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NOTE

Cryopreservation methods are effective for long-term storage of *Labyrinthula* cultures

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ABSTRACT: Marine heterotrophic protists of the Labyrinthulomycota are of interest for their biotechnological (e.g. thraustochytrid production of lipids) and ecological (e.g. wasting disease and rapid blight by pathogens of the genus *Labyrinthula*) applications; culture-based laboratory studies are a central technique of this research. However, maintaining such microorganism cultures can be labour- and cost-intensive, with a high risk of culture contamination and die-off over time. Deep-freeze storage, or cryopreservation, can be used to maintain culture back-ups, as well as to preserve the genetic and phenotypic properties of the microorganisms; however, this method has not been tested for the ubiquitous marine protists *Labyrinthula* spp. In this study, we trialled 12 cryopreservation protocols on 3 *Labyrinthula* sp. isolates of varying colony morphological traits. After 6 mo at −80°C storage, the DMSO and glycerol protocols were the most effective cryoprotectants compared to methanol (up to 90% success vs. 50% success, respectively). The addition of 30% horse serum to the cryoprotectant solution increased *Labyrinthula* sp. growth success by 20–30%. We expect that these protocols will provide extra security for culture-based studies, as well as opportunities for long-term research on key *Labyrinthula* sp. isolates.

KEY WORDS: Dimethyl sulfoxide · DMSO · Glycerol · Horse serum · Cryoprotectant · Wasting disease · Marine protist

INTRODUCTION

The members of the genus *Labyrinthula* are important heterotrophic aquatic protists in terrestrial and coastal marine ecosystems. They are surface microbiome members for macroalgae and seagrass hosts because of their roles in carbon and nutrient cycling, as well as the decomposition of senescent biomass (Armstrong et al. 2000, Raghukumar 2002). *Labyrinthula* also act as opportunistic pathogens that cause wasting disease in seagrasses and rapid blight in turf grass (Muehlstein et al. 1991, Martin et al. 2002). Seagrass wasting disease ecology was first described after a 90% die-off of seagrass along the North Atlantic coastlines in the 1930s (Petersen 1934, Renn 1935). In the late 1980s, *L. zosterae* was identified as the causative agent of seagrass wasting disease (Muehlstein et al. 1991), and more than a decade later, *L. terrestris* was identified as the pathogen causing rapid blight (Martin et al. 2002). Since then, research has focussed on what drives the incidence of disease as well as the virulence of *Labyrinthula* isolates (Brakel et al. 2014, Martin et al. 2016, Sullivan et al. 2017). For wasting disease, it

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has been hypothesised that disease occurrence is linked to elevated seagrass stress, to a genetic predisposition to virulence in some *Labyrinthula* clades, or to a combination of these 2 variables (Martin et al. 2016, 2017).

As with most marine disease research, isolating and maintaining pure cultures of *Labyrinthula* is a necessary procedure in many of the laboratory-based research techniques, which include pathogenicity and Koch's postulates tests (Muehlstein et al. 1988, Govers et al. 2016), as well as genomic sequencing and phylogenetic analyses (Martin et al. 2016). While the techniques for isolation in liquid- and agar-based media are well established, long-term maintenance of cultures can be a challenge due to the required labour and costs associated with frequent sub-culturing, the high risk of fungal contamination and the periodic and sometimes unexplained die-off of *Labyrinthula* in culture (Martin et al. 2016, Trevathan-Tackett et al. 2018). Cryopreservation is a technique for the long-term preservation of microorganisms (Hubalek 2003), including aquatic microalgal, protist and fungal preservation (Cañavate & Lubian 1995, Gleason et al. 2007, ATCC 2013), and thus may provide security against losing valuable cultures. Virulence of isolated microorganisms can be influenced by environmental conditions (e.g. Case et al. 2011), as well as artificial culture conditions. In the latter scenario, evolution or 'drift' of the genetic and phenotypic characteristics could occur when isolates are maintained in culture long-term (Dunham et al. 2002, Herring et al. 2006); therefore, cryopreservation could also help mitigate 'artificial' changes in pathogen virulence.

