Aquaculture 281 (2008) 138–144

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Artificial production of regenerable free cells in the gametophyte of *Porphyra pseudolinearis* (Bangiales, Rhodophyceae)

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ARTICLE INFO

Article history:
Received 14 November 2007
Received in revised form 27 May 2008
Accepted 11 June 2008

Keywords:
Allantoin
Development
Growth
Monospore
Porphyra pseudolinearis

ABSTRACT

A dioecious red alga, *Porphyra pseudolinearis*, which does not produce asexual spores such as archeospore, is a commercially valuable *Porphyra* seaweed. We obtained a large amount of regenerable free cells of *P. pseudolinearia* by gentle homogenization in seawater after culturing the thalli in nutrient-rich medium with 10 mM of allantoin at 15 °C and 20 °C. The obtained free cells were divided into three forms: globular, elliptic and 2-celled. These free cells formed cell walls slowly compared to conchospores, and they directly developed to thalli or filamentous germlings like conchocelis or conchosporangia. In particular, the occurrence of thalli was remarkable in free cells obtained from the thalli cultured with 10 mM allantoin at 20 °C. Once the free cells developed to the thalli, they showed the same rates of growth to the thalli generated from conchospores, and they grew to be more than 50 cm in blade length. This technique to produce regenerable free cells from the *Porphyra* species that do not produce archeospores is useful for vegetative propagation and breeding.

1. Introduction

Porphyra plants are generally called “Nori” in Japan, and they are industrially important macrophytes used as sea vegetable foodstuffs. The seaweeds have been widely cultivated in Korea, China and Japan using natural seedlings since the late 17th century when the cultivation was initiated in Japan. After Drew (1949) discovered the conchocelis phase of the life cycle, Hollenberg (1958) obtained liberated conchospores from conchocelis with conchosporangia.

Artificial seedlings of conchospores were first utilized in Japan in about 1960, and the production of *Porphyra* has increased with the development of the cultivation technique (Oohusa, 1984). Worldwide production reached 130,614 t dry weight for the year 1994/1995 (Zemke-White and Ohno, 1999). In 2004, world aquaculture production of *Porphyra* reached 1.4 million tones wet weight (FAO, 2006).

Alternation of the generation of *Porphyra* is generally accomplished with the macroscopic gametophyte and microscopic sporophyte (conchocelis) phases. There are some species, however, that form asexual reproductive spores, which are called archeospore. An archeospore is defined as a spore produced vegetatively from the parent thallus cell (Nelson et al., 1999). *Porphyra yezoensis* is a representative specimen that forms archeospores. Ecologically speaking, archeospores play an important role in the propagation of the biomass of the seaweed (Li, 1984). Therefore, this reproductive advantage is useful for seedling production, breeding and selection in the field of aquaculture.

On the other hand, there are a few industrially important species in *Porphyra* plants that do not produce archeospores such as *P. pseudolinearis* (Fukuhara, 1968; Kim, 1999). This plant is a valuable dioecious species that has a good taste and a resistance to the bacteria that induce Akagusare disease (Uppalapati and Fujita, 2001). Therefore, attempts to cultivate this alga have been made in Alaska (Stekoll et al., 1999) and Korea (Park et al., 2003). In Japan, this alga is distributed in the upper tidal zone of the coastal regions of the Japan Sea from Hokkaido to Honsyu, and its product is called “Iwa-nori,” meaning “*Porphyra* in rock.”

Vegetative propagation of *Porphyra* thalli is divided into two techniques, namely protoplast and archeospore production. Since selective breeding has been carried out, protoplast production was established (Polne-Fuller et al., 1984; Saga and Sakai, 1984; Waaland et al., 1990; Gall et al., 1993; Packer. 1994; Chen et al., 1994). Crossbreeding using cell fusion has also been reported by many investigators (Fujita and Migita, 1987; Fujita and Saito, 1990; Saito and Fujita, 1991; Araki and Morishita, 1990; Achiha and Fuseya, 1995; Achiha and Nakashima, 1995; Kito et al., 1998). At present, the protoplast production is generally carried out by a method in which enzymes, such as papain, mannanase, xylanase, agarase, etc., digest the cell wall. These procedures require great skill and are time-consuming for the regeneration. If the protoplast is produced at low cost and by a simple procedure, the technique is powerful for breeding. On the contrary, attempts have been made to utilize archeospores for breeding. Archeospore production was promoted by high light intensity in *P. yezoensis* (Li, 1984)

