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Seedling production using enzymatically isolated thallus cells and its application in *Porphyra* cultivation

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Abstract

On the basis of previous achievements in *Porphyra* seedling production using enzymatically isolated thallus cells, we have investigated the influences of water content of thallus before freezing, storing temperature and enzymes on the viability of isolated cells. We have also tried outdoor cultivation of seedlings produced enzymatically in different seasons. It has been found that survival rate of isolated cells of thallus stored for two months can reach 90% if water content is 30% before freezing and storing temperature is constant at −20 °C. The 80% cell survival rate can be reached after two years of storage under this condition. The fluctuation of storing temperature causes drastic decrease of cell survival rate. 84% of the cells have survived digestion of sea snail enzyme I, which is the highest among five enzymes used. Outdoor cultivation of seedlings produced on the first ten days of January can reach lengths of more than 20 cm upon harvesting in the middle of May. In addition, seedlings produced in spring can be stored frozen and cultivated in autumn. The harvesting date will advance 15 days in comparison with the conventional seedling producing approach. This promises multiple rounds of cultivation of *Porphyra* within a year.

Introduction

Traditional approach of *Porphyra* seedling production consists of the following steps: carpospore collection in spring, indoor cultivation of conch filament and outdoor cultivation of conchospore in autumn. The seedling production using this approach takes about 4–5 months (Fei, 1999). Since carpospores come from natural populations, traditional seedling production is restricted by seasons and plagued with diseases. In addition, both identity and quality of thalli harvested at different times from once seeded nets are low, reducing drastically the product quality and the economic benefit of laver cultivation.

In order to overcome such disadvantages of conventional seedling production, enzyme purification, single cell and protoplast isolation from thallus and *Porphyra* regeneration from isolated cells and protoplasts were conducted in the early 1980s in our laboratory (Tang, 1982; Liu et al., 1984). Following these preliminary trials, enzymatic isolation of cells and protoplasts from thallus and *Porphyra* regeneration from isolated cells were conducted in the United States, Japan, Korea and other countries and regions (Polne-Fuller & Gibor, 1984; Chen, 1987; Araki et al., 1987; Song & Chung, 1988; Gall et al., 1993). We reported for the first time seedling production using isolated cells and successful outdoor cultivation of the cell seedlings in 1986 (Fang et al., 1986). This is followed by studies on cell isolation, cultivation, seedling regeneration and outdoor cultivation of *Porphyra* by other researchers (Wang et al., 1986, 1987). From that time on, we have carried out a series of basic studies on enzymatic seedling production of *Porphyra* (Dai & Bao, 1988; Dai et al., 1988, 1993). In order to utilize enzymatic seedling producing technique in *Porphyra* cultivation, we have optimized important factors that influence the viability of enzymatically isolated cells, and tried multiple round outdoor cultivation of cell seedlings in recent years. Our main findings in cell seedling production and its application in laver cultivation are presented in this paper.

Materials and methods

Collection and storage of Porphyra *foliose thallus*

The thalli of *Porphyra yezoensis* Ueda at nutritional growth stage were collected, air dried for various periods of time, sealed in plastic bags and stored in freezers preset at different temperatures. The water contents of thalli were assayed at 1 h interval.

Cultivation condition

Boiled seawater supplemented with 10 mg KNO_3-N and 1 mg KH_2PO_4-P per liter was used as medium. The irradiation was 2000 $\mu Em^{-2}\ s^{-1}$ for indoor cultivation. For cell seedling production, filtered seawater was used as the medium, and natural light was used as the light source.

Enzyme and cell isolation

Sea snail enzymes I and II were obtained from Ocean University of Qingdao. Three abalone (red, pink and black) enzyme acetone powders were purchased from Sigma Company.

The viability of freeze stored *Porphyra* thalli was recovered through incubation in sterilized seawater. When normal physiological condition was reached, the thalli were cleaned with pre-cooled boiled seawater, cut into pieces, and digested in solution containing 2% enzyme and 2 mol l^{-1} glucose for 2 h at 20 °C. The digestion solution was filtered through nylon mesh. The single cells and protoplasts in solution were collected by centrifugation, cultivated in laboratory and used for survival rate assaying. Collected cells and protoplasts were also diluted to proper concentration, sprayed onto nylon nets, cultivated in seedling producing ponds for a short period and then used for outdoor cultivation.

Results and discussion

Storage of cell-generating thallus

The influence of water content of thallus before freezing on cell viability

The storage of cell-generating thallus is one of the key steps for enzymatic seedling production of *Porphyra*. Seedlings with high viability can only be produced using properly stored thallus. Improper storage of thallus may cause the failure of the whole seedling producing process.

The water content of thallus drastically influences the viability of the isolated cells. Thalli with different water contents before freezing generated cells with variable viabilities after 2 months storage at −20 °C. All the cells from fresh thalli without drying died. The ideal water content of thallus was 20–40%, and cell survival rate can reach more than 90% when the pre-freezing water content of the thallus was about 30%. The majority of the cells died when water content was lower than 10%. The water contents out of the range of 20–40% were found to be harmful. High water content in cells may crystallize at low temperature, damaging cell structure and causing cell death. If water content is too low, cells may not be able to maintain normal metabolisms, triggering cell death as well.

