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Review

Germplasm cryopreservation of macroalgae for aquaculture breeding and natural resource conservation: A review

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ABSTRACT

The expansion of the global macroalgal aquaculture and climate change creates the need for germplasm preservation of valuable aquaculture strains and maintenance of natural biodiversity. Compared to the large number of studies in fish and shellfish species, relative few studies have been conducted on the macroalgal germplasm cryopreservation. The first cryopreservation of macroalgae to −75 °C was reported on *Neopyropia tenera* (formerly called *Porphyra tenera*) in 1964. To date, a total of 34 studies reported germplasm cryopreservation in 33 species, including Chlorophyta (7 species), Ochrophyta (14 species), and Rhodophyta (12 species). The goal of this review was to summarize the published studies on macroalgal germplasm cryopreservation, compare the reported protocols for the cryopreservation process, and identify the factors affecting post-thaw viability. Overall, macroalgal germplasm cryopreservation included haploid or diploid thalli, spores, and gametes. Cryotubes (1.5-ml or 2-ml) have been widely used to package germplasm samples for cooling and storage in most studies, and the 0.5-ml straws and 5-ml cryotubes have been used in several studies. Two approaches (programmable controlled cooling and vitrification) were employed for macroalgal germplasm cryopreservation. A two-step programmable controlled cooling (e.g., from initial culture temperature to a frozen temperature, such as −40 °C, and then directly plunging into liquid nitrogen at −196 °C) was determined to be an effective cooling strategy. Vitrification, a super rapid cooling for a sample to form non-crystalline amorphous solid, was applied on macroalgal germplasm cryopreservation with sample encapsulation and dehydration. Survival of post-thaw samples varied significantly in different studies. Based on research updates, recommendations are made for future research. It is expected that this review can serve as a foundation for future germplasm banking of macroalgae for aquaculture and biodiversity preservation.

1. Introduction

Macroalgae, commonly known as seaweeds, are multicellular marine green, red, and brown algae consisting of complex life cycles, which include multicellular or siphonous macrothalli (Hurd et al., 2014). They vary in size from a few millimeters to ~60 m (e.g., *Macrocystis pyrifera*) (Schiel and Foster, 2015). Based on the pigments in the chloroplast, macroalgae are classified into three groups: Chlorophyta (green algae), Ochrophyta (brown algae), and Rhodophyta (red algae) (Baweja et al., 2016; Graham et al., 2019). Macroalgae play a significant role in the ecosystem as ecological engineers (Umanzor et al., 2019), primary producers (Rosenzweig et al., 2008), habitat and structure providers (Dayton et al., 1984), nutrient cyclers (Paine, 1969), ecosystem services (Neori et al., 2004; Kim et al., 2014, 2015; Kim et al., 2017; Park et al., 2018; Kim et al., 2019; Park et al., 2021; Racine et al., 2021); essential connectors in the food chain for invertebrates and pelagic organisms, and shoreline buffers from storms (Steneck et al., 2002; Smale et al., 2013). Furthermore, some macroalgae also have great economic value as direct food sources, being used as polysaccharide additives, or food ingredients for human consumption because of their nutritional value, richness in proteins, vitamins, minerals, and other organic substances (MacArtain et al., 2007; Hafting et al., 2015; Wells et al., 2017; Naylor et al., 2021). Additionally, macroalgae have been used in the industries as fertilizers (Pereira and Yarish, 2008; Kim et al., 2017; Buschmann and Camus, 2019), polysaccharides (Jönsson et al., 2020), oligosaccharides (Jiao et al., 2011), algal hydrocolloids (Roesijadi et al., 2010), minerals

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(Circuncisão et al., 2018), pharmaceuticals (McHugh, 2003), medical therapeutics (Vera et al., 2011), animal feeds (Vijn et al., 2020), and textile industries (Bixler and Porse, 2011). Overall, macroalgae are valuable and promising natural resources in diverse fields (Leandro et al., 2020, Capron et al., 2020).

To date, over 200 species of macroalgae have been harvested as food or for industrial uses (Sahoo et al., 2002; Ferdouse et al., 2018). Worldwide, macroalgal production in 2018 was 32.4 million tonnes (FAO, 2020) including the Japanese kelp *Saccharina japonica* (35.3% by production), *Eucheumoid* seaweeds (29.1%; *Kappaphycus alvarezii*, *Eucheuma denticulatum* and other *Eucheumoid* spp.), *Gracilaria* spp. (10.7%), nori *Neopyropia tenera* and *Neopyropia yezoensis* (formerly called *Porphyra tenera* and *Porphyra yezoensis, respectively*) (8.9%), *Sargassum fusiforme* (0.8%), and other algal species (Buschmann et al., 2017; Kim et al., 2017; Critchley et al., 2019; FAO, 2020). Macroalgal aquaculture is practiced in Asian countries (principally China, Indonesia, Korea, Philippines, Japan, and Malaysia), with total production being tripled from 2000 (10.6 million tonnes) to 2018 (32.4 million tonnes) (FAO, 2020). In recent years, seaweed aquaculture has been growing rapidly in European and North American countries for food, feed, bioenergy, nutrient bioextraction, and industrial uses (Grebe et al., 2019; Piconi et al., 2020; Vijn et al., 2020). With the steady growth of macroalgal aquaculture, breeding programs are being developed with different genetic manipulations to produce strains or lines suitable in different environments for improved productivity and quality (Mao et al., 2020). Germplasm from these aquaculture lines need to be preserved to promote sustainable seaweed aquaculture (Wade et al., 2020).

Similar to many other marine species, macroalgae are also facing biodiversity losses at alarming rates (De Paula et al., 2020) due to multiple stressors, such as warming sea surface temperatures, pollutants, overharvesting, and other anthropogenic disturbances (Smale et al., 2013; Krumhansl et al., 2016; Wade et al., 2020). Potential consequences include changes of ecological structure, loss of genetic diversity, ecological function and services provided by macroalgae, and eventually extinction (Díez et al., 2012; Assis et al., 2017; Steneck et al., 2019). Loss of genetic diversity in macroalgae was identified in several farmed seaweed species due to limited space in germplasm banks or continuous inbreeding (Cardinale, 2011; Valero et al., 2017).

The need for germplasm banking of macroalgae has been emphasized for the preservation of cultivars, biodiversity conservation and ecosystem restoration, and diverse research applications (Wade et al., 2020). Preservation of cultivated strains from the aquaculture industry is an important strategy to maintain this economically valuable germplasm in perpetuity (Wade et al., 2020) and serve as the repositories of genetic variation (Tanksley and McCouch, 1997). Preservation of wild types for genetic diversity can provide biological insurance against environmental stresses, natural weather disasters, and unpredictable accidents from maritime industries (Barrento et al., 2016). Cryopreservation is a technology referring to the preservation of biological materials, including germplasm, at extremely low temperatures (commonly in liquid nitrogen at −196 °C). Application of cryopreservation technology for germplasm has played a significant role in human infertility treatment, maintenance of biological diversity, preservation of genetic resources, assistance of breeding programs, and conservation of imperiled species (Yang and Tiersch, 2020). For macroalgae, germplasm cryopreservation is becoming an important and acceptable tool for long-term germplasm banking and conservation (O'Connell et al., 2020).

In this review, the development of germplasm cryopreservation in macroalgae is evaluated and summarized for macroalgae aquaculture production and natural resource conservation. Literature searches were performed in databases from Web of Science Core Collection and Google Scholar with keywords of "macroalgae (or macroalgal), vitrification, cryopreservation (or cryopreserve), Chlorophyta, Ochrophyta, and Rhodophyta". The findings and results in published studies on macroalgal germplasm cryopreservation will be summarized and compared at each step of the cryopreservation process (Tables 3–5 for Chlorophyta, Ochrophyta, and Rhodophyta). The factors affecting post-thaw viability and repeatability were evaluated, and directions for future research will be discussed. It is anticipated that this review can serve as a foundation for future germplasm banking of macroalgae for aquaculture and natural resource conservation. For microalgal cryopreservation, the interested readers are referred to other related review references or book chapters (Day et al., 1999; Day, 2004, 2007; Day et al., 2010; Fernandes et al., 2019).

2. Development of germplasm cryopreservation in macroalgae

2.1. Approach for germplasm cryopreservation in macroalgae

The history and principles of cryopreservation technology have been introduced and reviewed in other publications (Pegg, 2002; Yang and Tiersch, 2020). During the cooling process, two possible factors are responsible for cell injury: (1) solute effect, which injures cells by high solute concentrates when cells are cooled at a controlled cooling rate; and (2) intracellular ice formation, which injures cells via intracellular ice crystals when cells are cooled at fast cooling rates (Pegg, 2002). Based on experimental observations, a two-factor hypothesis was proposed and was illustrated with follow-up experiments on cryopreservation for different types of cells at a wide range of cooling rates (Leibo, 1976). Since then, this hypothesis has been recognized as the basis of cryopreservation, and two cooling approaches have been developed for germplasm cryopreservation (Pegg, 2002).

1) Cryopreservation by controlled cooling-rates

This approach was based on the two-factor hypothesis. The optimized cooling rates can be experimentally determined (Yang et al., 2012) or theoretically predicted (Thirumala et al., 2005) to increase post-thaw cell survival (Mazur, 2004). For macroalgae, most of the cryopreservation studies used a two-step cooling rate method. The detailed summary will be illustrated in Section 3.

