Bot. Bull. Acad. Sin. (1994) **35:** 45 – 51

Isolation and regeneration of protoplasts of *Monostroma latissimum* Wittrock (*Monostromataceae, Chlorophyta)*

Yean-Chang Chen and Young-Meng Chiang

Institute of Oceanography, National Taiwan University, Taipei, Taiwan, Republic of China

Abstract. Protoplasts of *Monostroma latissimum* were isolated enzymatically with 4% Cellulase Onozuka R-10 and 2% Macerozyme R-10. The yield of protoplasts increased as the sorbitol concentration of the enzyme solution was increased from 0.5 to 1.2 M, and then decreased as the sorbitol concentration was increased further. At the highest concentration (1.9 M) no protoplasts were produced. The highest number of normal protoplasts (9×10^6 per gram of fresh thallus) was obtained with 1.2 M sorbitol and 6 h incubation on a 50 RPM shaker. The number of isolated protoplasts increased as the speed of rotation increased, but the relative number of abnormal protoplasts increased when the rotation speed exceeded 50 RPM. The highest percentage (61.3%) of regenerated protoplasts was obtained by incubating freshly isolated protoplasts in 0.85 M mannitol-Provasoli's enriched seawater (PES) medium for 5 d and then transferring to PES medium. It was found that earlier (less than 5 d) transfer of protoplasts to PES medium, may cause the death or loss of viability of the protoplasts due to osmotic change. More than 5 d in mannitol-PES medium was also harmful to the protoplasts. Protoplasts usually began to form new cell walls within 5 h after isolation and began to divide from day 7 to day 9 in PES medium, then formed cell clusters from day 14 to day 18. A cell cluster can form a tubular frond then a leafy thallus after further cultivation in PES medium.

Keywords: Calcofluor White; Cell cluster; *Monostroma latissimum*; Protoplast.

Introduction

Monostroma is a widely distributed genus of green marine algae, growing as macroscopic, one-cell-thick thalli. Among the species of this genus, *M. latissimum* Wittrock is edible and has been successfully cultivated in Japan for many years on a large commercial scale (Shokita et al., 1991). In Taiwan, *M. latissimum* is used as a food supplement, but viable commercial aquaculture techniques for this plant are still being sought. In traditional cultivation of *Monostroma,* mature thalli are collected from the field during their growing season. After being washed to remove epiphytes, animals, and other material, they are treated to release gametes in a tank under strong illumination. The gametes are collected and conjugated to form zygotes, which are cultured for several months in indoor tanks to produce zoospores. The zoospores are collected and attached to nets, which are then hung in the sea. The traditional method is tedious and time consuming. Sometimes it is difficult to obtain sufficient quantities of mature thalli or of gametes and zoospores. Therefore, a new method of *Monostroma* cultivation needs to be developed.

The successful regeneration of plants directly from enzymatically isolated protoplasts of various species of seaweed (Tang, 1982; Zhang, 1983; Polne-Fuller et al., 1986; Chen, 1987) and cell-suspension cultures of *Porphyra* (Chen, 1989) provides a basis for improving aquacultural crops and for genetic manipulations. These techniques might be applicable in developing a new method of seaweed cultivation. Our research has accordingly been directed towards developing new techniques for mass cultivation of *M. latissimum.* The present report describes the production of protoplasts and stable, nonclonal suspension cultures of single cells and cell clusters that might provide an alternate propagation method for this alga.

Materials and Methods

Thalli of *Monostroma latissimum* were collected at Chiang-may, Peng-hu County, Taiwan on April 4, 1990. Freshly collected plants were wrapped in paper towels moistened with seawater, sealed in plastic bags, and packed in an ice-box for transport to the laboratory.

Preparation of Clean, Bacteria-Free Plant Material

Clean, bacteria-free material was obtained by a slight modification of the method of Reddy et al. (1989). Selected pieces of vegetative fronds (2 cm^2) were thoroughly cleaned in filtered seawater and placed in an ultrasonic cleaner (Branson 3200), twice for 5 min, with autoclaved seawater containing 1% KI-I_2 (2 g KI and 1 g I_2 dissolved in 300 ml distilled water) to remove animals and epiphytes. The pieces were then rinsed several times with autoclaved seawater. Finally they were incubated in 100 ml of autoclaved Provasoli's enriched seawater (PES) medium (35‰) (Provasoli, 1968) containing 10 ml of antibiotic mixture (Polne-Fuller and Gibor, 1984) for 24 h at 24 °C under a photoperiod of 12:12 L:D regime and a photon flux density of 20 $\mu E\ m^{-2}\ s^{-1}$ in a culture room.

