CHAPTER 4

Results

Chapter 4: Results

4.1 Sterilisation of Explants

The establishment of a culture for experimental purposes often requires axenic material, which is normally obtained through sterilisation of explants using physical and/or chemical treatment. Once the material is axenic, the induction of callus is based on optimising culture conditions (Mc Cracken, 1989). Six different sterilisation treatments described in Section 3.3 were tested for their suitability in generating axenic explants for tissue culture and protoplast isolation. Thalli of *Gracilaria changii* were cleaned with steriled seawater before being subjected to the different treatments. Sterility of treated thalli was assessed by streaking them on Marine Agar 2216E (Difco). The results obtained are summarized in Table 9.

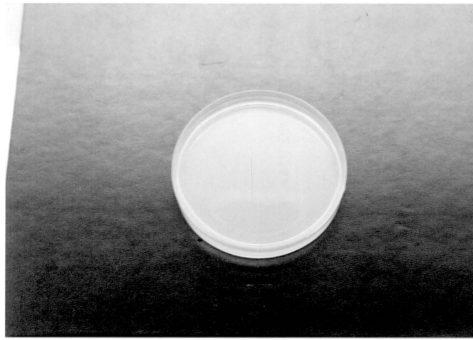
Table 9. Sterilization of *G. changii* explants under different treatments.

Treatment No.	Observation
1	Bacterial growth was noticed on Marine Agar surface. Thalli were bleached. Loss of dark pigmentation. No axenic explants were generated.
2	Bacterial growth was noticed on Marine Agar surface. Thalli were bleached. Loss of dark pigmentation. No axenic explants were generated.
3	No bacterial growth was observed on Marine Agar but thalli were severely bleached.
4	No bacterial growth was noticed on Marine Agar but thalli were severely bleached.
5	Bacterial growth was noticed on Marine Agar surface. Thalli remained healthy. No axenic explants were generated.
6	Thalli remained healthy and free of microbial contamination. Healthy and axenic thalli were generated.

Treatment 6 was found to be the most effective method for producing axenic explants. No bleaching agents such as hypochlorite or alcohol were used in the treatment. This treatment sterilised explants physically and chemically. The physical component consist of sonication while the chemicals used were 1% povidone iodine (PVP-iodine) in seawater (30 ppt) and a battery of antibiotics as described in Section 3.3. Healthy and axenic explants were produced from this treatment. No bacterial growth was observed on the surface of marine agar streaked with the treated thalli after three days as illustrated in Plate 5a.

Hypochlorite and alcohol were unsuitable for treating thalli of *G. changii* as they resulted in bleaching of the thalli. Treatments 1 and 2 employing only potassium iodide (KI) and alcohol were unable to produce axenic explants and resulted in either bleaching and/or loss of pigmentation. Treatment 5 employing KI and penicillin G was also unsuccessful in generating axenic explants. The thalli generated from this treatment remained non-axenic as bacterial growth were observed on Marine Agar after three days as shown in Figure 5b. No contamination were observed for Treatments 3 and 4 where both alcohol and hypochlorite were used, but severe bleaching on the thalli occurred rendering them useless for use in further experiments.

a.



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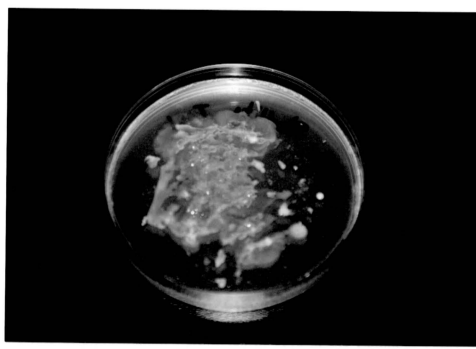


Plate 5. a. Plate of marine agar streaked with thalli from Treatments 3, 4 and 6 on Day 3; b. Plate of marine agar streaked with thalli from Treatments 1, 2 and 5 on Day 3.

