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Cryopreservation of brown algae gametophytes of *Undaria pinnatifida* by encapsulation–vitrification

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ABSTRACT

The cryopreservation of gametophytes of *Undaria pinnatifida* by encapsulation–vitrification was studied. The effect of the composition of the vitrification solution (VS), the composition and concentration of the loading solution (LS), the duration of loading and dehydration treatments and the washing method on viability of algae were investigated. The highest viability index for female and male gametophytes was 31.2% and 26.0%, respectively, which were obtained by loading alginate beads with a mixture of 2 M glycerol and 0.6 M sucrose for 120 and 90 min at 25 °C, dehydrated with PVS2 for 50 and 40 min at 0 °C and washed with a 1.2 M sucrose solution. Intact sporophytes developed from gametophytes cryopreserved have been observed.

1. Introduction

Cryopreservation offers a means of long-term, stable preservation of germplasm and experimental materials without the problems by routine subculture, which is time consuming, requires much labor and a large space, the possibility of genetic alteration during subculture (Sakai et al., 2000; Nawroth et al., 2002; Müller et al., 2007). A variety of organisms and cells were cryopreserved successfully in liquid nitrogen (LN) (Leunufna and Keller, 2003; Hongquan et al., 2004; Blanco et al., 2008; Cabrita et al., 2009). Marine algae represent a rich and diverse genetic resource and the interest in their biotechnology potential is increasing rapidly (Takashi and Takahiko, 2009). Seaweeds are extensively used as food and food additives by coastal peoples. Carrageenans, alginates and agaroses, together with other little-known macroalgal polysaccharides, also have several important biological activities or applications in biomedicine (Jens & Karlsson, 1996; Jin-Chywan et al., 2005; Takashi and Takahiko, 2009). These species are mainly red, green, and brown algae. Culture vigor, and nutritional value may change over time in culture and preservation of these isolates and strains is therefore desirable (Kuwano et al., 1993; Liu et al., 2004). Brown alga, *Undaria pinnatifida*, is widely used in aquaculture industries in China and worldwide (Arbault et al., 1990; Ginsburger-Vogel et al., 1992; Taylor and Fletcher, 1999; Qihua et al., 2005b). Among several cryopreservation protocols, the routine two-step cryopreservation method used for some algal strains requires special cooling equipment and cryoprotective additives toxic to biomaterials, thus restraining its application (Engelmann, 2004; Hirano et al., 2005). New cryopreservation methods, including encapsulation–dehydration, droplet freezing and vitrification which originate from the beginning of 90s have been used for germplasm maintenance (Hirai and Sakai, 1999; Sakai et al., 2000; Sakai and Engelmann, 2007; Harding et al., 2010). The encapsulation–vitrification method, which has been developed recently, has shown various advantages over encapsulation–dehydration and has been successfully used in 20 species of plants (Sakai and Engelmann, 2007; Kaczmarczyk et al., 2008; Quansheng et al., 2008), which shows great application potential, and has been applied mainly to cryopreservation of shoot tips with very few applications to cell suspensions (Yuanlong et al., 2005; Sakai and Engelmann, 2007). The present study was, therefore, undertaken to standardize an effective encapsulation–vitrification procedure for gametophytes of *U. pinnatifida*.

2. Materials and methods

2.1. Gametophytes of U. pinnatifida culture

Gametophytes of *U. pinnatifida* were obtained from the Institute of Oceanology of Chinese Academy of Sciences. The cultures of algae to be assessed for cryopreservation successfully were maintained in 250 ml flasks in standard PES media (Qihua et al., 2005a) at room temperature (about 23 ± 2 °C) under a light intensity of 25 μmol-photon m^{-2} s^{-1} from cool white fluorescent tubes with a 14 h light/

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10 h dark photoperiod. Filaments were harvested after 14 days by centrifugation (3450 rpm), and then immobilized in alginate beads for cryo-treatments after 1 d darkness and 3 d culture as above.

2.2. Encapsulation–vitrification

The scheme of encapsulation–vitrification method was outlined in Fig. 1.

A 1:1 (v/v) ratio of algal filament suspension (0.08 g FW/ml) and 6% sodium alginate solution with 30‰ (w/v) sodium chloride were mixed together. The mixture was dripped into a 0.1 M calcium chloride solution with a sterile syringe. Bead diameter was 2.5 mm. After 1 h, beads were retrieved with a spatula and washed twice with sterile seawater. They remained in seawater in the dark for 1 day before being subjected to the cryo-treatment.

2.2.1. Loading

Thirty beads were put in 20 ml of loading solution consisting of diluted vitrification solution (VS) in 50 ml flasks at 25 °C for 60 min. The composition of loading solution (LS) and VS employed is shown in Table 2. After the optimum LS was selected, the optimal duration of the loading treatment (0, 30, 60, 90, 120, and 150 min) was determined. The algae beads were dehydrated with VS, put in cryotubes, immersed in LN and stored at −196 °C. After 24 h in LN, algae beads were rewarmed by immersing the cryotubes in a water-bath at 40 °C for 1.5 min, transferred in the washing solution (WS), then put in culture medium for 8 days and viability determination was carried out thereafter.

2.2.2. Dehydration

The beads were dehydrated at 0 °C for 40 min with different VS, put in cryotubes, immersed in LN and stored at −196 °C. For determining the optimal dehydration duration, beads were dehydrated with the optimal VS for 0, 10, 20, 30, 40, 50 and 60 min, respectively.

2.2.3. Washing

Two types of washing procedures were used: (1) one step washing with 1.0, 1.2, and 1.4 M sorbitol or sucrose in PES medium for 20 min; or (2) two step washing. While washing time selected were 0, 15, 20, 25 min each step. Then algae beads were transferred to cultured medium.