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doi:10.1016/j.aquaculture.2008.06.012

and by high water temperature in *P. tenera* (Notoya et al., 1993). Kitade et al. (1998) reported in *P. yezoensis* that archeospore isolation achieved by a simultaneous change in water temperature and light conditions. Moreover, segmentation of the thallus was effective for releasing archeospores (Notoya, 1997). However, these environmental control of archeospore production is limited to some species that form and release archeospores.

Recently, we reported a simple method for production of a large amount of archeospores in *P. yezoensis* (Mizuta et al., 2003). This method is based on two processes, the induction of archeosporangium formation by culturing with allantoin in nutrient-rich medium, and the destruction of cell wall by mild homogenization. The objective of this work was to test the use of allantoin to obtain free cells from *P. pseudolinearis*. In addition, we observed the development of free cells and compared it to the general development of conchospores in this alga.

2. Materials and methods

2.1. Materials and pre-culture

Gametophytes (4–20 cm in blade length) of *Porphyra pseudolinearis* were collected in the upper tidal zone of the coastal regions near Hakodate, Hokkaido, Japan from November 2003 to January 2004 and in November 2004. The collected thalli were transported to our laboratory and washed with paper towels and sterilized seawater to remove the attached matter. After the mature male and female gametophytes with spermatangia and zygotosporangia, respectively, were selected in the collected samples, the thalli were placed in a polystyrene case (square type: 8.5 cm×17.5 cm×3.5 cm) and cultured in 10 °C and 60 μmol photons m^{-2} s^{-1} under a period of 12L:12D (Light:Dark) so zygotospores could be obtained. The culture of zygotospores and the collection of conchospores were done according to Baba et al. (2001). The zygotospores were cultured in vitamin-free Provasoli's enriched seawater (PES, Provasoli, 1968) at 10 °C and 60 μmol photons m^{-2} s^{-1} under 12L:12D for about 1 week. The developed conchocelis were cultured in 50 ml of vitamin-free PES at 20 °C and 60 μmol photons m^{-2} s^{-1} under 12L:12D until they produced conchosporangia. To release the conchospores, the conchocelis colonies were transferred to 15 °C and 60 μmol photons m^{-2} s^{-1} under 8L:16D and cultured under aeration. The obtained conchospores were cultured to form the thallus (ca. 1–2 cm in length) in the same conditions. The seawater used in all cultures was filtered with a glass fiber filter (GA-100, Advantec Co.) and autoclaved at 121 °C for 20 min. All culturing was done using vitamin-free PES medium with GeO_2 1.25 mg L^{-1} to inhibit the propagation of diatoms (Lewin, 1966) in an incubator (MIR-253, SANYO).

2.2. Induction of regenerable free cells

To investigate the optimal concentration of allantoin, immature thalli (ca. 2 cm in length) without spermatangia and zygotosporangia were collected under an inverted microscope (TMS, Nikon). Six discs (2 mm in diameter) were cut with a cork bore from the central and basal parts of the thallus. Prior to experimental culture, the discs were cultured at 15 °C and 60 μmol photons m^{-2} s^{-1} under 12L:12D for 3 days. Each disc was placed in 2 ml of vitamin-free PES medium adjusted with 0, 10^{-3}, 10^{-2}, 10^{-1}, 1, and 10 mM allantoin (Wako Pure Chemical Industries Ltd.), and they were then batch-cultured at 10 °C and 60 μmol photons m^{-2} s^{-1} under 12L:12D. The medium was renewed every week and the cells of the discs were observed with an inverted microscope (ALPHAPHOT YS, Nikon). Discs were photographed with a digital camera (CAMEDIA C-5050 ZOOM, OLYMPYS) in order to estimate the growth rate based on the expansion of area using computer software (CANBAS, Deneba Systems). After 6 weeks of culturing, six discs in each concentration were supplied for production of free cells in the lump as described below. The number of obtained free cells was then counted under an inverted microscope to estimate the average number of free cells. These free cells were cultured at 10 °C and 60 μmol photons m^{-2} s^{-1} under 12L:12D for 3 days, and they were observed every day under an inverted microscope.