4.2 Tissue Culture of *Gracilaria changii* Abbot, Zhang and Xia

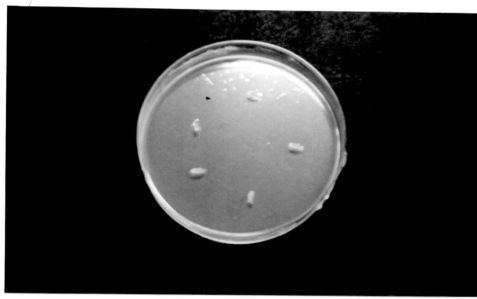
4.2.1 Solid Media

Five cross-sections of axenic explants were inoculated onto each 90cm diameter petri dish containing either solid Murashige and Skoog (MS) or Provasoli's Enriched Seawater (PES) media. Three different forms of MS media were used – the basic media (MS), basic media with added vitamins (MS + vitamins) and basic media with vitamins and added growth substances (MS + vitamins + growth substances). Two different forms of PES media were prepared for use – the basic media with added vitamins (PES + vitamins) and basic media with vitamins and added growth substances (PES + vitamins + growth hormones). The growth substances used were indole-3-acetic acid (IAA), α-naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin. The effects of these growth substances in the media were tested individually and combinations of different concentrations of auxins and cytokinin as described in Section 3.4.3.

Neither callus formation nor branching was observed for all explants cultured on all the different forms of solid media described in Section 3.4.2. The explants remained healthy (brown) for up to 14 days. Bleaching of the explants, indicating the beginning of death were detected from the 21st day onwards and on the 28th day, complete loss of pigmentation were noted in 90% of the explants. These explants were either green or colourless as illustrated in Plate 6a. The remaining 10% of the explants managed to survive up to the 56th day without forming callus (Plate 6b).

This experiment showed that solid MS and PES media were unsuitable for initiating callus regeneration. The addition of vitamins and growth substances in solid media, whether individually or in combination of auxins and cytokinin, were also ineffective in achieving the desired results.

a.



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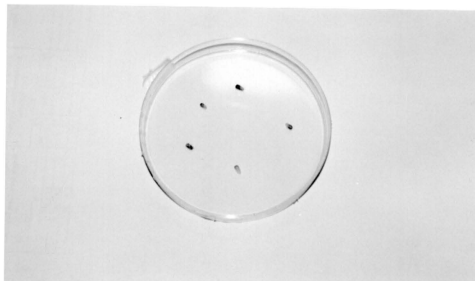


Plate 6. a. Explants on MS and PES solid media on the 28th day. Complete loss of pigmentation was observed on most of the explants. b. Some explants (10%) managed to survive up to the 56th days but without callus formation.

4.2.2 Semi-solid Media

Five cross-sections of axenic explants were inoculated into each 250 ml volume culture vessel containing either solid Murashige and Skoog (MS) or Provasoli's Enriched Seawater (PES) media. Three different forms of MS media were used as in Section 4.2.1 – MS, MS + vitamins MS + vitamins + growth substances. Two different forms of PES media were prepared for use as in Section 4.2.1 PES + vitamins and PES + vitamins + growth substances. The growth substances used were IAA, NAA, 2,4-D and kinetin. The effects of these growth substances in the media were tested individually and combinations of different concentrations of auxins and cytokinin as described in Section 3.4.3. The semi-solid media were prepared using 0.1% agar.

No observation of callus formation nor branching was made. However, the explants managed to remain healthy for a longer period than on solid media. Most of the explants remained healthy up to the 21st day when observation was made. Bleaching was observed in 80% of the explants on the 28th day. The remaining 20% managed to survive up to the 56th day but without callus formation or branching (Plate 7).

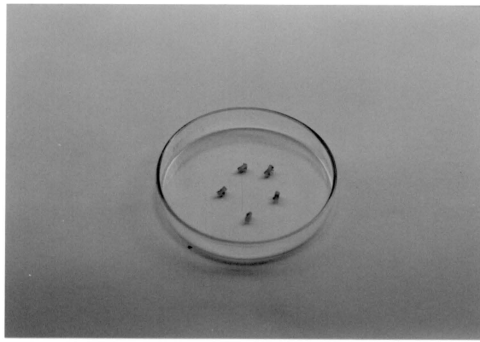


Plate 7. Sample of explants cultured in semi-solid MS, MS + vitamins, MS + vitamins + growth substances, PES + vitamins, PES + vitamins + growth substances media on the 56th day as shown on a petri dish.