2) Vitrification by cooling at ultra-rapid rates

This approach involves intracellular formation of a stable *glass* state (i.e., the solidification of a liquid in the absence of crystallization) (Rall and Fahy, 1985) and used for mammalian cryopreservation of oocytes (Kuwayama et al., 2005), embryos (Kasai and Mukaida, 2004), and blastocyst (Dal Canto et al., 2019). Vitrification can be achieved through osmotic dehydration by using penetrating cryoprotectants or cooling at ultrafast rates, and complemented with ultra-rapid warming (Mazur and Paredes, 2016). For macroalgae, vitrification was conducted by encapsulating samples into 3% calcium alginate beads, which are cooled in liquid nitrogen directly after dehydration (Wang et al., 2000; Zhang et al., 2008). The detailed summary will be illustrated in Section 3.

2.2. Germplasm materials for cryopreservation in macroalgae

Macroalgae come in many varieties of sizes and structures. The smallest macroalgae are only a few millimeters with a simple filamentous structure, while the largest macroalgae (e.g., giant kelp measures up to 60 m) could have complex structures with specific tissues (Mouritsen, 2013) and cells containing several nuclei and organelles (Baweja et al., 2016).

Generally, macroalgae have varying life history strategies which include gametic meiosis, sporic meiosis, or zygotic meiosis. Life histories of macroalgae that have sporic meiosis may have haploid and diploid generations that are isomorphic or heteromorphic life cycles (See detailed figures in Redmond et al., 2014; Graham et al., 2019). The sporophyte produces haploid meiospores, which upon germination grow directly into dioecious gametophytes. Gametophytes produce mitotically a range of gamete types depending upon the species (isogametes,

anisogametes or oogametes). After gametes fuse, a diploid zygote grows mitotically into diploid sporophytes. If the haploid gametophyte is morphologically similar to the diploid sporophyte, such organisms are characterized by an alternation of isomorphic generations. If the haploid and diploid stages are dissimilar, the organism exhibits an alternation of heteromorphic generations. In addition, algae characterized by an alternation of generations can reproduce asexually via mitosis - or gametophytes can produce haploid parthenogametes. Based on the life cycles, reproduction features, and capability for regenerations in macroalgae, germplasm materials for cryopreservation could be any stage of the algal thallus (sporophyte or gametophyte thalli, meiotic spores, mitotic spores, and/or parthenogametes).

2.3. History of macroalgal germplasm cryopreservation

Seed banking in terrestrial plants has been emphasized for several decades (Tanksley and McCouch, 1997). For algae, germplasm cryopreservation has been studied in many microalgae species, and germplasm repositories have been established in several species of economic importance (Barrento et al., 2016). Comparatively, fewer germplasm cryopreservation studies have been reported on macroalgae with limited success for long-term repository establishment (Taylor and Fletcher, 1999b; Day and Harding, 2008).

Macroalgal cryopreservation was firstly reported in 1960 on the survival of green alga *Ulva pertusa* after exposure to low temperatures at −5, −10, −15, −20, and − 28 °C by direct cooling without cryoprotectants (Terumoto, 1960). The results indicated that *Ulva pertusa* could tolerate low temperature to −10 °C for at least 24 h without cell injury. Later on, *Ulva intestinalis* (formerly called *Enteromorpha intestinalis*), was found to tolerate −20 °C for 24 h without cell injury, and 50% of cells survived after 3 days freezing at −20 °C (Terumoto, 1961), indicating the different resistance to low temperatures between *U. pertusa* and *U. intestinalis*. Therefore, direct comparisons of three green algae and five red algae in their resistance were conducted and significant differences were found (Terumoto, 1964). Since no efforts were made for long-term cryopreservation with cryoprotectants, those studies were categorized as frost resistance or cold storage (Table 1).

The first cryopreservation of macroalgae was reported in *Neopyropia tenera* (formerly referred to as *Porphyra tenera*) with glycerol or glucose at 2.5, 5, 10, and 20% as cryoprotectants (Migita, 1964). The findings were: 1) vegetative and rhizoidal cells of thalli showed higher resistance to freezing temperatures than carposporangial cells. Neutral spores showed higher resistance than carpospores and *conchocelis* filaments; 2) cells cryopreserved to −20 °C showed higher post-thaw survival than that to −75 °C; 3) half-dried cells remained fully viable after cryopreservation at −20 °C for 4 months; and 4) glucose, but not glycerol, showed protection of cells against freezing (Migita, 1964). Following this study, effects of cooling rate and water content in thalli were studied in *Neopyropia tenera* (= *Pyropia tenera*), *Neopyropia yezoensis* (=*Pyropia yezoensis*), and *Phycocalidia suborbiculata* (=*Pyropia suborbiculata*) (Migita, 1966). Additionally, sucrose, glycerol, NaCl, and ethylene glycol were used to cryopreserve *Neopyropia yezoensis*, NaCl and ethylene glycol did not cause cell injury, but sucrose, glycerol, and distilled water resulted in a fatal frost-injury to the cells (Terumoto, 1965). Since the early 1980s, a two-step cooling approach (van der Meer and Simpson, 1984) has been largely employed in many publications to cool macroalgae at cryogenic temperatures (−196 °C) with varied post-thaw survival. In recent studies, different pre-treatments before cooling, cryoprotectants, and cooling approaches were explored.

Through an intensive literature search, a total of 35 papers have been found on macroalgal cryopreservation (Table 2). These 35 publications addressed germplasm cryopreservation in a total of 33 species, including 7 green algae (6 publications, Table 3), 14 brown algal species (15 publications, Table 4), and 12 red algae (13 publications, Table 5). Because of their economic value as marine aquaculture crops, *Neopyropia yezoensis*, *Undaria pinnatifida*, and *Saccharina japonica* were the most studied species for germplasm cryopreservation (Table 2). Besides these research publications, one review paper (Taylor and Fletcher, 1999b), three book chapters or conference proceedings (Kuwano and Saga, 2000; Day, 2018; Paredes et al., 2021), and one perspective paper (Wade et al., 2020) were found on macroalgal germplasm cryopreservation.

Despite these publications on macroalgal germplasm cryopreservation, significant variations in post-thaw survival were reported and there have been no uniformed standardized protocols even for the same species. Therefore, long-term germplasm repositories in macroalgae are still lacking (Wade et al., 2020). Currently, macroalgal collections in many institutes are held as live collections with a few species cryopreserved, such as the Culture Collection of Algae and Protozoa (ccap.ca.uk), the Roscoff Culture Collection (http://roscoff-culture-collection.org/), and the Bigelow Laboratory for Ocean Sciences (https://www.bigelow.org/). Maintenance of live macroalgae needs space, manpower and is prohibitively expensive. Furthermore, there is always a risk of losing certain strains or species due to catastrophic failure of the environmental systems or contamination by human error. Therefore, further studies on standardization and quantification of cryopreservation procedures are needed to increase the protocol repeatability for successful application

Table 1
Summary about studies on macroalgal frost resistance or non-cryogenic cold storage. The species names used in original publications were annotated in parathesis following the new species names.

Species	Study topic	Reference
Chlorophyta		
Monostroma angicava	Frost resistance for 24 h to −20 °C with 50% survival	(Terumoto, 1964)
Ulothrix flacca	Frost resistance for 24 h to −25 °C with 50% survival	(Terumoto, 1964)
Ulva intestinalis (*Enteromorpha intestinalis*)	Frost resistance by cooling to −5 to 25 °C. Tolerance of −20 °C for 24 h	(Terumoto, 1961)
Ulva linza (*Enteromorpha linza*)	Frost resistance for 24 h to −20 °C with 50% survival	(Terumoto, 1964)
Ulva pertusa	Frost resistance by cooling from 0 °C to −28 °C. Tolerance of −10 °C for 24 h	(Terumoto, 1960, 1961)
Ochrophyta		
Fucus edentatus	Cold resistance of zygotes and embryos down to −25 °C for hours and days	(Bird and McLachlan, 1974)
Macrocystis pyrifera	Cold storage of gametophytes at 10 °C for 5 years	(Barrento et al., 2016)
Rhodophyta		
Bangia fusco-purpurea	Frost resistance for 24 h to −55 °C with 50% survival	(Terumoto, 1964)
Gloiopeltis furcata	Effects of different size, water content, and density on storage at 4 °C and − 18 °C)	(Chen et al., 2016)
Neopyropia tenera (*Porphyra tenera*)	Frost resistance of vegetative thalli, spores, and *conchocelis* filaments	(Migita, 1964, 1966, 1967)
Neopyropia yezoensis (*Porphyra yezoensis*)	Frost resistance for 24 h to −35 °C with 50% survival	(Terumoto, 1964)
	Effects of cooling rates and water content on frost resistance	(Migita, 1966)
	Freezing of thalli at −20 °C after dehydrated with 10%–40% H_2O	(Lin et al., 2010)
Phycocalidia suborbiculata (*Porphyra suborbiculata*)	Effects of cooling rates and water content on frost resistance	(Migita, 1966)
Porphyra umbilicalis	Freezing to −20 °C or − 80 °C after air dry to 5 or 30% H_2O (no cryoprotectants)	(Green and Neefus, 2014)
Pyropia pseudolinearis (*Porphyra pseudolinearis*)	Frost resistance for 24 h to −55 (female) to −70 °C (male) with 50% survival	(Terumoto, 1964)
Uedaea onoi (*Porphyra onoi*)	Frost resistance for 24 h to −10 °C with 50% survival, fatal at −15 °C	(Terumoto, 1964)

Table 2
Summary about studies on germplasm cryopreservation in macroalgae. The species names in original publications were annotated in parathesis following the new species names.