The immature thalli (ca. 1 cm in length, ca. 0.08 cm^2) were also cultured in 5 ml of vitamin-free PES medium containing 10 mM allantoin under four water temperature conditions (5, 10, 15 and 20 °C). The culture was done at 100 μmol photons m^{-2} s^{-1} under 12L:12D for 3 weeks. Every week, the medium was renewed. After 3 weeks, thalli were used for the production of free cells.

The discs of the thallus and the whole plants were placed in sterilized seawater containing 0.1% of popiyodone solution (Yoshida Pharmaceutical Co., Ltd.) for 2–3 min and washed with sterilized seawater. Each thallus was homogenized with 5 ml of sterilized seawater in a glass homogenizer (50 ml) in ice at about 100 rpm. The obtained cell suspension solution was filtered with nylon net (Mesh Opening 80 μm, TANAKA SANJIRO CO., LTD) to remove large-sized items such as mass of cell walls. The suspension solution of free cells was transported into 100 ml of medium in a polystyrene case and cultured at 10 °C and 100 μmol photons m^{-2} s^{-1} under a neutral day. After 10 days of culture, the early development of free cells was observed with an inverted microscope, and the numbers of non-germling cells, filamentous cells and thalli were counted in twelve randomly selected areas (1.3×1.3 cm^2) of the bottom of the vessel. The mean numbers were used to estimate the total number per disc or thallus by multiplying them by the ratio of the bottom area/observed area.

2.3. Early development and growth of conchospore and free cells

The free cells obtained by the above-mentioned procedure were collected with a pipette, and each 2–3 cells were inserted into 2 ml medium in a well of cell culture ware. In a similar manner, conchospores released from conchosporangia were also placed in other wells. After the sizes of the free cells and conchospores were measured using a microscope, they were cultured at 10 °C and 100 μmol photons m^{-2} s^{-1} under 12L:12D. The early development of these cells was observed with an inverted microscope, and photographs were taken with a digital camera (COOLPIX 5000, Nikon). To observe the formation of the cell wall, the cells were suitably transported onto a slide glass by a micropipette and were stained with 1% Calcofluor white M2R (Sigma Chemical Co.) (Darken, 1961; Paton and Ayres, 1964). The observation was carried out using a fluorescence microscope (VANOX-S, Olympus) with a digital camera (COOLPIX 990, Nikon).

The growth activities of the thalli gerenated from free cells were examined to evaluate their quality and health as seedling. Groups of 6 gametophytic thalli (ca.1 cm in length) generated from conchospores and free cells were cultured in 100 ml of medium at 15 °C under 116 μmol photons m^{-2} s^{-1} under neutral day conditions (12L:12D) for 5 weeks. The medium was renewed every day, and the gametophytes were observed with an inverted microscope. The area of the thallus was observed using a digital camera (COOLPIX 950, Nikon) and estimated using a software. The relative growth rate based on area was calculated with the same manner to the method described in Section 2.2.

After the experimental culture, we kept culturing the thalli, which originated from free cells, in 2-L medium at 15 °C at 40 μmol photons m^{-2} s^{-1} under 12L:12D using a simple culture vessel (Fukuda et al., 2004) for 3 months.

2.4. Growth measurement of thalli

Based on the data of the blade length and area, the relative growth rate was calculated by the following equation (DeBoer et al., 1978),

$$\text{Relative growth rate}\,(\%\,\text{day}^{-1}) = 100[\ln(N_t/N_0)]/t$$

where, N_0 represents the area or blade length at the start of culturing, N_t is the area or blade length t days after the start of culturing, and t is the culture period (days). Data were expressed as the mean±standard deviation, and statistical analysis was performed using Student's t-test. Results were significant for $p<0.05$.