Preparation of an Enzyme Solution for Cell Wall Digestion

The enzyme solutions were prepared by dissolving 4% Cellulase Onozuka R-10, 2% Macerozyme R-10 (Yakult Honsha Co. Ltd. Japan), and various concentrations of sorbitol (0.5 – 1.9 M), in 10 ml of distilled water. The pH was set at 6.0 using Na_2HPO_4-NaH_2PO_4 buffer.

The enzyme solution was centrifuged at 10,000 x *g* for 10 min at 0 °C to remove large particles. The supernatant liquid was sterilized by passing it through a 0.2 μm disposable syringe filter unit.

Isolation of Protoplasts

To establish the optimum concentration of osmoticum for isolation of protoplasts, healthy fronds were incubated in various concentrations of sorbitol (0.5 – 2 M) for 6 h, and then immersed in seawater-Evans Blue solution (0.01%) for 1 h (Millner et al., 1979) under a photon flux density of 20 $\mu E\ m^{-2}\ s^{-1}$ in a 24 °C incubator. The material was examined under a microscope to check whether the cells stained blue (Chen and Chen, 1991). This preliminary test showed that cells of *Monostroma latissimum* retained their plasmolysis activity after a 6-h incubation in 1.3 – 1.75 M sorbitol solution (1337.5 – 1803.7 milliosmol/kg) at 24 °C. A concentration higher than 1.75 M caused cell death, and a concentration lower than 1.3 M caused cells to swell and lose their viability. Aseptic material of *M. latissimum* was obtained by washing three times with autoclaved seawater, then cutting into pieces approx. 1-mm square with a sterile razor blade under a laminar flow hood. One-hundred milligrams of the material was incubated in each of 40 sterile 50 x 80-mm disposable plastic flasks (40 ml, Falcon) containing 10 ml of enzyme solution of various sorbitol concentrations (0.5, 0.7, 0.9, 1.1, 1.2, 1.35, 1.55, and 1.9 M) based on the plasmolysis range of the preliminary test, and placed on an orbital shaker (Firstek, Taiwan) rotating at different speeds (40, 50, 60, 70, and 80 RPM). The numbers of normal and abnormal protoplasts were counted. The abnormal protoplasts were distinguished from the normal ones by the shape of their surface. The former had concave or convex surfaces, while the latter had round and smooth ones. All procedures of isolation were carried out in the dark (Marchant and Fowke, 1977; Cheney et al., 1986; Reddy and Fujita, 1991) to promote the sinking of isolated protoplasts (Liu et al., 1992) at 24°C.

Purification of Protoplasts

Following incubation as above for 6 h, the solution was filtered through a 59 μm Nylon mesh to separate protoplasts from undigested cells and cell debris. The filtrate was layered over a 35% (w/v) density buffer (Ficoll-400, Sigma) solution and centrifuged at 100 x *g* for 30 min, to remove small debris. The protoplasts collected at the interface were retrieved with a sterile Pasteur pipette.

The freshly isolated protoplasts were counted with a hemacytometer (Bright-Line, Improved Neubauer, 0.1 mm deep) under a light microscope (Zeiss, Axioskop). The experiments were performed three times, and the average counts and standard errors were recorded.

Incubation of Protoplasts

Immediately after isolation, purified protoplasts were cultured in PES medium containing various concentrations (0.5, 0.7, 0.85 and 1 M) of mannitol and 0.01% of Evans-Blue dye to determine the most suitable concentration for cultivation of the protoplasts. The number of viable protoplasts was counted each day on an inverted microscope (Nikon, Diaphot-TMD). All protoplast cultures were incubated under a photoperiod of 12:12 L:D regime and a photon flux density of 166 $\mu E\ m^{-2}\ s^{-1}$ at 24°C. The regenerated protoplasts were cultured in 0.85 M mannitol-PES medium for 5 d, then transferred to pure PES medium for further cultivation under the conditions described above.

The cell clusters regenerated from the protoplasts were sprayed on fine strings made of synthetic fiber and cultured in an aquarium with PES medium for approx. 30 d. They were then hung in a fish pond for further cultivation.