Species	Study topic	Reference
Chlorophyta		
Ulva intestinalis (*Enteromorpha intestinalis*)	Crypreservation of zoospores to −20 °C to −40 °C with DMSO and glycerol	(Taylor and Fletcher, 1999a)
Ulva fasciata	Cryopresrevation of spores at 4 °C, −20 or − 70 °C with DMSO and glyerol	(Bhattarai et al., 2007)
Ulva lactuca	Application of cryopreservation protocol developed for *Gracilaria tikvahiae*	(van der Meer and Simpson, 1984)
Ulva lobata	Cryopreservation of vegetative thalli with 10% DMSO or glycerol	(Lalrinsanga et al., 2009)
Ulva pertusa	Cryopresrevation of spores at 4 °C, −20 °C and − 70 °C with DMSO and glyerol	(Bhattarai et al., 2007)
Ulva prolifera	Cryopreservation of gametophytic thalli with DMSO, glycerol, or proline	(Lee and Nam, 2016)
Ulva rigida	Cryopreservation of thalli, germlings, and gametes for up to 184 days	(Gao et al., 2017)
Ochrophyta		
Ecklonia kurome	Development of cryopreservation protocol for six species of Laminariales	(Kuwano et al., 2004)
Ecklonia stolonifera	Development of cryopreservation protocol for six species of Laminariales	(Kuwano et al., 2004)
Ectocarpus fasciculatus, E. siliculosus E. sp.	Cryopreservation of different strains with DMSO 10% (*v*/v) and sorbitol 9%	(Heesch et al., 2012)
Eisenia bicyclis	Development of cryopreservation protocol	(Kono et al., 1998)
Kjellmaniella crassifolia	Development of cryopreservation protocol for six species of Laminariales	(Kuwano et al., 2004)
Laminaria digitata	Vitrification by encapsulation dehydration	(Vigneron et al., 1997)
Saccharina japonica (*Laminaria japonica*)	Development of cryopreservation protocol for six species of Laminariales	(Kuwano et al., 2004)
	Cryopreservation of spores and gametophytes	(Zhang et al., 2007b; Zhang et al., 2007a)
	Cryopreservation of gametophytes using encapsulation-dehydration	(Zhang et al., 2008)
Saccharina longissima (*Laminaria longissima*)	Development of cryopreservation protocol for six species of Laminariales	(Kuwano et al., 2004)
Saccharina latissima	Development of cryopreservation protocol for gametophyte males and females	(Visch et al., 2019)
Scytosiphon lomentaria	Cryopreservation of filaments to −20 °C through a vitrification procedure	(Zhuang et al., 2015)
Undaria pinnatifida	Development of cryopreservation protocol for six species of Laminariales	(Kuwano et al., 2004)
	Ultrastructure observation of gametophytes during thawing process	(Ginsburger-Vogel et al., 1992)
	Effects of pre-incubation irradiance on post-thaw survival	(Nanba et al., 2009)
	Cryopreservation of gametophytes by encapsulation-dehydration	(Wang et al., 2005; Wang et al., 2011)
		(Arbault et al., 1990; Renard et al., 1992)
	Development of cryopreservation protocol for gametophytes	
Vaucheria sessilis	Elucidation of the metabolic and structural basis during cooling process	(Fleck et al., 1999)
Rhodophyta		
Chondrus crispus	Application of cryopreservation protocol developed for *Gracilaria tikvahiae*	(van der Meer and Simpson, 1984)
Devaleraea ramentacea	Application of cryopreservation protocol developedfor *Gracilaria tikvahiae*	(van der Meer and Simpson, 1984)
Gracilaria corticata	Cryopreservation of vegetative thalli with 10% DMSO or glycerol	(Lalrinsanga et al., 2009)
Gracilaria foliifera	Application of cryopreservation protocol developed for *Gracilaria tikvahiae*	(van der Meer and Simpson, 1984)
Gracilaria tikvahiae	Development of cryopreservation protocol, and application on other five species	(van der Meer and Simpson, 1984)
Hypnea musciformis	Cryopreservation of vegetative thalli with 10% DMSO or glycerol	(Lalrinsanga et al., 2009)
Neoporphyra dentata (*Porphyra dentata*)	Cryopreservation of conchocelis cells by use of a Styrofoam box	(Kuwano et al., 1994)
	Cryopreservation of sporothalli with 10% DMSO and 0.5 M sorbitol	(Jo et al., 2003)
Neoporphyra haitanensis (*Porphyra haitanensis*)	Cryopreservation of conchocelis cells by use of a Styrofoam box	(Kuwano et al., 1994)
	Cryopreservation of conchocelis using encapsulation-dehydration vitrification	(Wang et al., 2000)
Neoporphyra seriata (*Porphyra seriata*)	Cryopreservation of sporothalli with 10% DMSO and 0.5 M sorbitol	(Jo et al., 2003)
Neopyropia tenera (*Porphyra tenera*)	Cryopreservation of conchocelis cells by use of a Styrofoam box	(Kuwano et al., 1994, 1996)
	Development of cryopreservation protocol for sporothalli	(Migita, 1964; Jo et al., 2003)
Neopyropia yezoensis (*Porphyra yezoensis*)	Development of cryopreservation protocols gametophytic thalli	(Kuwano et al., 1996)
	Cryopreservation of sporothalli with 10% DMSO and 0.5 M sorbitol	(Jo et al., 2003)
	Development of cryopreservation protocol for conchocelis	(Kuwano et al., 1992; Kuwano et al., 1993; Kuwano et al., 1994; Zhou et al., 2007)
	Vitrification of gametophytic thalli and sporrothalli	(Liu et al., 2004; Choi et al., 2013)
Palmaria palmata	Application of cryopreservation protocol for *Gracilaria tikvahiae*	(van der Meer and Simpson, 1984)
Porphyra linearis	Development of cryopreservation protocol for conchocelis	(Arbault and Delanoue, 1994)
Pyropia pseudolinearis (*Porphyra pseudolinearis*)	Cryopreservation of conchocelis cells by use of a Styrofoam box	(Kuwano et al., 1994)
	Cryopreservation of sporothalli with 10% DMSO and 0.5 M sorbitol	(Jo et al., 2003)

Table 3
Summary about germplasm cryopreservation in Chlorophyta macroalgae (7 species, 6 publications). The species *Enteromorpha intestinalis* in original publications was changed into *Ulva intestinalis*.

Species	Germplasm	Cryoprotectant agent	Cooling process	Packaging container	Thawing temperature (°C)	Post-thaw viability	Reference
Ulva intestinalis	Settled zoospores	DMSO (5 and 10%) and glycerol (5 and 10%)	–1 °C min^{-1} to −20 °C or − 40 °C; −0.5 °C min^{-1} to −20 °C, and − 1 °C min^{-1} to −30 °C, then in −196 °C	None. Spores were on cover slips	Plunging the cover slips directly into VS culture medium at 37 °C	> 40% in samples frozen in 75% seawater at −20 °C for 5 weeks	Taylor and Fletcher, 1999a
Ulva intestinalis	gametophytic thalli (< 5 mm)	Dextran, DMSO, PVP, proline, glycerol, PG, EG separated or combined with sorbitol, glucose, or sucrose	–1 °C min^{-1} to −20, −30, −40, −50, and − 60 °C, and then in liquid nitrogen or not.	2-ml cryovial with 1.5 ml of samples	In water bath at 40 °C until just before the ice melted	DMSO 10% showed best protection and − 40 °C was the best temperature. Post-thaw growth and gamete release were observed.	Kono et al., 1997
Ulva fasciata Ulva pertusa	Suspended spores	DMSO: 5%, paraffin oil, glycerol: 5%–20%. Separated or combined	Treated spores were preserved at room T, 4 °C, −20 °C, or − 70 °C. Cooling rates were not stated	Micro tubes, no volume stated	Resuspended in 1 mL of f/2 culture medium and incubated for germination in 24-well plates at 18 °C	0–3% germination for samples preserved at −20 °C, and 0 for sample at −70 °C	Bhattarai et al., 2007
Ulva lactuca	Sporelings and apical segments	1.5 M DMSO	2 °C min^{-1} to −40 °C, then into liquid nitrogen −196 °C	1-ml freezing ampoules 1 ml	36 °C	100%	van der Meer and Simpson, 1984
Ulva lobata	Apical tips (1–2 mm)	DMSO, EG, glycerol at 5%, 10%, and 15%	<−1 °C min^{-1} to −20 °C, −30 °C, −40 °C, −50 °C, −60 °C, then −196 °C	4-ml sample in 5-ml cryovial	At 20 °C, 30 °C, 40 °C, 50 °C, and 60 °C; The best thawing temperature was 40 °C	8–29% at days 1–70	Lalrinsanga et al., 2009
Ulva prolifera	Gametophytes	DMSO, glycerol, and proline at 5, 10, 15, 20, or 25%, separated or combined	At a cooling rate of 1 °C min^{-1} from 15 °C to −40 °C, held at −40 °C for 5 min, and then into liquid nitrogen	1.5-ml cryovial	40 °C in water bath	92% in post-thaw samples frozen with 20% glycerol for 120 d	Lee and Nam, 2016
Ulva rigida	Gametophytes, germlings, and gametes	DMSO at 10 and 15% for thalli and germlings and 5 and 10% for gametes	1) Direct move to −20 °C or − 80 °C 2) At 1 °C min^{-1} to from 20 °C to −20 °C, then move to −80 °C	1.5-ml freezing ampoules	Plunging the ampoules in a 37 °C water bath	0% for gametophytic thalli at day 30; 0% for germling at day 1, and 4–19% for gametes at day 180	Gao et al., 2017

Note: DMSO: dimethyl sulfoxide; EG: ethylene glycol; PG: propylene glycol, and PVP: polyvinylpyrrolidone.

