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Short communication

Non-cryogenic preservation of thalli, germlings, and gametes of the green seaweed *Ulva rigida*

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ABSTRACT

There is a need to develop reliable and cost-effective preservation techniques that support the growing demand for *Ulva* cultivation. Using combinations of two dimethyl sulfoxide concentrations (DMSO; 10 and 15%), three non-cryogenic temperatures (4, −20 and −80 °C), and one- and two-step cooling, we evaluated the effectiveness of seven methods for preserving *Ulva* thalli, germlings, and gametes for periods of up to 184 days. Preservation success was assessed using post-thaw regrowth and a pigmentation index. Refrigeration at 4 °C without DMSO successfully preserved thalli for 184 days with little loss of plant quality. In contrast, the freezing methods produced low regrowth success and poor quality thallus tissue, with few plants surviving beyond 30 storage days. Freezing temperatures were lethal to germlings after storage for only one day, but germlings preserved at 4 °C had regrowth rates comparable to thalli preserved under the same conditions. In contrast, *Ulva* gametes were successfully preserved at freezing temperatures for 184 days although viability was relatively low (7.0–18.7% at −20 °C and 3.5–12.1% at −80 °C). Gamete viability at 4 °C decreased from 94.74 ± 4.30% to 26.12 ± 3.97% when the storage time was extended from one to 184 days. DMSO (10%) reduced gamete viability both at −20 °C and −80 °C. We demonstrate for the first time, the contrasting responses of *Ulva* thalli, germlings, and gametes to different non-cryogenic preservation methods.

1. Introduction

Having the capacity to reliably preserve live algae tissue and cell lines will advance seaweed cultivation, particularly in a hatchery context. Many microalgae have been successfully cryopreserved and the approach is now commonplace in culture collections (Day, 1998). In comparison, the preservation of seaweeds is less advanced and literature records are few.

Porphyra (both conchocelis and thalli) (Kuwano et al., 1992, 1993, 1994, 1996; Migita, 1964, 1966, 1967), *Undaria* (Arbault et al., 1990; Ginsburger-Vogel et al., 1992; Renard et al., 1992), and *Laminaria* (Vigneron et al., 1997) species have been preserved using two-step methods. Combination of cryoprotectants with a two-step cooling method have produced >60% survival of *Porphyra* conchocelis stored in liquid nitrogen for one day (Kuwano et al., 1993) and 54.6–70.9% for sporothalli (Jo et al., 2003). The viability of gametophytic thalli of *P. yezoensis* and *P. teneru* has exceeded 95% and in some cases 98% (Kuwano et al., 1996). Encapsulation-vitrification has also been used to preserve seaweeds (Wang et al., 2011; Zhuang et al., 2015). Few studies report seaweed preservation at non-cryogenic temperatures (above −150 °C), *e.g.* Migita (1966) and Kuwano et al. (1992), with viability being generally low and decreasing with storage time.

As far as *Ulva* is concerned, reports are mainly confined to preserving zoospores at both cryogenic and non-cryogenic temperatures (Bhattarai et al., 2007; Taylor and Fletcher, 1999b) with the best survival rates at 4 °C (1.4–5.8% after ten days storage). With respect to *Ulva* thalli, Van der Meer and Simpson (1984) cryopreserved *U. lactuca* for at least one hour with 100% survival, and Lee and Nam (2016) cryopreserved gametophytic *U. prolifera* thalli for 120 days achieving viabilities of over 90%.

Our understanding of *Ulva* preservation is poor and this may create a bottleneck for future expansion of the industry. This study used different methods to preserve *U. rigida* gametes, germlings and adults in an attempt to define an optimal non-cryogenic preservation method.

2. Materials and methods

Adult vegetative *Ulva rigida* was collected intertidally from Cullercoats, eastern coast of England (55.03° N, 1.43° W) after a spring tide in June 2014. The thalli were punched into 7 mm diameter disks after rinsing in 1 μm filtered seawater. Gametes were obtained by fragmentation (Hiraoka and Enomoto, 1998) and concentrated using a point light source. Some (1×10^6) of these gametes were used to

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produce germlings. After settlement and germination, 387 germlings of 2 mm length were selected for preservation trials.