This experiment proved that semi-solid media was unsuitable for callus regeneration. Neither the vitamins nor the growth substances had any effect on initiating callus formation.

4.2.3 Liquid Media

The types of media used were exactly the same as in Sections 4.2.1 and 4.2.2 except that no solidifying agent was added to them. Five cross-sections of axenic explants were inoculated into the liquid media contained in a culture vessel as shown in Plate 4.

When basic MS, MS + vitamins, MS + vitamins + growth substances and PES + vitamins media were used, no callus regeneration nor branching was observed. Most of the explants remained healthy up to the 35th day before loss of brown pigmentation began to occur on the 42nd day when observation was made. Only 40% of the explants remained brown on the 56th day (Plate 8).

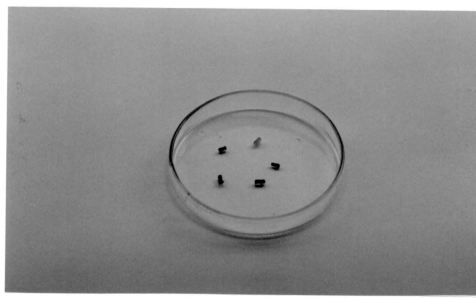


Plate 8. Sample of explants cultured in liquid MS, MS + vitamins, MS + vitamins + growth substances, PES + vitamins, PES + vitamins + growth substances media on the 56th day as shown on a petri dish.

4.2.4 Effects of Liquid Media with Growth Substances

Growth substances such as indole-3-acetic acid (IAA), α-naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin in concentrations ranging from 0.001 to 10 $mg.L^{-1}$ were added to MS + vitamins and PES + vitamins media. The effects of growth substances were tested individually and in combinations as described in Section 3.4.3.

Branching or shoot formation was only observed in liquid PES medium with vitamins and growth substances. No incidence of branching was observed in liquid MS + vitamins medium containing the said growth substances. Branches ranging from 5 mm to 10 mm extending from cross-sections of explants were observed on the 35^{th} day of culture (Plate 9). The results for explants cultured in liquid PES media containing vitamins and growth substances are summarized in Table 10. The explants that did not branch turned white at the end of the 56^{th} day.

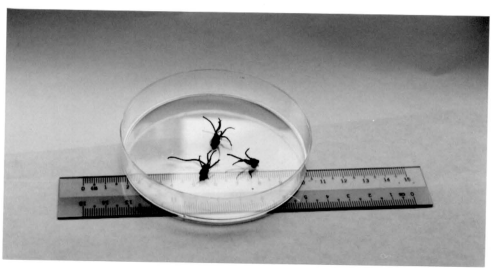
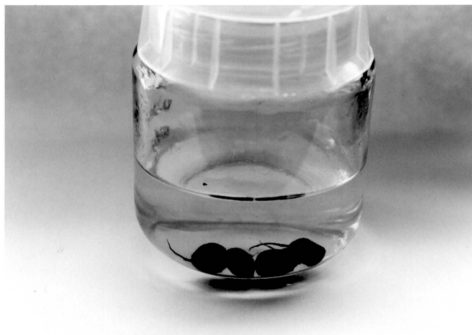


Plate 9. Explants that formed branches when cultured in liquid PES media containing growth substances.

Intermediate range of NAA concentrations (0.01 and 0.1 $mg.L^{-1}$) seemed to have more significant effects on branching compared to the lower and higher extremes. The incidence of branching with media containing IAA was shown to be reducing with the increase in concentration of this growth substance (from 20% branching in 0.001 $mg.L^{-1}$ to 0% in 10 $mg.L^{-1}$ IAA).