Table 4
Summary about germplasm cryopreservation in Ochrophyta macroalgae (14 species, 15 publications). The species *Laminaria japonica, Laminaria longissimi, and Kjellmaniella crassifolia* in original publications were changed into *Saccharina japonica, Saccharina longissimi,* and *Saccharina crassifolia.*

Species	Germplasm	Cryoprotectant agent	Cooling process	Packaging container	Thawing temperature (°C)	Post-thaw viability	Reference
Ectocarpus siliculosus *Ectocarpus* sp. *Ectocarpus fasciculatus*	Sporophytes	10% DMSO and 9% sorbitol	1 °C min^{-1} from 20 °C to −40 °C, then −196 °C	1-ml sample in 2-ml Cryovials	40 °C	25–50% for *Ectocarpus siliculosus*; >50% for *E.* sp. and *E. fasciculatus*	Heesch et al., 2012
Fucus edentatus	Zygotes and embryos	NA	Cooled to −2, −5, −10, and − 15 °C for 2 h (zygotes and embryos); to −25 °C (embryos)	NA	NA	34–92% for zygotes at −10 °C; nearly 100% for post-thaw embryos.	Bird and McLachlan, 1974
Eisenia bicyclis	Gametophytes	EG and 10% proline	Pre-freezing temperature was −40 °C, and then −196 °C	N/A	N/A	52.5% and 62.0% after thawing, 31.1% and 27.2% after 4 d post-thaw culture	Kono et al., 1998
Laminaria digitata	Gametophytes	0.3–0.5 M sucrose	Slowly from 19 °C to −40 °C and then −196 °C.	N/A	40 °C for 2 min	25–75%	Vigneron et al., 1997
Laminaria diabolica	Gametophytes	10% DMSO and 0.5 M sorbitol	Slowly <1 °C min^{-1} to −40 °C and then in liquid nitrogen.	2-ml vial with 0.75-ml sample	40 °C	Recovery growth was found	Sakanishi and Saga, 1994
Saccharina japonica *Saccharina longissimi* *Saccharina crassifolia* *Ecklonia stolonifera* *Ecklonia kurome* *Undaria pinnatifida*	Gametophytes	EG, glycerol, DMSO, L-proline, sorbitol, sucrose, and dextran T-500	Slowly cooling to −40 °C in 4 h, and then −196 °C.	1.5-ml sample in 2-ml cryovial	40 °C water bath and move to ice bath before melting.	3.1–73.3% for after thawing and 0–66.7% for after 4 d post-thawing culture	Kuwano et al., 2004
Saccharina japonica	Gametophytes	10% EG and 10% proline	At 0.5, 1.0, 2.0 °C min^{-1} cooled to −30, −60, −90 °C and holding for 0, 40, 80 min, then −196 °C	0.5-ml straws	26 °C water bath, then ice-bath before complete melting	69–84%	Zhang et al., 2007a
Saccharina japonica	Spores	DMSO, glycerol sorbitol, sucrose, and dextrose. Separated or combined	Direct cooling to −196 °C; to −20 °C within 30 min; at 0.25–5.0 °C min^{-1} to −60 °C, then −196 °C (straw only).	1.5-ml cryovial and 0.5-ml straws	26 or 39 °C water bath, transfer to ice-bath before complete melting	13–50%	Zhang et al., 2007b
Saccharina japonica	Gametophytes	0.4 M sucrose for 6 h	From 10 °C to −40 or − 60 °C within 30 min, then −196 °C	cryotubes	40 °C	22–43%	Zhang et al., 2008
Saccharina latissima	Gametophytes	DMSO, D-sorbitol PG, and methanol. Separated/combined	1 °C min^{-1} to −40 °C or − 80 °C, then −196 °C	1-ml sample in 2-ml cryovial	40 °C water bath	Up to 80% for male gametophytes and 20% for female.	Visch et al., 2019
Undaria pinnatifida	Gametophytes	Glycerol at 5–10% or 28%; DMSO	Cooled to −80 °C or − 196 °C	NA	NA		Arbault et al., 1990
Undaria pinnatifida	Gametophytes	Glycerol at 5–30%	5 °C min^{-1} from 22 °C to −30 °C or − 40 °C; Directly in −196 °C	NA	Rapid and slow thawing	Rapid thawing showed higher post-thaw viability than slow thawing	Renard et al., 1992
Undaria pinnatifida	Gametophytes	Glycerol at 5, 10, 15, and 20%	5 °C min^{-1} to −40 °C, then −196 °C	2-ml cryovial	32 °C water bath	NA. Ultrastructure was observed.	Ginsburger-Vogel et al., 1992
Undaria pinnatifida	Gametophytes	0.2–0.3 M sucrose for 9 h to dehydrate beads	Directly plunging at −196 °C	2-ml cryovials	40 °C for 2 min	7.3%-30.7%	Wang et al., 2005
Undaria pinnatifida	Gametophytes	10% L-proline and 10% Glycerol	<1 °C min^{-1} to – 40 °C in 4 h	2-ml Cryovials	40 °C	43–100%	Nanba et al., 2009

Note: DMSO: dimethyl sulfoxide; EG: ethylene glycol; PG: propylene glycol.

on germplasm banking.

3. Germplasm cryopreservation of macroalgae

Cryopreservation procedures involve a series of steps that are connected to each other and need to be optimized by experimental trials for the species of interest. These steps include: 1) germplasm sample collection; 2) selection of cryoprotectants by evaluating the acute toxicity and cryoprotective function during cooling process; 3) packaging of germplasm samples after mixing with cryoprotectants; 4) cooling process at suitable cooling rates by evaluating post-thaw survival; 5) thawing of frozen samples at different temperatures; and 6) viability assays or post-thaw amendments of samples before further use. Optimization of each cryopreservation step is crucial for protocol development with high post-thaw viability because these steps are interconnected and any error at any step could result in final failure (Leibo and Pool, 2011). In general, the optimized conditions at each step varied among different species, cell types (cell size, cell wall and cytoplasm membrane, etc.), and even the same samples when handling differently. The summary of macroalgal cryopreservation at each step was reviewed as follows.

3.1. Germplasm collection and preparation for cryopreservation

The targeted macroalgal germplasm for cryopreservation needs to be specific to their life cycle and reproduction characteristics (Wade et al.,

Table 5
Summary about germplasm cryopreservation in Rhodophyta macroalgae (12 species, 13 publications). The new Genus *Pyropia* was used for *Porphyra pseudolinearis, Porphyra seriata,* and *Porphyra umbilicalis,* Genus *Neoporphyra* was used for *Porphyra haitanensis* and *Porphyra dentata*, and Genus Neopyropia was used for *Porphyra tenera* and *Porphyra yezoensis.*

Species	Germplasm	Cryoprotectant agent	Cooling process	Packaging container	Thawing (°C)	Post-thaw viability	Reference
Gloiopeltis furcata	Germling	None	Direct preservation at 4 °C or − 18 °C	0.5 g per Sealed bag	12 °C, and 16 °C for cultivation.	0% (4 °C), and 16–72% (−18 °C)	Chen et al., 2016
Gracilaria corticata	Apical tips (1–2 mm)	DMSO, EG, glycerol at 5%, 10%, and 15%	<−1 °C min^{-1} to −20 °C, −30 °C, −40 °C, −50 °C, −60 °C, then −196 °C	4-ml sample in 5-ml cryovial	20, 30, 40, 50, and 60 °C. 40 °C was the best.	59%–85% at day 1–70	Lalrinsanga et al., 2009
Gracilaria tikvahiae	Sporelings and apical segments	1.5 M DMSO	Slow cooling rate to −40 °C, then −196 °C	1 ml freezing ampoules 1 ml	36 °C	60–100%	van der Meer and Simpson, 1984
Hypnea musciformis	Apical tips (1–2 mm)	DMSO, EG, glycerol at 5%, 10%, and 15%	<−1 °C min^{-1} to −20 °C, −30 °C, −40 °C, −50 °C, −60 °C, then −196 °C	4-ml sample in 5-ml cryovial	20 °C, 30 °C, 40 °C, 50 °C, and 60 °C	5.9–28.9%	Lalrinsanga et al., 2009
Neoporphyra haitanensis	Conchocelis	None	Dehydration the encapsulated beads, then −196 °C	2 ml cryogenic vials	40 °C	47.7–66.1%	Wang et al., 2000
Neoporphyra dentata *Pyropia pseudolinearis* *Pyropia seriata* *Neopyropia tenera* *Neopyropia yezoensis*	Conchocelis filaments	DMSO, glycerol, EG, proline, hydrochloride betaine, skimmed milk, sucrose, glucose, sorbitol, and mannitol	−1 °C min^{-1} to −40 °C, then −196 °C	1.5 ml of sample in 2-ml cryovials	40 °C	54.6–70.9% using 10% DMSO plus 0.5 M sorbitol	Jo et al., 2003
Pyropia umbilicalis	Small blades	None	Direct preservation at −20 °C or − 80 °C	1.7-ml centrifuge tubes	15 °C for removing 3 h	100%	Green and Neefus, 2014
Neopyropia yezoensis	Conchocelis	DMSO with EG, PEG, sorbitol, and sucrose	<−1 °C min^{-1} to −20 °C, −40 °C, then −196 °C	2-ml cryovials	20 °C, 30 °C and 40 °C	60–86% when using 10% DMSO plus 0.5 M sorbitol	Zhou et al., 2007
Neopyropia yezoensis	Conchocelis	10% DMSO and 0.5 M sorbitol in 50% seawater	0.1–1 °C min^{-1} from −20 to −80 °C at 0.1–1 °C min^{-1}, then −196 °C	2 ml cryogenic vials	40 °C	>60%	Kuwano et al., 1993
Neopyropia yezoensis	Gametophytes (5–10 mm)	DMSO, diglycerol, glycerol, PEG, PG, propanediol, butanediol at 5–50% in combination	Directly plunged into −196 °C	0.5-ml straw	40 °C water bath	> 60% with 5–25% glycerol or diglycerol plus 5% DMSO	Choi et al., 2013
Neopyropia yezoensis	Gametophytes	None	After dehydration, direct preservation at-20 °C	Self-sealing plastic bags	10 °C and 20 °C	93.10% with water content of 10–40%	Lin et al., 2010
Neopyropia yezoensis *Neopyropia tenera* *Porphyra pseudolinearis* *Neoporphyra dentata*	Conchocelis	5–15% DMSO and 0.5 M sorbitol	1. 1 °C min^{-1} to −20 to −60 °C, then −196 °C 2. Holding for 1 h at −40 °C, then −196 °C	2 ml cryogenic vials	40 °C	38.4–77.9%	Kuwano et al., 1994
Neopyropia yezoensis *Neopyropia tenera*	Gametophytes	5 or 10% DMSO, 5% dextran T-500, PVP K-30, Ficoll 400, PEG 6000, PG, EG, glycerol, sorbitol, sucrose, or glucose	1 °C min^{-1} to −20 to −60 °C, then −196 °C	2 ml cryogenic vials	40 °C	12–96% using dextran, PVP or Ficoll combined with DMSO	Kuwano et al., 1996

Note: DMSO: dimethyl sulfoxide; EG: ethylene glycol; PEG: polyethylene glycol; PG: propylene glycol.