DMSO concentrations of 10 and 15% were used for thalli and germlings and 5 and 10% for gametes. Single *Ulva* discs or germlings were placed into 1.5 ml freezing ampoules containing DMSO, with 54 ampoules per temperature treatment; nine of which were assessed for viability after every storage period (one, two, seven, 30, 92, and 184 days). DMSO pretreatment was conducted at 20 °C for 20 min. After pretreatment, two batches were immediately transferred to −20 and −80 °C respectively. Another batch was frozen to −20 °C at a cooling rate of −1 °C minute^{-1} and then immediately transferred to −80 °C. A batch of samples free from cryoprotectant or pretreatment was placed directly into a 4 °C refrigerator in 100 ml conical flasks and the medium was renewed monthly, and 75% ethanol cotton balls were used to remove any bacterial biofilm. Nine *Ulva* discs and germlings without any preservation treatment were set as a control. Gamete preservation was carried out using a similar method with the omission of the two-step cooling. Fifty four ampoules were used for every temperature treatment, nine of which were assessed for viability after every storage period. Each ampoule contained 2.5×10^6 gametes. Nine ampoules without any preservation treatment were set as a control. Cryopreservation with liquid nitrogen was not employed given its cost in long-term preservation and low success rates in preliminary experiments (data not shown).

After each storage period, the samples were thawed by plunging the ampoules into a 37 °C water bath. As soon as the ice had melted, the cryoprotectant was removed and the samples were repeatedly washed with fresh seawater. Afterwards, thalli and germlings were placed in 100 ml conical flasks (one thallus or germling per flask) and cultured at 18 °C, 80 μmol m^{-2} s^{-1} photon light density, with a 16L: 8D photoperiod for six days. The growth medium was natural seawater supplemented with 150 μM nitrate and 7.5 μM phosphate, and renewed every two days. Gametes were placed in Petri dishes in darkness for 24 h to ensure settlement and then incubated under the conditions described above.

Viability was assessed using two methods; a pigmentation index (PI) (Van der Meer and Simpson, 1984), and regrowth success. Part or all of the pigmentation of *Ulva* can be lost due to freezing damage. Regrowth was estimated by recording specific growth rate (SGR) over six days at 18 °C. SGR (%) = $[\ln (M_2/M_1)]/t \times 100$, where M_2 is the final mass, M_1 is the initial mass, and t is the number of culture days.

After 14 days of culture at 18 °C, gametes were examined by light microscopy and viability was determined by the level of germination (gametes that had divided). The mean percentage germination over nine fields of view (30–40 gametes per field) was recorded. Viability was expressed as a relative rate in order to eliminate effects of low gamete settlement. V = Gt/Gi × 100, where V is the viability of samples, Gt is the germination rate of treated samples and Gi is the germination rate of the reference treatment.

Results were expressed as means ± standard deviation for continuous data and medians ± interquartile range for categorical data. The effects of preservation time on PI were analysed by Friedman tests with Wilcoxon Signed Rank *post hoc* tests. Temperature and DMSO effects were analysed using Kruskal-Wallis tests with Mann-Whitney *post hoc* tests. Regrowth rates were normally distributed (Shapiro-Wilk, $P > 0.05$) and had equal variances (Levene's test, $F = 1.457$, $P > 0.05$ for thalli and $F = 1.329$, $P > 0.05$ for germlings). Differences in thalli regrowth were analysed by two-way ANOVA at different time points. The effects of preservation temperature, time and DMSO on thallus regrowth were analysed by three-way ANOVA with Tukey honest significant difference (HSD) excluding that at 4 °C which was analysed by one-way ANOVA with Tukey HSD. Viability of gametes was normally distributed (Shapiro-Wilk, $P > 0.05$) with equal variances (Levene's test, $F = 1.117$, $P > 0.05$). Differences in viability between treatments at different time points were analysed by two-way ANOVA with Tukey HSD with three-way ANOVA used to assess the effects of preservation temperature, time and DMSO, excluding that at 4 °C. Data were analysed using SPSS v.21.