Cryopreservation of thraustochytrids (marine heterotrophic protists related to *Labyrinthula*) in liquid media was successful using DMSO and horse serum (HS) as cryoprotectants (Cox et al. 2009); however, there is no published protocol on the successful cryopreservation of *Labyrinthula* spp. and other marine protist cultures growing on agar media. In this study, 3 *Labyrinthula* isolates in agar-media culture were cryopreserved for 6 mo in DMSO, glycerol and methanol, with and without added HS as an extra source of nutrients (Cox et al. 2009, ATCC 2013). The aim here was to develop a cryopreservation methodology for *Labyrinthula* that could be used in the formation of legacy cultures, to significantly reduce the risk of loss or contamination and to prevent the possibility of genetic or phenotypic change over time, thus further supporting disease ecology research on *Labyrinthula*.

MATERIALS AND METHODS

The *Labyrinthula* isolates used in this study were described by Trevathan-Tackett et al. (2018). Briefly, 2 isolates collected from San Remo, Victoria, Australia, were found to be pathogenic and were from 2 different haploytype groups: Aus4 (SR_Zm isolate C2) and Aus5 (SR_Ha isolate C). The third isolate from Lakes Entrance, Victoria, Australia (LAK_Zm isolate A), was also from the Aus4 haplotype but was found not to be pathogenic. The Aus4 haplotype was shown to be closely related to *L. zosterae* (Trevathan-Tackett et al. 2018), the most common and well-studied pathogenic species of *Labyrinthula* in the northern hemisphere (Sullivan et al. 2013). All 3 isolates had varying culture growth morphotypes, with the SR_Ha isolate showing the fastest colony area growth rate and in-agar colony growth characteristics.

DMSO (cell-culture grade, ChemCruz, Santa Cruz Biotechnology), glycerol (distilled analytical reagent, PROLABO) and MeOH (LC/MS grade, OPTIMA, ThermoScientific) were used as possible cryoprotectants at 5, 10 or 15% v/v concentration, with and without 30% v/v HS (Cox et al. 2009, ATCC 2013), for a total of 12 protocols (Table 1). Liquid media (agar culture media without agar; see Martin et al. 2009) was added to the cryoprotectant solution at a final salinity of 25 psu (final volume of 1 ml sample^{-1}; Table 1). Each *Labyrinthula* isolate culture was grown up for 12–22 d in order to build up enough surface area at the leading edge. For consistency, the leading edge, i.e. the youngest and growing edge of the culture made of vegetative cells (Muehlstein et al. 1991), was sampled with a 5 mm diameter, sterilised hole-punch to make standardised plugs of culture. The plugs from each isolate were added to each of the 12 protocol solutions (n = 3) in cryotubes (2 ml, internal thread; Greiner Bio-One). The cryopreservative solutions were allowed to penetrate the cells for at least 1 h before slow-freezing at 1°C min^{-1} (Mr. Frosty®, Nalgene) to −80°C (Cox et al. 2009).

After approximately 6 mo, the samples were quick-thawed in 50°C water baths. The plugs were removed from the cryoprotectant solutions with sterile forceps and placed on fresh agar media in 6-well plates. Since it was not possible to tell which side of the plugs contained the original culture, the plugs were cut in half with the forceps so that both ends of the plug were in contact with the agar. Lastly, 1 ml of fresh liquid media was added to each well to provide ample nutrients, as well as to dilute any remaining toxic effects of the cryoprotectants (Cox et al. 2009).