2020). See Section 2.2 the statement of life cycle and potential germplasm materials for cryopreservation.

3.1.1. Diploid or haploid thalli

Haploid and diploid thalli are present at different stages of the life cycle for macroalgae. For Chlorophyta and Ochrophyta macroalgae (Tables 3 and 4), the germplasm for cryopreservation included haploid gametophytic thalli (Wang et al., 2005; Zhang et al., 2007b; Zhang et al., 2007a; Zhang et al., 2008; Nanba et al., 2009; Barrento et al., 2016; Lee and Nam, 2016; Gao et al., 2017; Visch et al., 2019), and haploid parthenosporophyte thalli in *Ectocarpus* species (Heesch et al., 2012). For Rhodophyta, including *Neopyropia* and *Porphyra* spp. (Table 5), diploid conchocelis was the most common germplasm for cryopreservation (Kuwano et al., 1993; Wang et al., 2000; Zhou et al., 2007; Choi et al., 2013), and gametophytic thalli and free-living diploid conchocelis filaments were cryopreserved with relatively higher post-thaw viability (Kuwano et al., 1996; Jo et al., 2003) (Table 5). For other Rhodophyta macroalgal species, such as *Gracilaria corticata*, *Gracilaria tikvahiae* and *Hypnea musciformis*, apical segments of mature thalli were used for cryopreservation (van der Meer and Simpson, 1984; Lalrinsanga et al., 2009).

Preparation of diploid or haploid thalli were performed by cleaning with sterilized seawater and cutting into 1–2 mm fragments using blenders or single edge razor blades. In some reports, the fragments after cutting were cultured in sterilized medium with or without antibiotics for a period of days or months before being used for cryopreservation.

3.1.2. Spores, gametes, and zygotes

For most animals, germplasm such as gametes, embryos, and larvae are the primary focus for cryopreservation. For macroalgae, spores, gametes, and zygotes were suitable germplasm for long-term cryopreservation. To date, several studies reported successful cryopreservation

or cold storage of meiospores from *Saccharina japonica* (Zhang et al., 2007b), *Ulva intestinalis* (Taylor and Fletcher, 1999a), *Ulva fasciata* and *Ulva pertusa* (Bhattarai et al., 2007), gametes from *Ulva rigida* (Gao et al., 2017) and zygotes (embryos) from *Fucus edentatus* (Bird and McLachlan, 1974). In addition, meiospores of kelps were reported cryopreserved successfully in the Bigelow Laboratory (www.bigelow.org, personal communication with M. Lomas).

For Chlorophyta (Table 3), zoospores of *Ulva fasciata* and *Ulva pertusa* cooled down to 4 °C in f/2 seawater medium with ampicillin (100 μg mL^{-1}) showed a viability of 42–61% after storage for 100 days (Bhattarai et al., 2007). Zoospores of *Ulva intestinalis* cooled to −20 °C showed a viability of over 40% after storage for 5 weeks (Taylor and Fletcher, 1999a), and gametes of *Ulva rigida* cooled to −20 °C and −80 °C showed 7.0 − 18.7% and 3.5–12.1% post-thaw viability (Gao et al., 2017). Released gametes and zoospores in *Ulva* species were collected by concentrated them using a point light source (Hiraoka and Enomoto, 1998). For Ochrophyta (Table 4), meiospores of *Saccharina japonica* cryopreserved in liquid nitrogen by gradual cooling at 0.25–5.0 °C min^{-1} to −60 °C followed by plunging directly into liquid nitrogen showed a 13–50% viability after 24 h (Zhang et al., 2007b). For *Saccharina japonica*, meiospores were released from 10-cm^2 pieces of sorus tissue in sterilized seawater at 8 °C and were collected by filtering through a 30-μm cell strainer (Zhang et al., 2007b). For Rhodophyta macroalgae (Table 5), there has been no study on spores or gametes cryopreservation.

3.2. Choice of cryoprotectants and evaluation of acute toxicity

The use of cryoprotectants is essential for cryopreservation technology since the first application of glycerol (20%) as cryoprotectant for fowl semen cryopreservation (Polge et al., 1949). Since then, cryopreservation has been investigated in many hundreds of different species, and the use of cryoprotectant is almost universal for cryopreservation technology. For different cell types and species for cryopreservation, effective cryoprotectant types and concentrations need to be determined through systematic experimentation based on cryoprotectant toxicity, molecular weight, and permeability (See reviews in Pegg, 2002; Elliott et al., 2017; Yang and Tiersch, 2020). Depending on cryoprotectant toxicity, cell sensitivity, and osmotic pressures, methods to mix cryoprotectants and biological cell suspension could be conducted in different ways at different temperatures. After mixing with cryoprotectants, sample suspensions usually require a specific amount of time, which is called “equilibration time”, to allow cryoprotectants and samples to interact with one another before cooling. Systematic assessment of the acute toxicity of cryoprotectants on fresh samples is an effective way to screen cryoprotectant types, concentrations, and equilibration time for germplasm cryopreservation.

For macroalgae, choice of cryoprotectants in most studies were based on peers' results with DMSO, glycerol and sugars. Acute toxicity evaluation for screening cryoprotectants were reported in only two studies (Zhang et al., 2007b; Choi et al., 2013)). For *Neopyropia yezoensis*, a total of 10 cryoprotectants were evaluated on gametophytic thalli, and a combination of 5% DMSO and 20% diglycerol showed the least toxicity with a 94.6% post-exposure viability (Choi et al., 2013). For *Saccharina japonica*, five cryoprotectants (DMSO, glycerol, sucrose, dextrose, and sorbitol) were showed toxicity on meiospores, and DMSO showed the lowest level of toxicity. After exposure to 5% DMSO for 50 min or 10% DMSO for 15 min, about 70% of the meiospores developed into gametophytes (Zhang et al., 2007b).

3.2.1. Types and concentrations of cryoprotectant

The cryoprotectant types used for macroalgae germplasm cryopreservation include DMSO, glycerol, methanol, ethylene glycol, polyethylene glycol, propylene glycol, dipropylene glycol, 3-methyl-1,3-butanediol, 1,3-propanediol, 1,2-butanediol, 1,3-butanediol, and 2,3-butanediol (see summaries in Tables 3–5) with DMSO reported in majority of the publications (Taylor and Fletcher, 1999a). Additionally, amino acids (proline and L- proline) and sugars (sorbitol, sucrose, mannitol, glucose, dextrose, and dextran T-500), and others (hydrochloride betaine and skimmed milk) have been used together with the cryoprotectants for macroalgal cryopreservation (Jo et al., 2003; Lee and Nam, 2016).

Different cryoprotectants showed varied effects in different macroalgae species because cell sizes, types of tissues, cell wall construction, and cytoplasm membrane composition. For example, DMSO at 10% showed effective protection for *Ulva lobata* (highest post-thaw survival) (Lalrinsanga et al., 2009), but was considered to be harmful to the fresh mature thalli and germlings of *Ulva rigida* (Gao et al., 2017). Glycerol at 20% showed the highest protection during cryopreservation for *Ulva prolifera* (Lee and Nam, 2016).

Cryoprotectants can be used independently or in combination for germplasm cryopreservation (Taylor and Fletcher, 1999b). DMSO, the most commonly used cryoprotectant for macroalgae, is often combined with other cryoprotectants such as sugars and amino acids for germplasm cryopreservation in many species (Tables 3–5) (Kuwano et al., 1993; Kuwano et al., 1994, 1996; Bhattarai et al., 2007; Zhou et al., 2007; Nanba et al., 2009; Choi et al., 2013; Lee and Nam, 2016; Visch et al., 2019). Additionally, combinations of different cryoprotectants (e.g., DMSO combining diglycerol; DMSO combing glycerol and proline) have been reported in several studies with effective protection of germplasm materials were identified with high post-thaw viability (Bhattarai et al., 2007; Choi et al., 2013; Lee and Nam, 2016).

The concentrations of cryoprotectants is another important factor which affects the post-thaw viability of cryopreserved germplasm. For example, high concentration of glycerol caused high mortality of cryopreserved gametophytes of *Undaria pinnatifida* due to cytotoxic leakage of vacuolar contents to the cell cytoplasm and destruction of thylakoids in plastids (Ginsburger-Vogel et al., 1992). For macroalgae, the cryoprotectant concentrations used for germplasm cryopreservation ranged from 5% to 25% (Tables 3–5). For example, the highest post-thaw viabilities for male and female gametophytes of *Saccharina latissima* were found in samples cryopreserved with DMSO 10% (*v*/v) using controlled-rate cooling methods (Visch et al., 2019). Gametophytic thalli of *Ulva prolifera* had a viability of 91.6% using a two-step controlled-rate cooling method with 20% glycerol (Lee and Nam, 2016). Optimal concentrations varied depending on different cryoprotectants and macroalgal species. These conditions could be determined through systematic evaluation of cryoprotectant toxicity on fresh cells and protection during cooling process (examined on post-thaw viability).