Protoplast Cell Wall Formation

A fluorescent brightener reagent, Calcofluor White ST (Sigma), was used to investigate the course of cell wall resynthesis (Galbraith, 1981; Roberts et al., 1982; Chen and Chen, 1993). We stained the protoplasts with

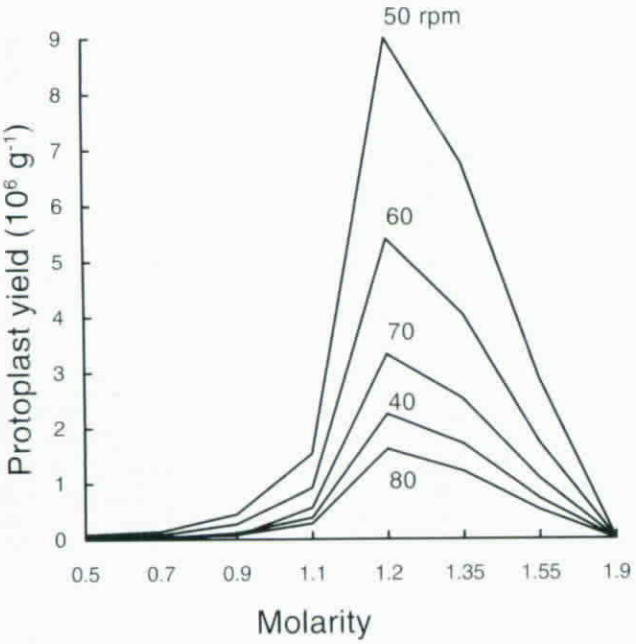

Fig. 1. Influence of agitation rate and sorbitol concentration on production of protoplasts

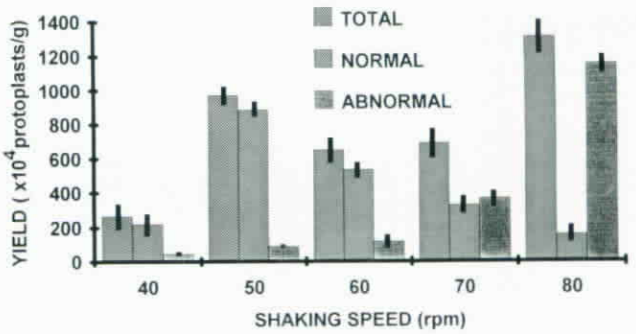

Fig. 2. Agitation rate and the yield of normal and abnormal protoplasts. Sections of *M. latissimum* fronds were incubated in 1.2 M sorbitol-enzyme solution at 24 °C for 6 h in darkness. The yield of protoplasts were observed on an inverted microscope, and the normal and abnormal protoplasts were counted.

Calcofluor White and found that those with resynthesized walls appeared green, while those without cell walls were red. About 0.01% of Calcofluor White (w/v) was added to each culture of purified protoplasts in 0.85 M mannitol-PES medium after 0, 2, 4, 8, 12, and 24 h in culture. During the first 24 h, each culture was examined hourly on an inverted microscope with fluorescence equipment (Nikon, Diaphot-TMD, BG-12 filters with wave length of 320 – 400 nm), and the green and red protoplasts in five randomly selected microscope fields were counted.

Results and Discussion

As shown in Fig. 1, protoplasts of *Monostroma latissimum* can be isolated from 0.5 to 1.55 M sorbitol-enzyme mixtures containing 4% Cellulase R-10 and 2% Macerozyme R-10 (ca. equal to the osmolarity of 0.6 – 1.7 M pure sorbitol solution) incubated for 6 h at 24 °C on an orbital shakers at speeds of 40 – 80 RPM. At 50 RPM the number of isolated protoplasts increased to its maximum yield of 9 x 10^6 protoplasts per gram as the sorbitol concentration of the enzyme solution increased from 0.5 to 1.2 M, and then decreased as the sorbitol concentration increased further. At the highest concentration (1.9 M) no protoplasts were produced. Fujita and Migita (1985) isolated approx. 10^6 *Monostroma nitidum* and *Ulva pertusa* protoplasts per gram of thallus with 10% cellulase (Onozuka R-10) in 0.8 M mannitol at 18 – 20 °C for 2 and 4 h, respectively. The differences in the yield of *Monostroma* and *Ulva* protoplasts could be due to differences in the enzyme components, osmoticum concentration, pH, or the physiological status and growth stage of the plant materials (Fujita and Migita, 1985; Reddy et al., 1989).