3. Results

The effect of preservation method on the pigmentation index (PI) is given in Table 1. There was a significant difference in PI between preservation times but patterns were different for each temperature (Friedman test, $K > 35.000$, $df = 5$, $P < 0.001$). The PI of samples preserved at −80 °C reduced to zero (dead) by day seven (Wilcoxon Signed Rank test, $Z < -2.700$, $n = 9$, $P < 0.05$) whereas it was above 6.0 for samples stored at −20 °C and 10 at 4 °C. The PI of samples stored at −20 °C decreased to zero (Wilcoxon Signed Rank test, $Z < -2.750$, $n = 9$, $P < 0.05$) by day 30 while samples stored at 4 °C maintained a high PI by day 184. There were significant differences in PI between preservation temperatures regardless of preservation times (Kruskal-Wallis test, $K > 50.557$, $df = 6$, $P < 0.001$). In contrast, samples stored at −80 °C for one day exhibited an extremely low PI. There were no significant differences between the thalli PIs between the two DMSO concentrations except for samples preserved at −20 °C for one day (Mann-Whitney *U* test, $U = 17.500$, $Z = -2.136$, $n = 9$, $P < 0.05$). The 15% DMSO concentration enhanced the PI of thalli preserved at −20 °C for one day (Table 1). The two-step cooling process to −80 °C did not enhance the PI compared with rapid cooling.

Thallus regrowth data are presented in Table 2; treatment and time had an interactive effect on regrowth (Supplemental Table 1), indicating the differences in regrowth between times were not the same for different preservation treatments. For instance, the regrowth of thalli preserved at −80 °C decreased to near zero by day seven, whereas thalli preserved at −20 °C regrew by day 30. In contrast, regrowth of thalli preserved at −4 °C had a high value even after 184 days (Table 2). The highest regrowth rate (23.09 ± 2.23%) was at −4 °C and it decreased to 3.59 ± 3.98% (15% DMSO) and 2.60 ± 3.10% (10% DMSO) at −20 °C. There were no significant differences between two-step and rapid cooling. The 15% DMSO concentration enhanced regrowth more noticeably at −20 °C (Tables 2 and Supplemental Table 2).

Germlings were more vulnerable to freezing temperature than thalli. The PI was zero at −20 and −80 °C after one day of storage although the PI of germlings preserved at 4 °C remained high until day 30 (Supplemental Table 3). PI was also affected by storage duration (Friedman test, $K = 25.159$, $df = 5$, $P < 0.001$).

Regrowth of germlings preserved at −20 °C, −20 °C then −80 °C, and −80 °C was not measured due to the PI score of zero (Table 3). Germlings preserved at 4 °C had significant differences in regrowth between storage times (ANOVA, $F = 50.698$, $df = 5, 48$, $P < 0.001$); it had increased by 18.42% after day seven (Tukey HSD, $P < 0.001$) and had a further 10.46% rise by day 30 (Tukey HSD, $P < 0.001$). However, it decreased to 21.8 ± 3.0% for 92-day preservation (Tukey HSD, $P < 0.001$).

Preservation treatment and time interacted to affect gamete viability (Fig. 1, Supplemental Tables 4 and 5). For instance, the viability of gametes preserved for one day at 4 °C, −20 °C with 5% DMSO, and −80 °C with 5% DMSO were 94.74 ± 4.30%, 56.14 ± 5.26%, and 12.48 ± 3.97%, respectively (Fig. 1). The highest viability (60.00 ± 23.32%) was at 4 °C.

4. Discussion

Successful preservation of seaweed thalli has been documented for relatively short-term storage duration (Jo et al., 2003; Kuwano et al., 1993, 1996). Long-term preservation is poorly documented, although sporelings and apical segments of mature thalli of *Gracilaria foliiferae* were successfully stored in liquid nitrogen for four years (Van der Meer and Simpson, 1984). In the present study, cryopreservation with liquid nitrogen was not employed due to its failure in a preliminary experiment (data not shown). Instead, *Ulva rigida* was preserved at 4 °C and the freezing temperatures of −20 and −80 °C. Thallus survival decreased with time at −20 or −80 °C; all thalli were dead after seven