Water content of thallus is adjusted by air-drying. Air-drying time influences cell viability as well. Since it was inconvenient to assay water content, we optimized air-drying time for practical use. At 20 °C and in normal air with moisture content ca. 65%, no significant changes of cell viability were observed within 12 h of drying. However, nearly half of the nutritional cells died if drying time was more than 24 h. An even larger proportion of these cells died when drying time was 48 h. The proper air drying time was found to be 4–8 h.

The influence of storing temperature on cell viability

Even when water content was properly maintained, changes of storing temperature could have drastic influences on cell viability. Almost all the cells of fresh thallus died within 3–5 days when storing temperature was about 20 °C, so did the cells of the thallus stored at 4 °C in about 15 days and at above −10 °C in 2 months. In contrast, the cell survival rate was very high when the thallus was stored at −20 °C. The storing temperature can vary between −20 °C and −30 °C without obvious cell survival rate changes. The proper storing temperature was found to be constant −20 °C. For example, the majority of the cells from thallus stored at −20 °C on Jan 10, 1996 were viable and could regenerate into blades when they were isolated 2 years later in October of 1998. However, the fluctuation of temperature during storage can cause drastic cell death.

Table 1. Comparison of average number of cell lines released and percentage survival rate in average of released cells among different enzymes

Enzymes	Number of cell lines released		Survival rate of cells after 2 days cultivation	
	5% concentration 1 h digestion	1% concentration 1 h digestion	5% concentration 3 h digestion	1% concentration 16 h digestion
Sea snail enzyme I	5–6	2–3	84	72
Sea snail enzyme II	3–5	1–2	55	45
Red abalone enzyme	6–7	2–3	81	68
Pink abalone enzyme	3	1	41	36
Black abalone enzyme	1	0	25*	48

*16 h digestion.

Effects of different enzymes on cell isolation

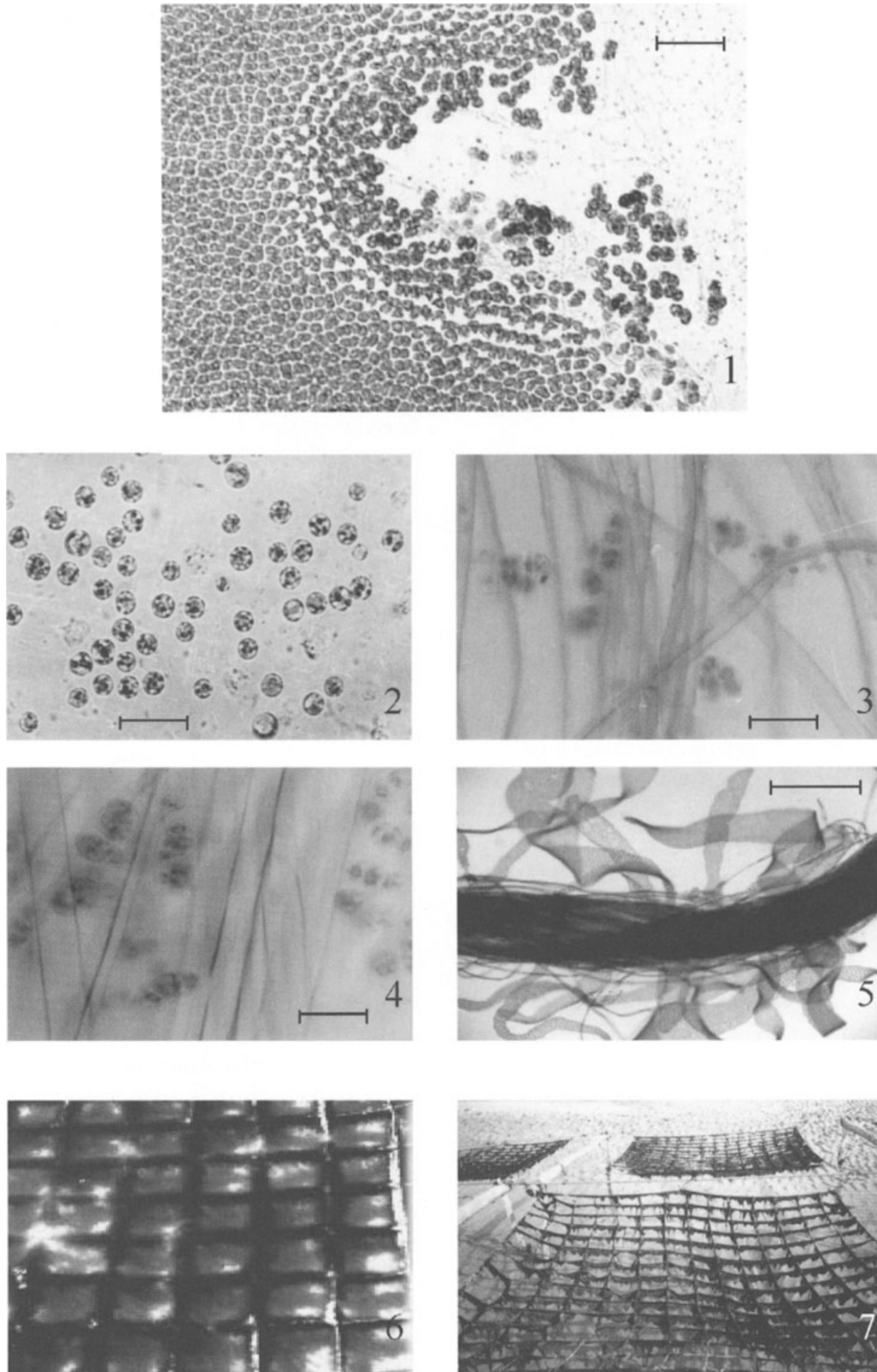
The cell isolation efficiencies of the five enzymes at different concentrations are shown in Table 1. At the same concentration, these five enzymes released different number of cell lines and produced variable number of viable cells. Among these five enzymes, red abalone enzyme and sea snail enzyme I showed best performances in releasing cells and retaining cell viability, while black abalone enzyme did worst. For the same enzyme, more cells would be released at high concentrations and these cells would retain high cell viability. In contrast, less cells would be released at low concentration and these cells also had decreased cell viability. The greatest number of viable cells was isolated with 5% red abalone enzyme and sea snail enzyme I in 3 h digestion.