It is necessary to purify the released protoplasts for subsequent study, but filtration through Nylon mesh (Saga, 1984; Reddy et al., 1989) often resulted in contamination by large amounts of cell debris and broken protoplasts. We tried various methods and found that the utilization of Ficoll-400 (a density buffer for centrifugation) has proven to be most effective in obtaining clean protoplasts due to its low osmotic potential and high density (Chen and Chen, 1991).

Table 1. Percent of *M. latissimum* protoplasts with new cell walls after staining with Calcofluor White for various lengths of time

Stain time (h)	Age of culture (h)										
	4	5	6	7	8	12	24	48	72	96	120
0	0.0 ± 0.01	0.0 ± 0.01	0.0 ± 0.01	5.1 ± 0.5	10.8 ± 0.6	20.4 ± 2.3	34.9 ± 7.1	48.1 ± 6.8	51.8 ± 3.8	53.7 ± 4.6	56.0 ± 2.9
2	0.0 ± 0.01	0.0 ± 0.01	6.6 ± 1.10	10.4 ± 1.4	12.8 ± 1.8	28.2 ± 2.0	51.8 ± 3.7	53.9 ± 4.2	55.3 ± 3.3	60.5 ± 4.6	64.2 ± 5.2
4	2.0 ± 0.40	11.2 ± 2.3	21.5 ± 1.4	23.0 ± 3.2	23.6 ± 3.1	40.0 ± 4.8	57.0 ± 3.9	58.2 ± 5.0	61.3 ± 5.8	67.1 ± 6.1	75.1 ± 5.5
8	–	–	–	–	29.0 ± 2.6	49.5 ± 3.2	58.4 ± 4.2	59.2 ± 4.6	65.3 ± 4.1	70.7 ± 4.2	75.5 ± 3.8
12	–	–	–	–	–	51.9 ± 4.4	60.9 ± 5.1	63.1 ± 3.2	65.4 ± 4.2	71.5 ± 3.2	76.1 ± 5.7
24	–	–	–	–	–	–	63.0 ± 4.9	65.9 ± 6.0	66.2 ± 3.6	72.7 ± 2.6	85.3 ± 5.2

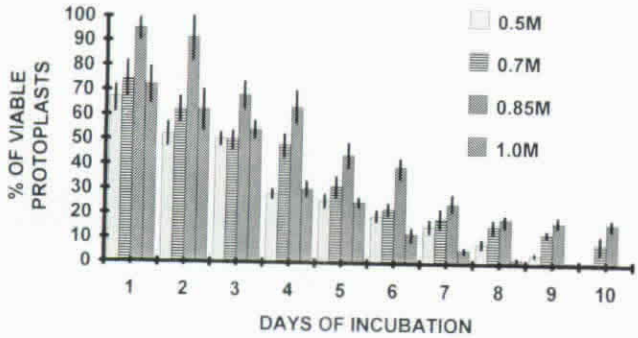

Fig. 3. Percentage of viable protoplasts. The protoplasts were incubated in 0.5, 0.7, 0.85, and 1.0 M mannitol in PES (salinity of seawater = 35‰) at 24 °C for 10 d under a photon flux density of 166 μE $m^{-2} s^{-1}$ and a photoperiod of 12:12 L:D.

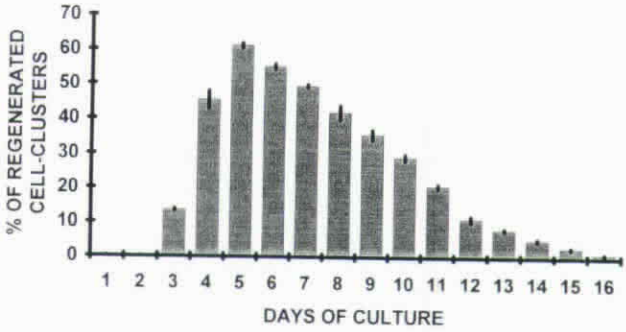

Fig. 4. Percentage of regenerated cell clusters. Protoplasts were incubated in 0.85 M mannitol-PES (salinity of seawater = 35‰) for 1 to 16 d at 24°C under a photon flux density of 166 μE $m^{-2} s^{-1}$ and a photoperiod of 12:12 L:D, then transferred to PES medium for 12 d under the same conditions.