2.3. Viability determination and development observation after cryopreservation

After storage in LN, algal beads were placed in the dark for 1 day, then for 8 days cultured in PES medium at room temperature (about 23 ± 2 °C) under a light intensity of 25 μmol photon m^{-2} s^{-1} from cool white fluorescent tubes with a 14 h light/10 h dark photoperiod for viability determination. After algae beads were recultured, the Chlorophyll a concentration of the immobilized cells was determined by extraction with acetone. The value of the extraction solution was determined at wavelength of 666 nm and 730 nm by spectrophotometer. Viability was calculated as viability index according to the Chlorophyll a levels as a percent of control (Qihua et al., 2005b). After 14 days of recovery culture, gametophytes of *U. pinnatifida* were mixed at the ratio of ♀:♂ = 1:1 (v/v). Then gametophytes were cultured under a light intensity of 40 μmol photon m^{-2} s^{-1} for 7–14 days at 18 °C with 12 h light/12 h dark photoperiod. Ggametophytes and the developing sporophytes were determined microscopically.

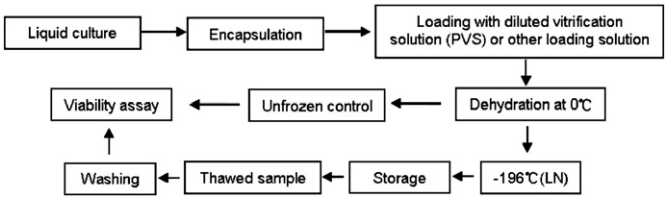

Fig. 1. Procedure for the encapsulation–vitrification technique for cryopreservation.

Table 1
Effect of vitrification solutions on the viability index (%) of gametophytes of *U. pinnatifida*.

Loading solution	Female	Male
25% PVS2	9.0 ± 0.9^{e}	5.8 ± 0.9^{e}
60% PVS2	15.5 ± 0.8^{b}	12.8 ± 0.8^{b}
70% PVS2	9.3 ± 1.0^{e}	8.0 ± 0.5^{d}
2 M GLY + 0.4 M SUC	13.9 ± 0.6^{c}	10.0 ± 0.5^{c}
2 M GLY + 0.6 M SUC	19.0 ± 1.1^{a}	17.1 ± 1.4^{a}
2 M GLY + 0.8 M SUC	10.8 ± 0.6^{d}	9.1 ± 0.7^{c}

Encapsulated gametophytes of *U. pinnatifida* were loaded with different vitrification solution 60 min, dehydrated with 100% PVS2 for 40 min, frozen and rewarmed rapidly, washed with 1.2 M sucrose for 20 min. Values indicate the mean of viability index (%) ± SD; numbers followed by the same letter in lines are not significantly different ($P<0.05$).

The chlorophyll a content data were subjected to analysis of variance and multiple comparison tests for the three independent experiments performed. Numbers followed by the same letter in viability index determination tables are not significantly different ($P<0.05$).

3. Results

3.1. The influences of loading procedure on viability

The optimum LS for gametophytes of *U. pinnatifida* is shown in Table 1. The highest viability indexes achieved were 19.0% (female gametophytes) and 17.1% (male gametophytes) loading with 2 M glycerol and 0.6 M sucrose. When loaded with 60% PVS2, the highest viability indexes were 15.5% and 12.8% for female and male gametophytes of *U. pinnatifida*.

Female and male gametophytes of *U. pinnatifida* both obtained higher viability index with glycerol and sucrose with fitting Me_2SO concentration of 9% (Tables 1 and 2). With 4% and 11% Me_2SO concentration, lower index was obtained. Too high or too low Me_2SO concentration can lead to low viability.

The viability index of gametophytes of *U. pinnatifida* increased in line with increasing durations of the loading treatment (Fig. 2).

Table 2
Effect of various vitrification solutions on the viability index (%) of gametophytes of *U. pinnatifida and* composition of the vitrification solutions employed during the encapsulation–vitrification experiments.

Vitrification solution	Components	Female	Male
VS1	30% GLY + 15% EG + 10% Me_2SO*	9.8 ± 0.6^{d}	4.8 ± 1.0^{e}
PVS2	30% GLY + 15% EG + 15% Me_2SO*	26.1 ± 1.2^{a}	22.1 ± 2.0^{a}
VS3	30% GLY + 15% EG + 20% Me_2SO*	16.8 ± 1.9^{c}	9.4 ± 1.2^{d}
VS4	30% GLY + 15% EG + 25% Me_2SO*	15.5 ± 0.6^{c}	11.8 ± 1.5^{c}
VS5	50% GLY + 50% SUC**	19.7 ± 1.3^{b}	14.4 ± 1.7^{b}
VS6	30% GLY + 10% Me_2SO + 10% SUC***	5.7 ± 2.3^{e}	5.5 ± 2.3^{e}

Encapsulated gametophytes of *U. pinnatifida* were loaded with 2 M GLY plus 0.6 M SUC 120 min (female), 90 min (male) at 25 °C, dehydrated with different vitrification solution for 40 min, frozen and rewarmed rapidly, washed with 1.2 M sucrose twice for 20 min. Values indicate the mean of viability index (%) ± SD; numbers followed by the same letter in lines are not significantly different ($P<0.05$). GLY, glycerol; EG, ethylene glycol; Me_2SO, dimethyl sulfoxide; SUC, sucrose.

* Plus 0.4 M SUC in PES culture medium.
** Plus 0.6 M SUC in PES culture medium.
*** In PES culture medium;% w/v.