Seedling production and outdoor cultivation

Cells in the outermost line around tissue pieces were released first, followed by those in the adjacent inner lines (Fig. 1). Within 2 h, somatic cells of *Porphyra* thallus pieces were digested completely into single cells and protoplasts (Fig. 2). Once being sprayed, the isolated cells started to adhere onto nylon nets in one day and to divide in 3–4 days (Fig. 3), forming young seedlings with 3–4 cells (Fig. 4). At the beginning of year 1994, we produced enzymatically seedlings from fresh *Porphyra* thallus and cultivated these seedlings in seedling producing ponds for about one month. On the last 10 days of January, the newly produced seedlings were transplanted into the sea and cultivated until the end of February. We checked the developmental performance of seedlings on Feb 28, and found that the seedlings had grown into sizes visible through naked eyes. At the beginning of May, all tender thalli reached harvesting sizes. In April 1994, we produced 1600 m^2 (net area) of seedlings, and reared these seedlings outdoor for 2 weeks. The seedling size reached lengths of 1–2 mm (Fig. 5). We harvested these seedlings, stored them in $-20\,^{\circ}$C cold room, and transferred them into the sea again on Oct 8. Four weeks later, the thalli size reached 7.5–8 cm length and 2.5 cm width on average, with 12 cm as the longest (Fig. 6). The thalli reached sizes of harvesting standard within 5 weeks of cultivation (Fig. 7). As control, the conchospore seedlings reached only 3.5–4 cm length within the same period. These trials have shown that high quality *Porphyra* could be produced in advance of normal growing season of *Porphyra* by using freeze stored nets with seedlings. It is also feasible that seedlings produced in winter can be cultivated in spring, which is not the normal growing season of *Porphyra*.

Conclusions

Seedling production using enzymatically isolated thallus cells of *Porphyra* is a new and advanced method in comparison with the conventional approach of conch filament cultivation. It has omitted filament cultivation, thus shortening the seedling producing time from 4–5 months to about 5 days. It is an asexual and clonal process, which can avoid genetic recombination and facilitate the retaining of desirable characters of parental thalli and the identity of the adult thalli. It is noticeable that enzymatic method can get rid of seasonal restriction, producing seedlings throughout the year and meeting the demand for seedlings for cultivation anytime. It makes multiple round cultivation of *Porphyra* possible, and utilizes the natural light and temperature more efficiently. The shift from traditional multiple harvesting from once seeded nets to



Figures 1–7. Seedling production and outdoor cultivation of *Porphyra*. 1. *Porphyra* cells released in one hour digestion using sea snail enzyme I, scale = 100 μm. 2. Isolated single cells and protoplasts, scale = 50 μm. 3. Cells divided into 2 cells in 2–3 days after adhering onto nylon nets, scale = 50 μm. 4. Young thalli with 3–4 cells after 3–4 days cultivation, scale = 50 μm. 5. Young seedlings with 1–2 mm lengths after 2 weeks cultivation, scale = 2 mm. 6. Thalli with 7–8 cm lengths after 40 days cultivation. 7. Lengths of *Porphyra* reaching 20–30 cm after 50 days cultivation.

newly developed multiple harvesting from repeatedly seeded nets promises high quality, high yield and high economic benefit of *Porphyra* cultivation. Seedling production using enzymatically isolated somatic cells is a revolutionary advancement for *Porphyra* cultivation. With its consummation and popularization, this approach will certainly bring us the most economic benefits in the future.

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