Friable callus-like structures (CLS) were initiated, in addition to shoot formation (branching), from cross-sections of explant in liquid PES + vitamins medium supplemented with five different combinations of 2,4-D (an auxin) and kinetin (a cytokinin) (Plate 10). All the explants cultured in the medium mentioned that developed CLS and shoots did so within 35 to 42 days in culture. The phenomenon of branching was identical to that observed when single growth substance was used and is independent from CLS regeneration. Plate 11 shows the microscopic examination of the CLS cross-sections revealed that the callus cells were derived mainly from both the epidermal and cortical cells. Their sizes ranged between 10 to 50 μm in diameter and were pigmented red. Explants that formed CLS and shoots were able to survive and remained healthy even after the end of the 56^{th} day experimental period and continued to survive up to 20 months with periodical medium replacement. The results are summarised in Table 11. Other auxins that were tested in combination with kinetin (IAA and NAA) did not initiate either branching or callus formation. The explants that failed to form callus, turned white at the end of the 56^{th} day.

a.



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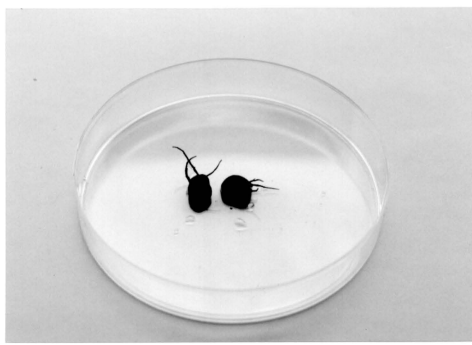
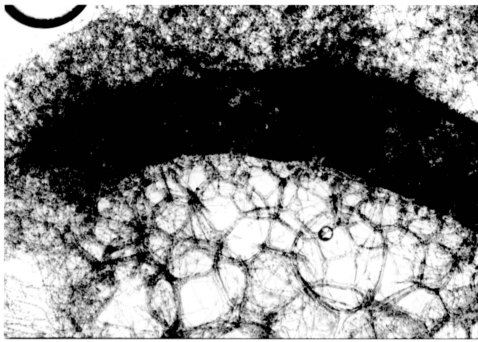


Plate 10. a. Ball-like fraible CLS developed from explants in liquid PES media containing a combination of 2,4-D and kinetin. b. CLS as shown on a petri dish.

a.



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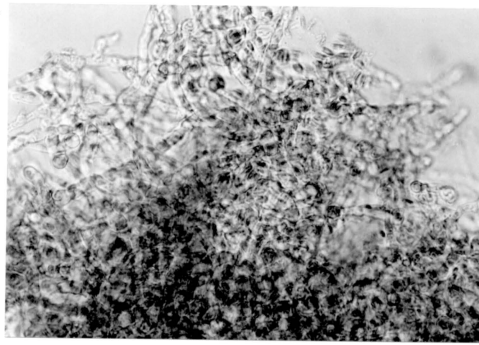

Plate 11. a. Cross-section of the CLS under microscope (200× magnification).
b. Cells of the CLS (400× magnification)

Table 11. Culture of explants in liquid PES medium (pH 7.8, 30 ppt salinity) with combinations of growth substances. Each replicate (R) consisted of a culture vessel with five cross-sections of explants inoculated.

Combination No.	Medium Composition	Number Explants Developed CLS in Each Replicate			Average Percentage of CLS and branching from Three Replicates (%)
		R1	R2	R3	
1	0.001 mg.L^{-1} 2,4-D + 10 mg.L^{-1} Kinetin	2	3	1	40
2	0.01 mg.L^{-1} 2,4-D + 1 mg.L^{-1} Kinetin	5	3	4	80
3	0.1 mg.L^{-1} 2,4-D + 0.1 mg.L^{-1} Kinetin	4	5	5	93.3
4	1 mg.L^{-1} 2,4-D + 0.01 mg.L^{-1} Kinetin	3	1	2	40
5	10 mg.L^{-1} 2,4-D + 0.001 mg.L^{-1} Kinetin	5	5	5	100

The results show that a combination of 10 mg.L^{-1} 2,4-D and 0.001 mg.L^{-1} kinetin in liquid PES medium (Combination 5) was the most successful in generating CLS and shoots in all explants. Except for Combination 4, the percentage of CLS and shoot formation in explants increased in correspond to the increase in 2,4-D concentrations and in the decrease in kinetin concentrations, i.e. in order of Combinations 1, 2, 3 and 5. Thus a trend was observed in which high concentrations of 2,4-D and low concentrations of kinetin stimulated CLS and shoots development.