3.2.2. Equilibration time

Before cooling, germplasm cells need to be mixed with cryoprotectants at optimal concentration and equilibration time in culture medium to minimize intracellular water (Pegg, 2002). Overall, most publications used only one equilibration time, usually ranging from 15 to 60 min. Equilibration time used for macroalgae germplasm cryopreservation varied for different species and germplasm materials, and even for the same species in different reports.

For *Gracilaria tikvahiae*, equilibration time between 5 and 90 min had no effects on germplasm cell viability (van der Meer and Simpson, 1984). For *Neopyropia yezoensis,* gametophytic thalli were found sensitive to cryoprotectants and a 5-s equilibration time yielded the best post-thaw viability (Choi et al., 2013). The optimal equilibration time has been investigated in several other macroalgal species, including the meiospores of *Saccharina japonica* (Zhang et al., 2007b) and vegetative thalli of *Gracilaria corticata*, *Hypnea musciformis*, and *Ulva lobata* (Lalrinsanga et al., 2009). For the meiospores of *Saccharina japonica*, equilibration with 10% DMSO for 15 min was optimal (Zhang et al., 2007b). For vegetative thalli of *Gracilaria corticata*, the equilibration time of 60 min was found to be the most optimal. For vegetative thalli of *Hypnea musciformis* and *Ulva lobata* equilibration time of 45 min was sufficient (Lalrinsanga et al., 2009). For (spore producing thallus)

sporothalli filaments of *Neoporphyra* and *Pyropia* species, the equilibration time of 45 min was found optimal (Jo et al., 2003).

Temperature for the equilibration process can change the optimal equilibration time. For macroalgae that inhabit temperate regions, such as *Ulva prolifera* and *Gracilaria tikvahiae*, equilibration time was optimal at room temperature (usually 20–23 °C), while for macroalgae that inhabit colder regions, such as *Neopyropia yezoensis* and *Saccharina latissima,* equilibration was usually performed at lower temperatures at 0–10 °C (Zhou et al., 2007; Visch et al., 2019). As mentioned above in this section, evaluation of acute toxicity at different temperatures could be an effective approach to determine the optimal equilibration temperature.

3.2.3. Mixing of cryoprotectants with germplasm materials

The addition of cryoprotectants to germplasm materials could cause abrupt osmotic changes because most cryoprotectants have high osmolarity. For example, DMSO has an osmolarity over 2000 mOsmol/kg. Therefore, the method of mixing cryoprotectants and germplasm materials is critical to avoid sudden osmotic changes to germplasm cells. For macroalgae, cryoprotectants were added gradually to macroalgae germplasm in majority of publications, including the cryopreservation of conchocelis of *Neopyropia, Neoporphyra* and *Porphyra* spp. (*P. seriata*, *Neopyropia yezoensis*, *Neopyropia tenera*, *Porphyra pseudolinearis* and *Neoporphyra dentata*) (Kuwano et al., 1993; Jo et al., 2003), gametophytic cells of six species of Laminariales (*Saccharina japonica*, *S. longissima*, *Kjellmaniella crassifolia*, *Ecklonia stolonifera*, *E. kurome*, and *Undaria pinnatifida*) (Kuwano et al., 2004), vegetative thalli of *Gracilaria corticata*, *Hypnea musciformis* and *Ulva lobata* (Lalrinsanga et al., 2009), gametophytic thalli of *Ulva prolifera* (Lee and Nam, 2016), and gametophytes of *Undaria pinnatifida* (Nanba et al., 2009). Generally, addition of cryoprotectants to germplasm materials were performed drop by drop within 15 min, and the mixture was left for 45 min of equilibration time at specific temperatures. Rapid mixing of cryoprotectants with target germplasm samples was also reported for macroalgal cryopreservation, for example, gametophytic thalli of *Ulva rigida* (Gao et al., 2017), gametophytes of *Saccharina latissima* (Visch et al., 2019), parthenosporophytes of *Ectocarpus* (*E. siliculosus, Ectocarpus sp., E. fasciculatus*) (Heesch et al., 2012) and meiospores (suspended or settled) of *Saccharina japonica* (Zhang et al., 2007b). Comparisons of mixing DMSO with conchocelis cells of *Neopyropia yezoensis* with different times from 0 to 30 min revealed that post-thaw survival was higher when DMSO was added gradually to reach its final concentration than that when DMSO was added rapidly (Kuwano et al., 1993).

To compensate the osmotic change, cryoprotectant media in some studies were made in 50% of seawater (van der Meer and Simpson, 1984) or even distilled water (Bhattarai et al., 2007). To keep the pH balance after mixing of cryoprotectant and germplasm materials, HEPES (0.01 M, pH = 8) was employed to make cryo-media in many studies (Kuwano et al., 1994, 1996; Jo et al., 2003; Kuwano et al., 2004; Lalrinsanga et al., 2009; Nanba et al., 2009; Lee and Nam, 2016). Similarly, the removal of cryoprotectant from post-thaw samples could be performed either gradually or rapidly. The details were stated in Section 3.7.

3.3. Packaging of macroalgal germplasm samples

Packaging containers are important for germplasm cryopreservation because the cooling and warming of germplasm samples is directly related to the volume, shape, and material type of packaging containers. Currently, the commercially available containers include straws (0.25 ml, 0.3 ml, 0.5 ml, 5 ml) made of different materials (polyvinyl chloride for French straw, and ionomeric resin for CBS™ straw) for sperm and embryos, cryopreservation bags for blood and stem cells, cryovials made of different materials (polypropylene for the Corning™ cryovials) for cell lines, and other custom containers.

For macroalgae germplasm cryopreservation, cryogenic vials (1.5 ml or 2 ml) were used in almost all published studies as packaging containers (Tables 3–5). Large volume cryovials (5 ml) were sometimes used for germplasm cryopreservation of *Gracilaria corticata, Hypnea musciformis*, and *Ulva lobata* (Lalrinsanga et al., 2009). Occasionally, straws (0.5-ml) were used for macroalgal cryopreservation with high post-thaw viabilities, such as gametophytes and meiospores of *Saccharina japonica* (Zhang et al., 2007b; Zhang et al., 2007a) and gametophytic thalli of *Neopyropia yezoensis* (Choi et al., 2013). To date, no comparison of different packaging containers has been reported for macroalgal germplasm cryopreservation.

3.4. Cooling process

Based on the two-factor hypothesis (Mazur et al., 1972), cooling rate was considered a critical factor of post-thaw cell viability. The optimal cooling rate could be empirically determined depending on germplasm cell types, cryoprotectants, packaging containers etc. (Mazur, 1977; Pegg, 2002). Cooling of samples can be conducted to a temperature (−40 °C or − 80 °C) at which samples and the cryo-medium should be completely frozen, and then frozen samples can be directly immersed liquid nitrogen at −196 °C for long-term storage. This approach is often called controlled cooling cryopreservation. Another method of cryopreservation, called 'vitrification.' This process cools samples at ultrafast cooling rates, which yield a glass-like ice transformation rather than ice crystallization to avoid injuring cells during cooling process (Fahy et al., 1984). Vitrification has been successfully used for human egg and embryo cryopreservation as routine for artificial fertilization (Loutradi et al., 2008; Rienzi et al., 2017).

3.4.1. Cryopreservation with controlled cooling rates

For controlled cooling, a two-step cooling procedure was commonly applied for macroalgal cryopreservation (Tables 3–5). Cooling rates, initial temperature, final temperature, and holding time of the first step and second-step cooling were different for different macroalgae species. Overall, a controlled cooling rate at around 1 °C min^{-1} was applied for the first step cooling procedure using a computer-programmed freezer or cooling at 1–6 cm above the surface of liquid nitrogen. This probably related to the large cell size of macroalgal germplasm which need more time for intracellular water transport during cooling process (Grout and Morris, 1987) to avoid intracellular ice crystal formation (McLellan, 1989). For some macroalgal species such as *Gracilaria tikvahiae*, slow and fast cooling rates (0.2 °C min^{-1} to 32 °C min^{-1}) did not yield significant differences in post-thaw survival (van der Meer and Simpson, 1984). While in other macroalgal species, such as *Saccharina japonica*, controlled cooling rates at 0.25 °C min^{-1} and 5.0 °C min^{-1} resulted in the loss of preserved meiospores. The post-thaw viability of meiospores, immersed directly in liquid nitrogen, was 34% (Zhang et al., 2007b).

Initial temperature of the first step cooling process was usually the temperature for equilibration of germplasm with cryoprotectants. For *Saccharina latissima,* a cold temperate species, cooling started at 10 °C (Visch et al., 2019) while for *Ectocarpus* species (*E. siliculosus, Ectocarpus* sp., and *E. fasciculatus*), which inhabit warm temperate regions, cooling started from 20 °C (Heesch et al., 2012). The final temperature of the first step cooling process was usually −40 °C, which was demonstrated as optimal in majority of the reports for macroalgal germplasm cryopreservation (Tables 3–5). Cooling to a temperatures higher than −40 °C would increase cell death because of insufficient intracellular dehydration (Kuwano et al., 1994). For cryopreservation of gametophytes of *Saccharina japonica*, cooling of samples to −60 °C at a cooling rate of 1 °C min^{-1} yielded a 59% post-thaw survival (Zhang et al., 2007a) .

