria tissue. Bar = 2 mm

Plate 2



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