Table 1. Composition of cryopreservation solution (µl) for each protocol (1–12). Stock media were adjusted so that final salinity of the cryopreservation solution was 25 psu. MeOH: methanol; HS: horse serum

	DMSO + HS		DMSO only		Glycerol + HS		Glycerol only		MeOH + HS		MeOH only	
	1	2	3	4	5	6	7	8	9	10	11	12
10% DMSO	100		100									
15% DMSO		150		150								
10% Glycerol					100		100					
15% Glycerol						150		150				
5% MeOH									50		50	
10% MeOH										100		100
30% HS	300	300			300	300			300	300		
Media	600	550	900	850	600	550	900	850	650	600	950	900

Fresh liquid media were reapplied throughout the 13 d of growth to prevent desiccation before colony growth was established. *Labyrinthula* growth on the fresh agar and in the liquid media was monitored during the next 2 wk using an inverted microscope with phase-contrast (CK30/CK40 RPSL, Olympus). Positive culture growth, and thus cryopreservation success, was identified by the characteristic colony formation within an ectoplasmic network (Tsui et al. 2009), as well as continued growth throughout the experiment. Clumps of cells with no visible network formation were considered non-viable.

Culture growth was measured by manually tracing the culture edges on the bottom of the well-plate (Trevathan-Tackett et al. 2015), followed by culture area quantification using Image-J (https://imagej.nih.gov/ij/). Growth rates were also calculated as $mm^2\ d^{-1}$. A preliminary 2-way ANOVA indicated an insignificant protocol × isolate interaction for both final colony area size and growth rate. Therefore, statistically different effects among isolate and protocol (independent variables) on growth rates and final area (dependant variables) were tested separately with a 1-way ANOVA. If the data were not normal even after square-root transformation, a non-parametric Kruskal-Wallis test was used. A Bonferroni correction was applied to the post hoc tests ($\alpha = 0.05$), and all statistical tests were performed on SPSS v25 (IBM Analytics)

RESULTS AND DISCUSSION

All samples were retrieved after freezing, except 1 replicate from Protocol 10 (LAK_Zm) (see Table 1 for protocols) and 1 replicate from Protocol 12 (SR_Zm), which were lost or damaged. Positive *Labyrinthula* growth was identified as full colony formation within an ectoplasmic network, while 'non-viable' samples were characterised by clumps of intact cells growing from the plug but never fully revived. In general, the DMSO (Protocols 1–4) and glycerol (Protocols 5–8) treatments had the highest success at reviving *Labyrinthula* isolates (up to 90% success vs. 50% success, respectively; Fig. 1). For these protocols, there was little difference between the 10 and 15% concentrations of DMSO and glycerol, so these were combined for statistical tests of growth rate and area. Additionally, the cryopreservation solutions containing additional HS increased the survival success by 20–30% (Fig. 1), possibly due to additional nutrient resources and protection from chemical toxicity (Cox et al.

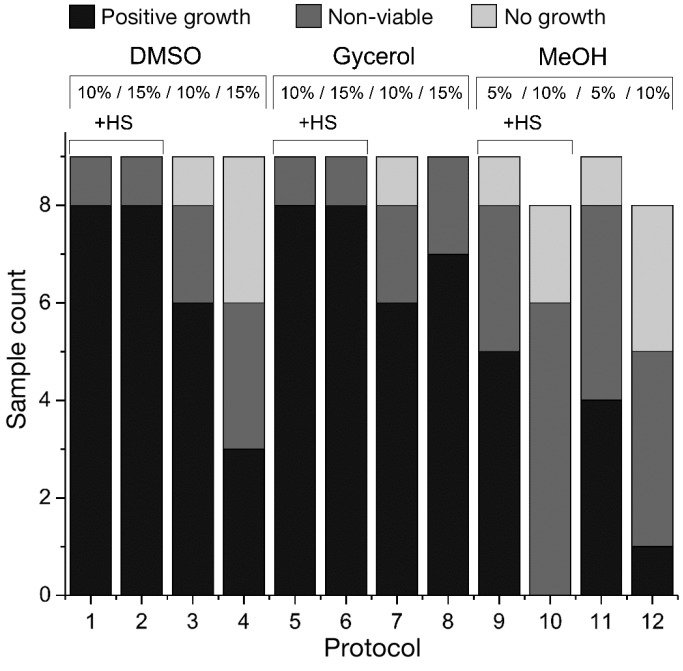

Fig. 1. Categorical survival success of the 12 cryopreservative treatments (see Table 1 for treatment details)

2009). Overall, the MeOH treatments had the least success as a cryoprotectant, despite adding fresh liquid media to the cultures in order to counteract the evaporative effect of the MeOH. In fact, the MeOH treatments in Protocols 10 (no positive growth) and 12 (1 positive growth) performed so poorly, these treatments were removed from the statistical tests in order to have enough power to perform post-hoc tests.