Holding time between the first-step and second-step cooling procedure was different among reports, e.g., 5 min for *Ulva prolifera* gametophytic thalli cryopreservation (Lee and Nam, 2016), and 10–15 min for cryopreservation of gametophytes of *Saccharina latissima* (Visch et al., 2019). The effect of holding times after the first step cooling on post-thawed gametophyte viabilities was investigated in *S. japonica* and

40 min proved to be the optimal holding time (Zhang et al., 2007a). In some reports, no holding time was reported after the first cooling step.

The second cooling step was to immerse samples directly into liquid nitrogen (−196 °C) or liquid nitrogen vapor. Samples were then kept in liquid nitrogen (or vapor phase) for long-term storage. This cooling procedure was reported in majority of macroalgae for germplasm cryopreservation studies. However, for green macroalgae *Ulva intestinalis*, *Ulva fasciata*, *Ulva pertusa*, and *Ulva rigida*, the second cooling step was to −80 °C rather than −196 °C. The reports suggested high post-thaw survival for gamete and zoospore cryopreservation (Taylor and Fletcher, 1999a; Bhattarai et al., 2007; Gao et al., 2017).

3.4.2. Vitrification – Ultrafast cooling

Vitrification is the process of liquid "solidification" without ice crystallization but transformation into a glass state (Harding et al., 2004). Complete vitrification requires ultra-fast cooling rates and high sample viscosity by use of evaporative desiccation and/or employing osmotic dehydration with penetrating cryoprotectants (Harding et al., 2004; Day, 2018). Another method to achieve vitrification is encapsulation-dehydration, which involves in encapsulation and dehydration of encapsulated materials by immersion and storage in liquid nitrogen (Sakai and Engelmann, 2007). There are no toxic cryoprotectants required for samples, therefore post-thaw samples can be cultured upon thawing without the need for cryoprotectant removal (Taylor and Fletcher, 1999b).

Vitrification has been extensively applied to germplasm cryopreservation of animals, humans, and higher plants (Harding et al., 2004; Sakai and Engelmann, 2007). To date, only a few studies were reported on vitrification of macroalgal germplasm. Mostly vitrification was conducted by simply immersing samples, with or without cryoprotectants, directly into liquid nitrogen (Tables 3–5). For *Neopyropia yezoensis*, gametophytic thalli were vitrified by directly immersing into liquid nitrogen after equilibration for 30 s with 5% DMSO and 15–25% diglycerol, yielded over 80% post-thaw viability (Choi et al., 2013). Haploid protoplasts were vitrified by immediately immersing in liquid nitrogen after mixing with 25% vitrification solution (10% *w/v* DMSO, 30% w/v glycerol, and 10% sucrose in seawater) and had a post-thaw viability as high as 66.5% (Liu et al., 2004). For *Undaria pinnatifida*, gametophytes were vitrified in liquid nitrogen by encapsulating into calcium alginate beads after mixing with 2 mol $^{-1}$ glycerol and 0.6 mol L^{-1} sucrose for 90–120 min at 25 °C. Dehydration with vitrification solution (30% glycerol +15% ethylene glycol +15% DMSO) for 40–50 min at 0 °C, and washing with 1.2 mol L^{-1} sucrose solution, had a post-thaw viability of 26–31% (Wang et al., 2011). For *Scytosiphon lomentaria,* filaments were vitrified after mixing with a solution (2 mol L^{-1} glycerol +0.4 mol L^{-1} sucrose) for 30 min. Dehydration followed with a vitrification solution (10% DMSO +10% glucose +10% polyethylene glycol 6000) for 30 min at 0 °C. A rinse followed with 1.2 M sucrose for 20 min yielded a post-thaw survival rate of 38% (Zhuang et al., 2015).

3.5. Storage of cryopreserved germplasm samples

Theoretically, cryopreserved samples need to be stored and maintained at temperatures below −135 °C/−140 °C, the glass transition temperature. Commonly, liquid nitrogen or nitrogen vapor were employed for cryopreserved sample storage in Dewars. Most evidence proved that there was little or no detectable decline of viability of cells after many years storage at −196 °C (Huang et al., 2019). Routine care of filling liquid nitrogen of the storage tanks needs to be taken. Alternatively, electric ultra-freezers at −150 °C can be used for storage of cryopreserved samples. These ultra-freezers are commercially available, and many types have built-in liquid nitrogen back-up systems, which can be self-activated if a power outage occurs.

Besides cryopreservation at −196 °C, germplasm samples could be occasionally preserved at non-cryogenic temperatures (mostly at 4 °C) for a relatively short storage time for aquaculture use, such as extending the growing season (Oohusa, 1984; Lobban and Harrison, 1994). For example, thalli of *Neopyropia yezoensis* (1–3 cm) with 10–40% water content without cryoprotectants was frozen and stored at −20 °C for 40 d, and survival after thawing was 93.10% (Lin et al., 2010). Cultured blades of *Porphyra umbilicalis* (4.8 ± 0.22 mg) were directly frozen for 1, 3, 6, or 12 months at −80 °C or − 20 °C without cryoprotectants after being air-dried to 5% or 30% absolute water content, and 100% survival rates were obtained after thawing and rehydration (Green and Neefus, 2014) (Table 1).

3.6. Thawing of cryopreserved samples

For cryopreserved macroalgal germplasm, relatively few studies have been conducted on the effects of thawing rate on the post-thaw survival (Tables 3–5). Theoretically, factors causing cell injury during cooling process would potentially cause cell injury during warming process. Therefore, optimal thawing temperature or ultra-rapid warming rate (similar with vitrification) needs to be determined.

Slow thawing has been used in recovery of non-cryogenic preserved macroalgal germplasm. For example, thawing of blades of *Porphyra umbilicalis* preserved at −20 °C and − 80 °C were conducted by immersing into 125-mL of aerated sterile culture medium at 15 °C to recover for 3 h, and a 100% survival was achieved and continued to grow after rehydration (Green and Neefus, 2014). Different thawing temperatures (30, 40, and 50 °C) on the post-thaw survival of *Ulva lobata*, *Gracilaria corticata* and *Hypnea musciformis* were tested, and thawing at 40 °C yielded the highest post-thaw survival (Lalrinsanga et al., 2009). For *Neopyropia yezoensis* conchocelis, thawing temperatures (20 °C, 30 °C and 40 °C) on post-thaw survival were tested, and again, 40 °C yielded the highest recovery rate of 87% (Zhou et al., 2007). In a majority of the published studies, thawing of cryopreserved samples has been conducted merely by immersing the frozen samples into a warm water bath at a temperature around 40 °C until samples had been completely thawed.

Ultra-rapid warming has been emphasized in recent years to address the low post-thaw survival especially for large-sized cells such as mouse embryos and oocytes after vitrification. It was proposed that intracellular ice could be recrystallized during warming process and affect post-thaw survival (Mazur and Paredes, 2016). With an infrared laser pulse and Indian ink at μm sizes as heat-transfer medium, ultra-rapid warming at 10,000,000 °C min^{-1} was achieved. Application on thawing of vitrified mouse oocytes yielded nearly 100% post-thaw survival (Jin et al., 2014), even on thawing of vitrified mouse oocytes without permeable cryoprotectants (Jin and Mazur, 2015). Application of ultra-rapid warming yielded a 10% post-thaw survival vitrified zebrafish *Danio rerio* embryos (Khosla et al., 2017) and 43% in vitrified coral *Fungia scutaria* larvae (Daly et al., 2018). Additionally, ultra-rapid warming was also achieved by using inductive heating system and nanoparticles, and have been successfully applied on thawing vitrified tissues and organs (Manuchehrabadi et al., 2017).

For macroalgae, the germplasm materials are usually large-sized, ultra-rapid or rapid warming could have a significant effect on post-thaw survival. Rapid thawing of *Undaria pinnatifida* gametophytes yield a 50–100% survival, which was significantly higher than that from slow warming (Renard et al., 1992; Taylor and Fletcher, 1999b). Further investigation is warranted for application of ultra-fast warming.

3.7. Post-thaw sample amendments

One of the common post-thaw sample amendments was to remove the cryoprotectants in post-thaw samples to avoid the toxicity of cryoprotectants on germplasm cells or tissues. This amendment has been applied for human, livestock, poultry, and fish sperm cryopreservation (Elliott et al., 2017). For macroalgae, removal of cryoprotectant in post-thaw samples has been reported in majority of the publications before conducting viability assays. The first approach to remove

cryoprotectants was to dilute post-thaw samples with culture medium, for example, the vegetative thalli of *Gracilaria corticata*, *Hypnea musciformis* and *Ulva lobata* (Lalrinsanga et al., 2009). The gametophytic thalli of *Ulva prolifera* (Lee and Nam, 2016), the gametophytes of *Undaria pinnatifida* (Nanba et al., 2009) and the gametophytic thalli of *Neopyropia yezoensis* (Choi et al., 2013) required removal of cryoprotectants as well. The second approach to remove cryoprotectants was to centrifuge the post-thaw sample followed by discarding of the supernatant. This approach has been applied on conchocelis cells of *Neopyropia yezoensis* (Zhou et al., 2007) and free-living conchocelis filaments of five species of *Neopyropia* and *Porphyra* (Jo et al., 2003). So far, no differences were found in post-thaw viabilities between post-thaw macroalgal samples with or without removal of cryoprotectants in post-thaw samples.

4. Viability assays of post-thaw samples

Easy, quick, and accurate viability assays are essential for development of cryopreservation protocols. For macroalgae, post-thaw samples require several days to weeks to germinate or grow into mature thalli for viability determination. A summary of the most widely methods for viability assay in macroalgae germplasm is listed in Table 6. Based on the macroalgal cell characteristics, the three commonly used viability assays were cell staining, measurement of cell pigmentation index, and evaluation of re-growth.