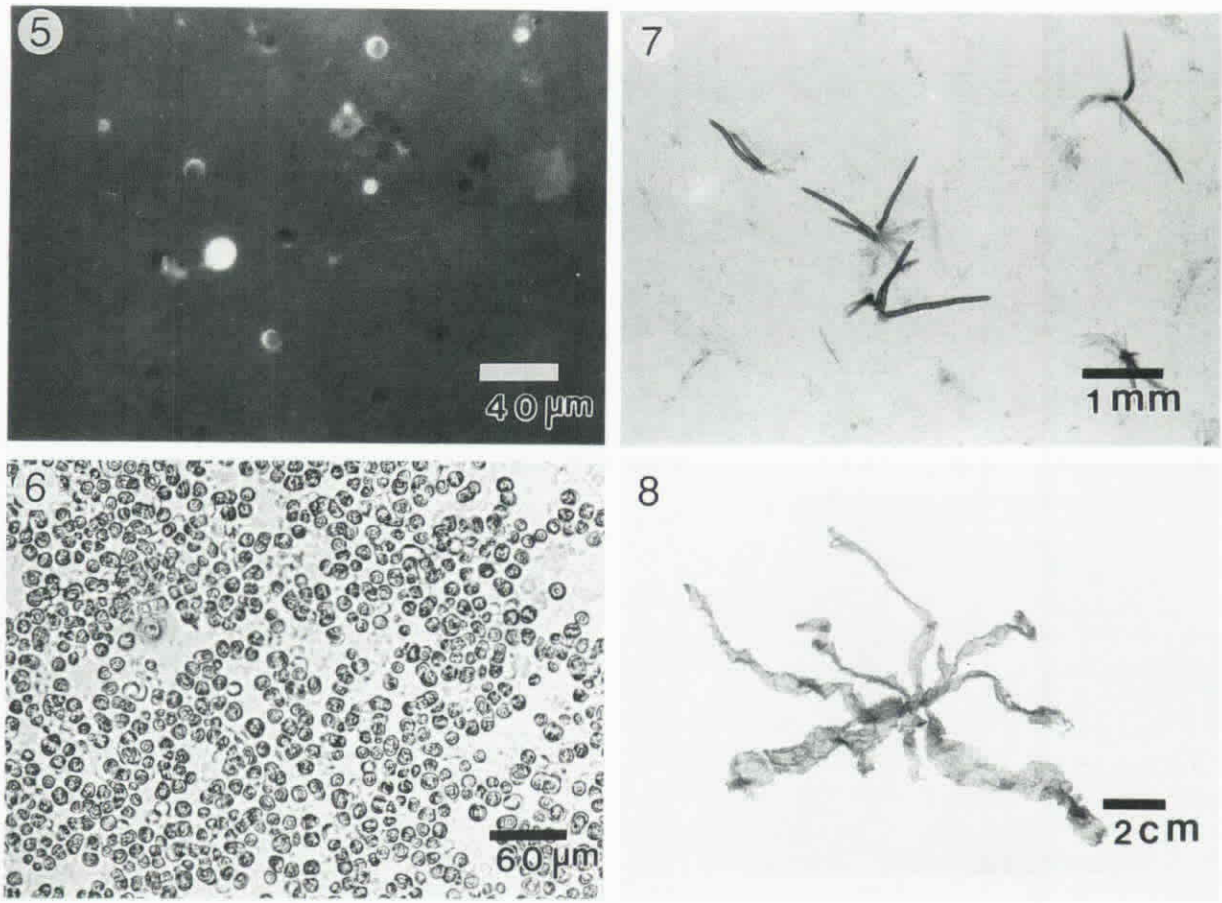

Fig. 5. Protoplasts, stained with calcofluor white after 24 h of culture, on a dark field, inverted fluorescent microscope. Fluorescence indicates new cell walls.

Fig. 6. Cell clusters regenerated from protoplasts of *M. latissimum* after incubating for 30 d in PES at 24 °C under a photon flux density of 166 μE $m^{-2} s^{-1}$ and a photoperiod of 12:12 L:D.

Fig. 7. Tubular thalli regenerated from protoplasts of *M. latissimum* after 45 d in PES at 24 °C under a photon flux density of 166 μE $m^{-2} s^{-1}$ and a photoperiod of 12:12 L:D.

Fig. 8. Leafy thalli regenerated from a protoplast of *M. latissimum* after incubating in PES for 60 d at 24 °C under a photon flux density of 166 μE $m^{-2} s^{-1}$ and a photoperiod of 12:12 L:D.