Similarly to survival success, the cryoprotectant treatments affected *Labyrinthula* culture growth rate and colony area. There was a significant effect of the cryopreservative solution on both *Labyrinthula* growth rates ($F_{5,97}$ = 5.116, p < 0.001) and final area (K-W p < 0.001) after the 2 wk growth period (Fig. 2). The growth rates of the MeOH treatments (Protocols 9 and 11) were ~3- to 10-fold lower than the other protocols, while DMSO (Protocols 1+2, 3+4) and glycerol (Protocols 5+6, 7+8) had the highest growth rates (Fig. 2). We noted that, while the growth rates for the DMSO + HS and glycerol only treatments were ~9 times higher compared to the MeOH only treatment, the post hoc analysis indicated the glycerol only treatment was statistically the same (Fig. 2). This treatment had half the number of samples, and so the reduced power of this comparison has likely influenced these contradictory results, i.e. a false positive. Overall, we noted similar patterns for the total colony area, although the MeOH + HS serum treatment was statistically similar to the DMSO only treatment. In contrast to this study, Cox et al. (2009) found that thraustochytrid cultures, i.e. related marine heterotrophic protists, had more success with the DMSO + HS cryopreservation solution compared to the glycerol solution when revived in liquid media. While we saw a similar positive effect of HS on growth success for both DMSO and glycerol, the poor performance of glycerol as a cryoprotectant in Cox et al. (2009) could be linked to the omission of HS in the glycerol treatments. Furthermore, *Labyrinthula* move within an ectoplasmic network that is involved in nutrient absorption and adhesion to substrate, while thraustochytrids do not (Tsui et al. 2009). We hypothesise that this adaptation could indirectly help buffer the effect of toxic cryoprotectants. Lastly, we noticed a slight delay in *Labyrinthula* growth after thawing. On average, *Labyrinthula* from the glycerol treatments took ~4 d to start growing away from the cryopreserved plug, while the DMSO and MeOH treatments took 5–7 and 6–13 d, respectively. This delay in cell growth after thawing has been found for other Labyrinthulomycota, but was an isolate- rather than protocol-dependent effect (Cox et al. 2009).

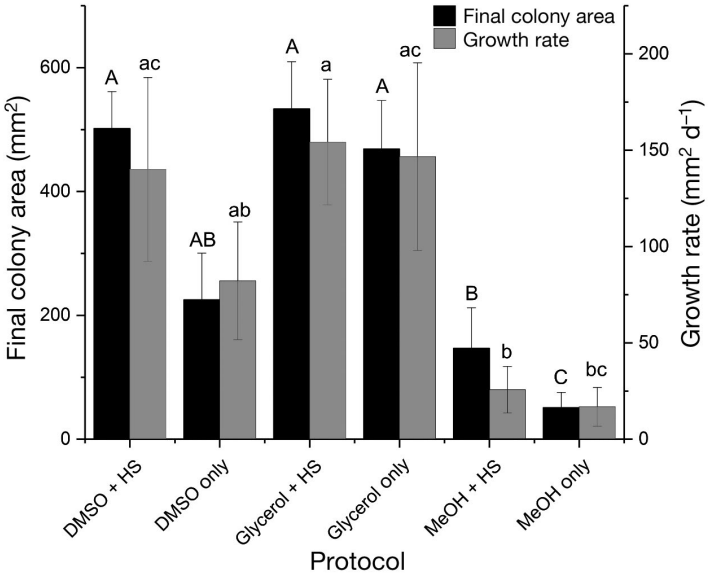

Fig. 2. Effect of cryoprotectant and horse serum (HS) on *Labyrinthula* growth rate and final colony area. Letters indicate significant differences in post hoc tests for area (upper case) and rate (lower case). MeOH: methanol. Bars represent means ± 1 SEM (n = 17–18)