4.3 Determination of Optimal Mannitol (osmoticum) concentration.

The optimal concentration of mannitol, the osmoticum that was used in the enzyme solution, was determined by soaking three thin cross-sections of the thallus in seawater (30 ppt) containing different concentrations of mannitol (0.5 to 1.2M) for 30 minutes as described in Section 3.5.1. The state of cellular plasmolysis in the cortical and epidermal (or outer cortical) cells was observed at the end of 30 minutes under light microscope after staining with Neutral Red (1% w/v). The optimal mannitol concentration was determined based on the percentage of plasmolysed cells per sample of 10 cortical or epidermal cells from each cross-section soaked in a given mannitol concentration. The results are tabulated in Appendix 1 and presented graphically in Figure 1. When observed under the light microscope, the size range of the epidermal cells was between 10-30 μm and the cortical cells, 50-150 μm.

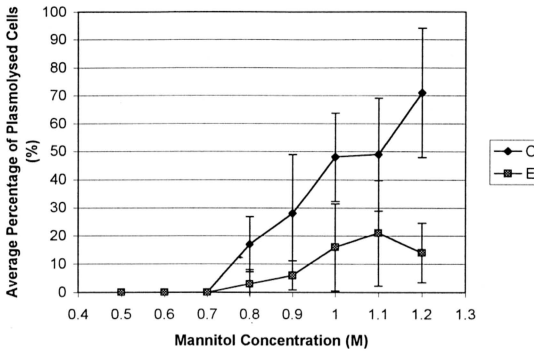

Figure 1. Average percentage of plasmolysed cortical (C) and epidermal (E) cells of the thallus cross-sections in different mannitol concentrations.

No plasmolysed cells were observed with mannitol concentration below 0.8M. The percentage of plasmolysed cortical cells began to increase with mannitol concentration from 0.8 to 1.2M. The number of plasmolysed epidermal cells showed almost similar trend over the same range of mannitol concentrations except for a decline at 1.2M (Figure 1). From this experiment, 1M mannitol was chosen as the optimal osmoticum concentration for the induction of plasmolysis as concentration above this resulted in over-plasmolysed cells.

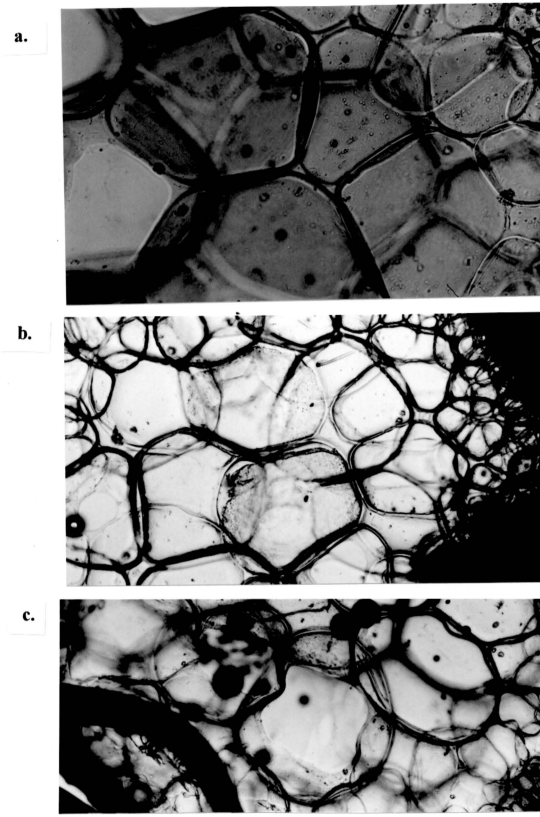

Plate 12. a. Cells remained unplasmolysed for mannitol concentrations below 0.7 M (× 100 magnification). b. Sufficient plasmolysis was induced for cell in 1 M mannitol (× 100 magnification). c. Over-plasmolysed cells in mannitol concentration above 1.1 M (× 100 magnification).