The agitation rate can affect the yield of protoplasts, irrespective of the concentration of sorbitol-enzyme solutions (Fig.1). As shown in Fig. 2 the number of normal protoplasts increased from 2.21 to 8.86 x 10^6 protoplasts per gram as the rate of rotation increased, but the relative number of swollen or shrunken protoplasts increased from 1.16 x 10^6 (17.8 %) to 11.57 x 10^6 (88.2%) protoplasts per gram when the rate exceeded 60 to 80 RPM. This phenomenon was also found with *Ulva fasciata* Delile protoplasts (Chen and Chen, 1991).

We found that sorbitol did not crystalize in concentrations as high as 1.2 M, though mannitol did. We therefore used sorbitol for the isolation of protoplasts. Later, we found that the protoplasts lose their viability if kept in sorbitol-enzyme solution for more than 6 h; this might be due to a cumulative toxicity of the sorbitol-enzyme. We therefore used mannitol for the incubation of algal protoplasts during this study, as many others have (Saga and Sakai, 1984; Saga, 1984; Cheney et al., 1986; Polne-Fuller and Gibor, 1987; Ducreux and Kloareg, 1988; Reddy et al., 1989, 1992; Fujimura et al., 1989; Fujita and Saito, 1990). The purified protoplast should be transferred to mannitol-PES medium as soon as possible. We also found that high concentrations of mannitol can affect the viability of the protoplasts (Fig. 3). Of the mannitol concentrations tested, 0.85 M mannitol in the PES medium produced the largest number of viable protoplasts in every incubation stage (from day 1 to day 10).

We incubated freshly isolated protoplasts in 0.85 M (ca. 1800 milliosmol/kg) mannitol-PES medium for 1 to 16 d, and then transferred them to pure PES medium (ca. 998 milliosmol/kg) for 12 d. The percentage of regenerated cell clusters (Fig. 4) increased from day 1 to day 5, and decreased from day 6 to day 16. The protoplasts incubated in 0.85 M mannitol-PES for 5 d and then transferred to PES medium produced the highest percentage of regenerated cell clusters (61.3%). For those incubated in 0.85 M mannitol-PES for 1, 2, 3, and 4 d, the percentage of regenerated cell clusters was 0.3%, 0.2%, 13.6%, and 45.5%, respectively. This might be due to the fact that the protoplasts had not completely formed cell walls in 4 d (Table 1), and so the sudden transfer to a lower osmotic condition caused death or loss of viability.

Protoplasts incubated in 0.85 M mannitol-PES for more than 5 d also showed a lower percentage of regeneration. This may indicate that mannitol is toxic to the protoplasts over an extended period (Chen and Chen, 1991). The same results were described by Reddy et al. (1989) and Reddy and Fujita (1991), who indicated that prolonged cultivation of cells in hyperosmotic solutions (0.2 – 0.8 M mannitol) impaired normal cell division and resulted in abnormal cell structures. The inhibition of cell division by hyperosmotic conditions might be due to the higher concentration of inorganic salts in the cytoplasm inhibiting various growth processes, such as photosynthesis and respiration.

Fluorescent staining showed that the protoplasts began to form new cell walls after 5 h of isolation, and the number of protoplasts with a cell wall increased over time (Table 1). At 4 h, about 2% of the protoplasts showed the presence of a cell wall, but this could be a remnant of the old cell wall. Earlier staining of protoplasts with calcofluor white can result in a lower percentage of protoplasts forming new cell walls; the negative influence of calcofluor white on cell wall formation seems to be long lasting. Protoplasts stained with calcofluor white immediately after isolation had the lowest percentage (56%) of new cell walls after 5 d of incubation (Table 1). Those stained after 24 h (Table 1, Fig. 5), however, had the highest percentage (85.3%). This is higher than in Fujita and Migita's 1985 study of *M. nitidum* (68.3%). The differences in cell wall resynthesis could be due to environmental stresses, such as osmotic pressure and temperature, that affect the physiology of the protoplasts (Galun, 1981).

Protoplasts usually began to divide from day 7 to day 9, and to form cell clusters (Fig. 6) from day 14 to day 18. Some of them formed tubular fronds (Fig. 7) from day 25 to day 30. Further cultivation in the PES medium for another 30 d induced tubular fronds to become leafy forms (Fig. 8). Saga and Kudo (1989) obtained cell clusters from protoplasts of *M. angicava*, but Fujita and Migita (1985) reported that the protoplasts of *M. nitidum* produced gametes after regeneration of the cell wall. The differences in the growth pattern of the protoplasts in the species of *Monostroma* may be influenced by the original location of the protoplasts on the frond, as well as by culture conditions. This behavior was also reported in *Porphyra* spp. (Polne-Fuller et al., 1986).