4.1. Cell staining

The stains for determination of living macroalgal cells include 0.05% (*w/v*) erythrosine (Nanba et al., 2009; Lee and Nam, 2016), 0.1% (w/v) fluorescein diacetate (Zhang et al., 2007b; Zhang et al., 2007a), and 0.02–0.1% (w/v) neutral red (Wang et al., 2000; Zhou et al., 2007). After staining for about 20– 30 min, macroalgal samples were rinsed with fresh seawater, and observed immediately by use of light microscopy. The stained cells were determined as living cells, while un-stained cells were dead cells. This method is easy to conduct, quick, and effective, but may over-estimate the percentage of living cells. To date, neutral red staining is the most widely used assay for macroalgae cell viability.

4.2. Measurement of cell pigmentation index

Measurement of cell pigment index is another fast method for macroalgal viability assay (Table 6), which was called the method of damage assessment (van der Meer and Simpson, 1984). Small pieces of thalli damaged by freezing lose all or part of their pigmentation and this observation was used to devise a 'pigmentation index' where fully pigmented thalli were given a score of 10 whereas completely white, dead thalli received a score of 0. Partially damaged thalli were given intermediate scores reflecting both the area of dead tissue and the degree to which surviving tissue had lost pigmentation. The pigments for evaluation include chlorophyll levels (van der Meer and Simpson, 1984; Wang et al., 2005; Wang et al., 2011) and the ratio of viable cells with brown color out of total cells for Ochrophyta macroalgae (Zhang et al., 2008; Visch et al., 2019). The effectiveness of this method was confirmed for seaweed survival estimates (Lalrinsanga et al., 2009; Gao et al., 2017).

4.3. Evaluation of re-growth

The re-growth of post-thaw samples would be the gold standard for viability assay. This viability assays were extensively used in majority of studies (Table 6). Comparison of this direct method with other indirect methods (staining and pigment index) showed similar results, indicating the effectiveness of indirect methods for viability assays (Leeson et al., 1984). However, whenever time allows, the re-growth method should be employed for viability assays of post-thaw macroalgal samples.

Table 6
Viability assays of macroalgae in germplasm cryopreservation studies.

Methods	Viability assay	Reference
Staining and/or examination of cells for ultrastructural effects	Neutral red (1:10000)	Terumoto, 1960, 1961, 1964, 1965
	Neutral red (0.1%); Erythrosine (0.05%)	Kuwano et al., 1992, 1993, 1994, 1996, 2004
	Neutral red (0.02%)	Wang et al., 2000
	Neutral red (0.1%)	Jo et al., 2003
	Neutral red (0.1%)	Zhou et al., 2007
	Neutral red (0.1%)	Zhuang et al., 2015
	Cell ultrastructural examination	Migita, 1964, 1966
	Cell ultrastructural observation using double fixation with glutaraldehyde (4%)-osmium tetroxide (1%)	Ginsburger-Vogel et al., 1992
	Fluorescein diacetate (3.6×10^{-5} M)	Liu et al., 2004
	Fluorescein diacetate (0.1%)	Zhang et al., 2007a, 2007b
	Erythrosine (0.05%)	Nanba et al., 2009
	Erythrosine (0.05%)	Choi et al., 2013
	Erythrosine (0.05%)	Lee and Nam, 2016
Measurement of photosynthetic rate	Fluorescence/photosynthesis measurement	Dudgeon et al., 1989, 1990
	Chlorophyll fluorescence measurements	Lin et al., 2010
	Photosynthetic efficiency of PSII measurement	Green and Neefus, 2014
Ability of cell division, regeneration, and re-growth	Percentage regeneration after 6-week cultivation	van der Meer and Simpson, 1984
	Re-growth rate	Renard et al., 1992
	Cell division and formation of gametophytic thallus	Kuwano et al., 1994
	Developmental capacity	Wang et al., 2005
	Thallus regeneration	Lalrinsanga et al., 2009
	Developing sporophytes determination	Wang et al., 2011
	Regrowth rate after 6–8-week cultivation	Heesch et al., 2012
	Regrowth rate after 6-week cultivation	Green and Neefus, 2014
	Regrowth rate after 1-month cultivation	Zhuang et al., 2015
	Regrowth determination	Barrento et al., 2016
	Regrowth rate after 7-day cultivation	Chen et al., 2016
Measurement of spore liberation/gamete release	Liberation of spores from conchocelis	Migita, 1967
	Gamete release	Vigneron et al., 1997
Measurement of cell pigmentation	Pigmentation index	van der Meer and Simpson, 1984
	Pigmentation index	Vigneron et al., 1997
	Chlorophyll measurement	Wang et al., 2005
	Cells with brown color (living) to the total	Zhang et al., 2007a, 2007b, 2008
	Pigmentation index	Lalrinsanga et al., 2009
	Chlorophyll a level after 8-day incubation	Wang et al., 2011
	Pigmentation index	Gao et al., 2017
	Cells with brown color (living) to the total	Visch et al., 2019
Spore/gamete germination	Germination rate of zoospores	Taylor and Fletcher, 1999a
	Germination rate of spores	Bhattarai et al., 2007
	Germination rate of gametes	Gao et al., 2017
Rate of gamete formation	Rate of gametogenesis	Lee and Nam, 2016

4.4. Other viability assays

Additional methods for macroalgal viability assay included the observation of ultrastructural damage, liberation of spores or gametes, photosynthetic efficiency (PSII), and evaluation of gamete formation. For macroalgal spores or gametes, germination rates would be the gold standard for viability assay immediately after thawing (Taylor and Fletcher, 1999a; Bhattarai et al., 2007; Gao et al., 2017).

Overall, any methods stated above could potentially be used for macroalgal viability assays (Taylor and Fletcher, 1999b). However, large variation in viability may exist among these viability assay methods (McLellan et al., 1991). Therefore, extreme caution should be exercised when comparing the viabilities estimated by different methods among different publications. Furthermore, even with the same viability assay, comparison of results from different publications needs to pay attention to the definition of viability.

5. Outlook and future research

Overall, macroalgal germplasm cryopreservation has been studied mostly in economically or ecologically important species. Most of these studies were conducted empirically, and limited application has been reported on the establishment of germplasm repository or commercial macroalgal production. The majority germplasm types used for cryopreservation were sporophyte or gametophyte thalli. Post-thaw viability showed significant variations among different publications even for the same species. Depending on specific germplasm type and studied species, the cryopreservation protocols showed variable results (Tables 3-5).

Based on the review of the current research updates, further investigation topics on macroalgal germplasm cryopreservation should include:

1) Standardization of research protocols need to be improved through systematic evaluation of some factors, such as addition of proteins, lipids, vitamins, or antioxidants to cryo-medium; osmolality and pH change of cryo-medium; equilibration time and interaction with cryoprotectants. Standardization of protocols would allow direct comparison of results from different publications and could provide easy operation and assure quality control for the application of macroalgal cryopreservation protocols;
2) Different packaging containers with potential for high-throughput processing need to be evaluated and compared on protocol development for macroalgal germplasm repositories;
3) Research cryopreservation protocols for different types of germplasm need to be developed including thalli (gametophytes or sporophytes), spore type (flagellated or amoeboid), and gametes (isogametes, anisogametes or oogametes). Cryopreservation of spores and gametes could facilitate preservation of numerous strains and species of macroalgae with less requirement of labor and space;
4) Vitrification technology, such as encapsulation-dehydration with no toxin cryoprotectant, needs to be developed for cryopreservation of macroalgal germplasm with direct use of post-thaw samples without repeated washing; and
5) Use of ultra-rapid warming technology showed promising improvement on post-thaw viability of large-cell or tissue vitrification (see Section 3.6). There is a potential that ultra-rapid warming could be applied on macroalgal cryopreservation or vitrification.

Overall, germplasm cryopreservation for marine macroalgae have been majorly focused on development of research protocols in the laboratory. These research protocols could be applied to establish macroalgal germplasm repositories for commercial aquaculture and natural resources conservation. Establishment of germplasm repository (seed banking) requires reliable research protocols, infrastructure, repository management strategy for sample storage and inventory, database management for phenotype, genotypes, and germplasm collection information, and policy for use of cryopreserved samples.

Author statement

Term	Definition
Conceptualization	Ideas; formulation or evolution of overarching research goals and aims
Methodology	Development or design of methodology; creation of models
Software	Programming, software development; designing computer programs; implementation of the computer code and supporting algorithms; testing of existing code components
Validation	Verification, whether as a part of the activity or separate, of the overall replication/reproducibility of results/ experiments and other research outputs
Formal analysis	Application of statistical, mathematical, computational, or other formal techniques to analyze or synthesize study data
Investigation	Conducting a research and investigation process, specifically performing the experiments, or data/evidence collection
Resources	Provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation, computing resources, or other analysis tools
Data Curation	Management activities to annotate (produce metadata), scrub data and maintain research data (including software code, where it is necessary for interpreting the data itself) for initial use and later reuse
Writing - Original Draft	Preparation, creation and/or presentation of the published work, specifically writing the initial draft (including substantive translation)
Writing - Review & Editing	Preparation, creation and/or presentation of the published work by those from the original research group, specifically critical review, commentary or revision – including pre-or postpublication stages
Visualization	Preparation, creation and/or presentation of the published work, specifically visualization/data presentation
Supervision	Oversight and leadership responsibility for the research activity planning and execution, including mentorship external to the core team
Project administration	Management and coordination responsibility for the research activity planning and execution
Funding acquisition	Acquisition of the financial support for the project leading to this publication

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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