4.4 Protoplast Isolation Using Single Enzyme and Combination of Enzymes.

Protoplasts were isolated from vegetative thalli of *G. changii* using single enzyme as described in Section 3.5.3. The protoplast isolating solution contained 1M mannitol, the optimal osmoticum concentration for the induction of plasmolysis as verified in Section 4.3. Five different enzymes were tested individually in different concentrations – 1, 2 and 3% w/v respectively. They were abalone acetone powder, cellulase, pectinase, pectolyase and agarase. Finely chopped fresh vegetative thalli were incubated in the isolating solution containing a given enzyme for 0.5, 1.0, 1.5 and 2.0 hours. The effectiveness of each enzyme was measured by the number of viable protoplast isolated at every 30 minutes interval over a period of two hours. The number of protoplast isolated were enumerated using haemocytometer after staining with Evans Blue as described in Section 3.5.3. The number of viable protoplast isolated over time in different concentrations of a given enzyme is as illustrated in Figures 2 to 6. Table 12 summarises the maximum number of protoplast obtained for using each enzyme. Most of the viable protoplasts isolated were between the size range of 10 – 50 μm suggesting that they were derived from the cortical cells.

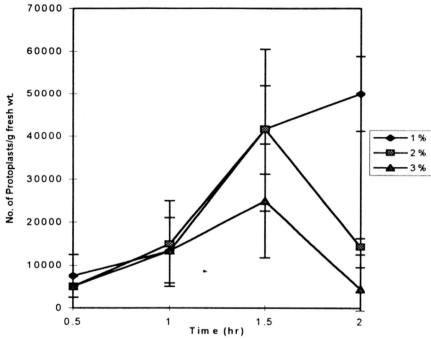

Figure 2. Number of protoplast isolated per gram fresh weight at different time of incubation using 1%, 2% and 3% w/v abalone acetone powder.

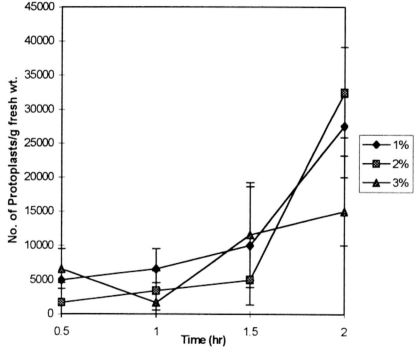

Figure 3. Number of protoplast isolated per gram fresh weight at different time of incubation using 1%, 2% and 3% w/v cellulase.

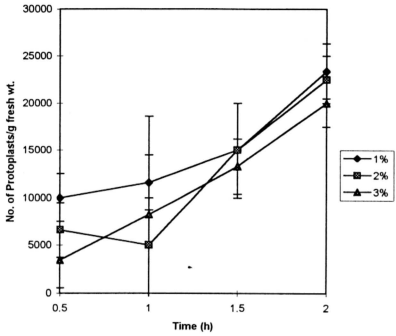

Figure 4. Number of protoplast isolated per gram fresh weight at different time of incubation using 1%, 2% and 3% w/v pectinase.

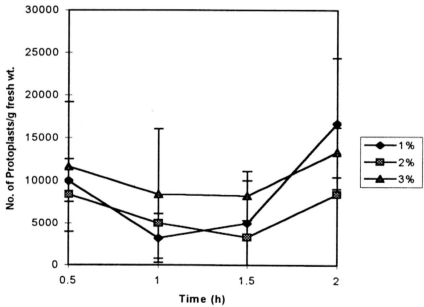

Figure 5. Number of protoplast isolated per gram fresh weight at different time of incubation using 1%, 2% and 3% w/v pectolyase.