The cell clusters of *M. latissimum* can be easily propagated in large quantities in PES medium. We sprayed them on fine synthetic strings and cultured them in fish ponds. Thalli appeared within two weeks, but the yield of thalli varied from pond to pond and from season to season. Apparently, the yield of thalli was affected by natural conditions of the ponds. Further study is needed of the environmental factors which affect the formation of thalli by cell clusters before we can develop techniques for the large-scale cultivation of *Monostroma* with cell clusters regenerated from protoplasts .

Acknowledgements. We wish to express our sincere thanks to Dr. L. C-M. Chen, Institute for Marine Biosciences, National Research Council of Canada for reading the manuscript and for useful suggestions. This study was supported by the National Science Council (81-0418-B-002A-503-BG) and the Council of Agriculture (81AC-12.1-F-67(59)), Republic of China.

Literature Cited

Chen, L. C-M. 1987. Protoplast morphogenesis of *Porphyra leucosticta* in culture. Bot. Mar. **30:** 399 – 403.

Chen, L. C-M. 1989. Cell suspension culture form *Porphyra linearis* (Rhodophyta), a multicellular marine red alga. J. Appl. Phycol. **1:** 153 – 159.

Chen, C. S. and Y. C. Chen. 1991. Isolation and regeneration of protoplasts from the green alga *Ulva fasciata* Delile. Proceedings of the National Science Council, ROC. Part B: Life Science **15**(4): 244 – 250.

Chen, Y. C. and C. S. Chen. 1993. Use of fluorescent staining to monitor the temporal pattern of cell wall resynthesis in *Ulva fasciata* (Chlorophyta: Ulvales, Ulvaceae) protoplasts. Jpn. J. Phycol. **41:** 237 – 242.

Cheney, D. P., E. Mar, N. Saga, and J. van der Meer. 1986. Protoplast isolation and cell division in the agar-producing seaweed *Gracilaria* (Rhodophyta). J. Phycol. **22:** 238 – 243.

Ducreux, G. and B. Kloareg. 1988. Plant regeneration from protoplasts of *Sphacelaria* (Phaeophyceae). Planta **174:** 25 – 29.

Fujimura, T., T. Kawai, M. Shiga, T. Kajiwara, and A. Hatanaka. 1989. Regeneration of protoplasts into complete thalli in the marine green alga *Ulva pertusa*. Nippon Suisan Gakkaishi **55**(8): 1353 – 1359.

Fujita, Y. and S. Migita. 1985. Isolation and culture of protoplasts from some seaweeds. Bull. Fac. Fish. Nagasaki Univ. No. 57. pp. 39 – 45.

Fujita, Y. and M. Saito. 1990. Protoplast isolation and fusion in *Porphyra* (Bangiales, Rhodophyta). Hydrobiologia **204/205:** 161 – 166.

Galun, E. 1981. Plant protoplasts as physiological tools. Ann. Rev. Plant Physiol. **32:** 237 – 266.

Galbraith, D.W. 1981. Microfluorimetric quantitation of cellulose biosynthesis by plant protoplasts using Calcofluor White. Physiol. Plant. **53:** 111 – 116.

Liu, Q, Y., L. C.-M. Chen, and A. R. A. Taylor. 1992. Ultrastructure of cell wall regeneration by isolated protoplasts of *Palmaria palmata* (Rhodophyta). Bot. Mar. **35:** 21 – 33.

Marchant, H. J. and L. C. Fowke. 1977. Preparation, culture, and regeneration of protoplasts from filamentous green algae. Can. J. Bot. **55:** 3080 – 3086.

Millner, P. A., M. E. Callow, and L. V. Evans. 1979. Preparation of protoplasts from the green alga *Enteromorpha intestinalis* (L.) Link. Planta **147:** 174 – 177.

Provasoli, L. 1968. Media and products for the cultivation of marine algae. pp. 6375. *In* A. Watarabe and A. Hattori (eds.), Cultures and collections of algae. Jap. Soc. Plant Physiol., Tokyo.

Polne-Fuller, M. and A. Gibor. 1984. Developmental studies in *Porphyra* I. blade differentiation in *Porphyra perforata* as expressed by morphology, enzymatic digestion, and protoplast regeneration. J. Phycol. **20:** 609 – 616.