The *Labyrinthula* isolates showed varying responses to the cryoprotectants. The LAK_Zm isolate had a significantly higher growth rate (2-fold higher; $F_{2,97}$ = 4.793, p = 0.01) and colony area (2–3-fold higher; $F_{2,104}$ = 12.664, p < 0.001) than SR_Ha and SR_Zm (Fig. 3). The LAK_Zm isolates grew in the added liquid media more frequently than on the agar media compared to the other isolates (11 samples vs. 3–5

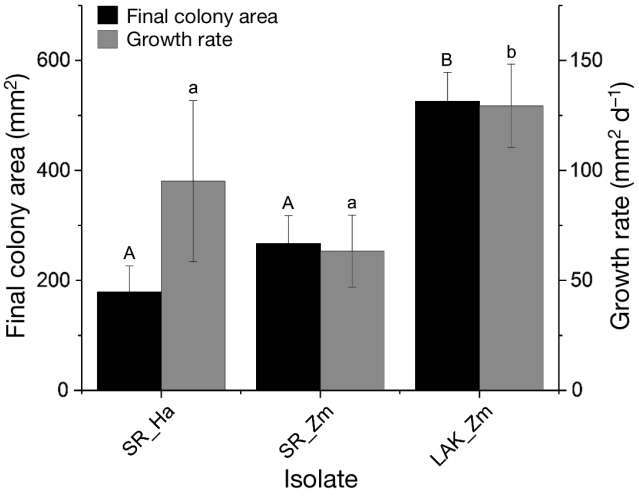

Fig. 3. Variation in *Labyrinthula* isolate growth rate and final colony area. Letters indicate significant differences in post hoc tests for area (upper case) and rate (lower case). Bars represent means ± 1 SEM (n = 35–36)

samples for the others). This resulted in most LAK_Zm isolates covering their respective well plates in 1–2 d, and thus an overall faster growth rate. This variation is likely related to the in-agar growth that seems to be preferred by SR_Ha and SR_Zm (Trevathan-Tackett et al. 2018), which is generally a slower method of movement than growing in liquid media. We also compared the growth rates in this study to the original stock *Labyrinthula* cultures growing in 90 mm diameter plates (10 d). The stock LAK_Zm cultures typically grew 2–3-fold slower than in this study (~45 mm^2 d^{-1}), which was also attributed to the differences in liquid media versus agar-based media. In contrast, the stock SR_Ha and SR_Zm cultures typically grew 2–4-fold faster than seen post-cryopreservation (~475 and 155 mm^2 d^{-1}, respectively). This is likely an artefact of the cryopreservation stress, and while we did not keep track of all of the samples beyond the well-plate quantification, we would expect at least partial, if not full, recovery of culture growth characteristics. This variation highlights how different culture morphologies and growth strategies of *Labyrinthula* isolates could affect the post-cryopreservation success, and suggests that choice in post-thaw care as well as cryoprotectant are important to reviving *Labyrinthula*.

Both DMSO and glycerol solvents showed the most success as cryoprotectants across the different *Labyrinthula* isolates. Whilst the addition of HS seemed to increase growth success, it is also the most expensive media ingredient. In cases where its use is cost-prohibitive, we recommend using the glycerol-only protocols, which were less dependent on HS for viability success, in addition to having the added benefit of glycerol being less toxic to the user and relatively inexpensive compared to DMSO. In conclusion, cryopreservation is an effective way to store *Labyrinthula* cultures long-term and can help prevent loss of cultures and save in costs of long-term culture maintenance. While further research is needed to identify changes in genes and virulence after cryopreservation, we have identified the growth strategies and phenotypic responses to different cryoprotectants and the cryopreservation method overall.

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