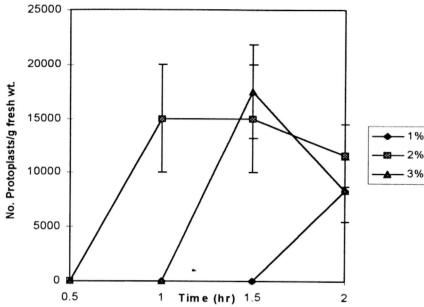

Figure 6. Number of protoplast isolated per gram of fresh weight at different time of incubation using 1%, 2% and 3% w/v agarase.

Table 12. Maximum number of protoplast obtained using each enzyme at the respective isolating conditions.

Enzyme	Isolating Condition	Maximum number of Protoplast isolated per g fresh weight.
Abalone acetone powder	1% (w/v), 2 hours incubation	50000
Cellulase	2% (w/v), 2 hours incubation	32000
Pectinase	1% (w/v), 2 hours incubation	23400
Pectolyase	1% (w/v), 2 hours incubation	16700
Agarase	3% (w/v), 1.5 hour incubation	17500

Except for agarase, all the enzymes tested functioned most effectively at 2 hours incubation with the highest maximum number of viable protoplast isolated using 1% w/v abalone acetone powder (5.0×10^4 protoplast g^{-1} fresh weight). Pectolyase and agarase were less efficient as low number of viable protoplast was isolated using these enzymes (Table 12, Figures 5 and 6).

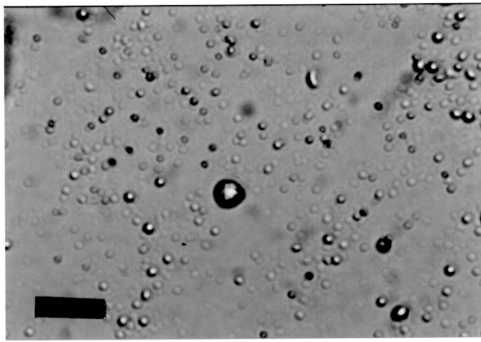

Plate 13. Membrane of live protoplasts not stained with Evans Blue after isolation. Bar: 50μm.

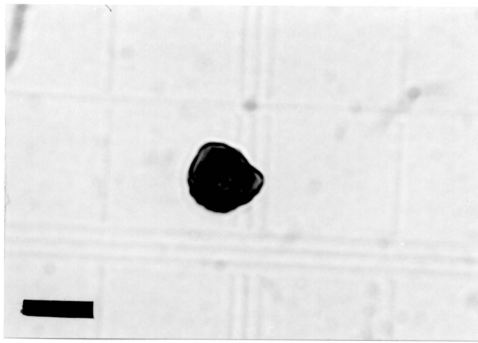

Plate 14. Membrane of dead protoplast stained with Evans Blue after isolation. Bar: 50μm.

After the efficiency of protoplast isolation was determined, the effects of a combination of all the enzymes were investigated. Protoplast isolating solution containing 1% w/v each of abalone acetone powder, cellulase, pectinase, pectolyase and agarase and 1 M mannitol were used for protoplast isolation. The efficiency of using a combination of these enzymes was assessed as in 3.5.3. Figure 7 illustrates the number of protoplast per gram of fresh weight isolated over time.

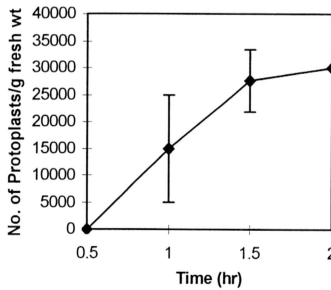

Figure 7. Number of viable protoplast isolated per gram of fresh weight at different time of incubation using 1% w/v each of abalone acetone powder, cellulase, pectinase, pectolyase and agarase.

The number of viable protoplast per gram of fresh weight isolated using a combination of 1% w/v each of the five different enzymes increased with the time of incubation until a maximum of 3.0×10^4 protoplast g^{-1} fresh weight was obtained at time, 2 h. However, this maximum figure was way below that obtained using 1% w/v abalone acetone powder alone under the same incubation period (Table 12).