Polne-Fuller, M., N. Saga, and A. Gibor. 1986. Algal cell, callus, and tissue cultures and selection of algal strains. Beihefte Zur Nova Hedwigia. **83:** 30 – 36.

Polne-Fuller, M. and A. Gibor. 1987. Tissue culture of seaweeds. *In* K. T. Bird and Benson, P. H. (Eds.), Seaweed cultivation for renewable resources. pp. 218 – 239. Elsevier.

Roberts, E., R. W. Seagull., C. H. Haigler, and R. M. Brown, Jr. 1982. Alteration of cellulose microfibril formation in eukaryotic cells: Calcofluor White interferes with microfibril assembly and orientation in *Oocystic apiculata*. Protoplasma **113:** 19.

Reddy, C. R. K., S. Migita, and Y. Fujita. 1989. Protoplast isolation and regeneration of three species of *Ulva* in axenic culture. Bot. Mar. **32:** 483 – 490.

Reddy, C. R. K. and Y. Fujita. 1991. Regeneration of plantlets from *Enteromorpha* (Ulvales, Chlorophyta) protoplasts in axenic culture. J. Appli. Phycol. **3:** 265 – 275.

Reddy, C. R. K., M. Iima, and Y. Fujita. 1992. Induction of fast-growing and morphologically different strains through intergeneric protoplast fusion of *Ulva* and *Enteromorpha* (Ulvales, Chlorophyta). J. Appli. Phycol. **4:** 57 – 65.

Saga, N. and Y. Sakai. 1984. Isolation of protoplasts from *Laminaria* and *Porphyra*. Bull. Jap. Soc. Sci. Fish. **50**(6): 10 – 85.

Saga, N. 1984. Isolation of protoplasts from edible seaweeds. Bot. Mag. Tokyo. **97:** 423 – 427.

Saga, N. and T. Kudo. 1989. Isolation and culture of protoplasts from the marine green alga *Monostroma angicava*. J. Appl. Phycol. **1:** 25 – 30.

Shokita, S., K. Kakazu, A. Tomori, and T. Toma. 1991. Aquaculture in tropical areas. 360 pp. Midori Shobo Co. Ltd. Japan. (English edition prepared by M. Yamachi)

Tang, Y. L. 1982. Isolation and cultivation of the vegetative cells and protoplasts of *Porphyra suborbiculata* Kjellm. J. Shandong College of Oceanog. **12:** 37 – 50. (in Chinese with English abstract).

Zhang, D. L. 1983. Studies on the protoplast preparation, culture and fusion of somatic cells from two species of green algae *Ulva linza* and *Monostroma angicava* Kjellm. Journal of Shandong College of Oceanology **13**(1): 57 – 64.

青海菜原生質體之分離及培養

陳衍昌　江永棉

國立台灣大學海洋研究所

爲發展青海菜養殖之新技術，本研究使用酵素做青海菜原生質體之分離。研究發現青海菜原生質體可以4% Cellulase 和 2% Macerozyme 之 sorbitol 溶液分離出來。原生質體之產率隨著 sorbitol-enzyme 之濃度，由 0.5M 增加至 1.2 M 而增加，然後隨著前者之繼續增加而減低。在 1.9 M sorbitol-enzyme中則無原生質體產生。在1.2 M sorbitol-enzyme 溶液中，以50 rpm 速度旋轉六小時者產量最高（9×10^6個原生質體／每公克新鮮藻體）。原生質體產量亦隨著橢圓旋轉式培養器旋轉速度之增加而增加，但是若超過 50 rpm，則其不正常形狀原生質體數目，將隨著旋轉速度之增加而增加。原生質體分離出後必須儘速移至含有 mannitol之Provasoli's enriched seawater (PES)之培養液中，否則將喪失其分生能力。在 0.85M mannitol-PES培養液中五天後移至純 PES 培養液繼續培養，則可獲得最高比率 (61.3%) 之具分生能力之原生質體。早或晚於五天移置則會導致原生質體之死亡或喪失活力。原生質體分離出後五小時即開始形成細胞壁。在 PES培養液中七至九天開始分裂，十四至十八天則形成細胞群。細胞群在 PES 培養液中可大量繁殖並產生少數葉狀體。本研究結果似可做爲青海菜養殖之另一方法。

關鍵詞：螢光劑；細胞團；青海菜；原生質體。