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Open encapsulation-vitrification for cryopreservation of algae

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ABSTRACT

Vitrification offers a cost-effective solution for the preservation and management of genetic resources with, low-cost international movement of selected genetic materials and for long-term maintenance of stable stocks of a wide variety of microorganisms. However, its success is limited by the wide range of algal species. Here, we report a simple open encapsulation-vitrification protocol of cryopreservation. Results showed that ~58% and ~27% of *Oocystis* sp. survived vitrification-warming after the open and closed system of vitrification respectively when compared to non-cryopreserved controls. The improved success in an open system of vitrification was also observed in *Anabaena* sp. Furthermore, with the addition of 2-mercaptoethanol or glutathione the post-warming viability of vitrified algae in both open and closed system of vitrification was significantly improved ($p < 0.05$). The present case study aimed to develop a vitrification-based cryopreservation protocol and confirms an improvement in survival percentage over conventional encapsulation-vitrification method.

1. Introduction

A large number of algae have been isolated, characterized, selected, mutated and/or genetically modified worldwide to address the shortage of fossil fuels and to understand the ecological response patterns to climate change. Algae are also considered to be useful as agro foods, human supplements, source of preservatives, and bio-fertilizers [32,35,40]. Several research communities and various industries, therefore, maintain a rich genetic diversity of different algal forms including microalgae and cyanobacteria. Many of the microalgal species have been successfully cryopreserved but several of them have either showed low post-thawing viability or failed to survive altogether [4,13]. On the other hand, most macroalgal species such as filamentous alga and seaweeds have not shown satisfactory post-thawing viability [11,12]. Alternative options include, algal culture banks which are practicing non-axenic culture storage or density dependent preservation protocols [1,29].

Long-term maintenance of algal collections in liquid or solid media have not guaranteed their availability as these processes are labour intensive, costly and subject to contamination and genetic drift [6,21,43]. However, cryopreservation offers a cost-effective solution for long-term maintenance and transport of stable stocks of a wide variety of microorganisms including bacteria, fungi and algae [9,11]. Among different methods of cryopreservation, vitrification offers the advantage of being simple to perform and additionally cost effective as it does not require any expensive devices such as programmable freezer [16,34,36]. However, success of vitrification is limited in most algal species [5,7]. Moreover, procedural steps such as the concentration and timing of loading and unloading of cryoprotective agents (CPA), cell density can influence the success of algal cryopreservation [37,44,46]. Recent studies have also shown that CPA-exposure and cooling can generate intracellular free radicals which can cause oxidative stress and induce apoptosis [10]. The free radicals may also cause genetic alterations [2]. Accordingly, use of antioxidants during vitrification was proposed to help improve cryotolerance and viability of vitrified-warmed algae [14]. However, studies on the possible beneficial effects of antioxidants on viability of vitrified-warmed algae are scarce.

Studies in mammalian oocytes and embryos have shown that, direct contact of the biological samples with liquid nitrogen (LN_2) during vitrification can greatly improve their post-warming

Abbreviations: liquid nitrogen, LN_2; cryoprotective agents, CPA; open encapsulation vitrification, OEV; closed encapsulation vitrification, CEV; 2-mercaptoethanol, 2-ME; glutathione, GSH; field emission scanning electron microscope, FESEM; 3′6′diacetyl fluorescein diacetate, FDA; Phosphate-buffered saline, PBS.

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viability [16,17,38]. This open vitrification system permits rapid rate of cooling due to a minimal volume of vitrification solution surrounding the biological sample and absence of an insulating wall of the cryo-containers. Consequently, this open system has become a popular method for vitrification of animal oocytes and embryos. In contrast to an open system of vitrification there exists a closed system of vitrification. In closed system of vitrification, samples are loaded and sealed in a cryo-container prior to vitrification to eliminate direct contact of the biological sample with LN_2. The latter system is advantageous for human samples to avoid possible cross-contamination with bacteria and viruses such as human immunodeficiency virus and hepatitis B and C viruses which are known to survive in LN_2 [20]. Hence, for the first time our study was designed to investigate the possible benefits of open system of vitrification over closed system of vitrification on the post-warming viability of algae. An encapsulation-vitrification procedure that was used previously for cryopreservation of algae [24,42,44] was evaluated in open and closed system for vitrification by using model organisms of green oleaginous microalgae *Oocystis* sp. and filamentous cyanobacteria *Anabaena* sp. Furthermore, dose-dependent effects of two antioxidants such as 2-mercaptoethanol (2-ME) and glutathione (GSH) on post-warming viability and re-growth of vitrified algae was also evaluated.

2. Materials and methods

2.1. Isolation and culturing of algae samples

Water samples were collected from local water deposits (22′15′19.5″N 84′54′14.5″E) and microalgae axenic culture was isolated by serial dilution and streak plating as described previously [31]. Isolated pure cultures were identified as per Prescott [30]. Obtained strains (nitrkl/ch/5c and nitrkl/ch/4b) were maintained in modified Bold Basal Media in an incubator shaker with constant shaking of 120 rpm, at 25 ± 2 °C under light intensity of 50 ± 5 μmol m^{-2} sec^{-1} and 18:6 (light: dark) hours photoperiod. Cells were harvested by centrifugation (5000 × g for 5 min). The morphological study was performed under inverted microscope (Olympus Corporation Tokyo, Japan) and field emission scanning electron microscope (FESEM).

2.2. Field Emission Scanning Electron Microscopy (FESEM)

Algae samples were vortexed and appropriate concentrations were dropped on glass slides. The specimen was fixed with glutaraldehyde (2–3%) followed by osmium tetroxide (1–2%). Dehydration steps included ethanol washing and overnight drying at room temperature (26–28 °C). Protocols for sample preparation were previously described by Dananjaya et al. [8]. The digital images of carbon-coated specimen were taken at 10,000-fold magnification by FESEM (Nova Nano SEM 450, USA).

2.3. Molecular identification

Total genomic DNA was extracted from fresh samples as described by Park et al. [28]. The PCR reaction was carried out in a total volume of 10 μl containing 100 ng DNA, 5 pmol of forward and reverse primers, 1 × PCR reaction buffer containing 5 mM Tris-HCl [pH9.1], 1.6 mM $(NH_4)_2SO_4$, 15 μg μl^{-1} BSA, 2.5 mM $MgCl_2$ and 1 U Taq polymerase. PCR products were visualized on 2% agarose gels by ethidium bromide staining. The 16S rRNA gene for cyanobacteria and 18S rRNA gene for green algae were amplified using universal primers i.e. F: 5′-GAGTTTGATCCTGGCTCAG-3, R: 5′-AGAAAGGAGGTGATCCAGCC-3′ and F: 5′- TGATCCTTCYGCAGGTTCAC −3′ and R: 5′-ACCTGGTTGATCCTGCCAG-3′ carried out with the following cycle: initial denaturation for 3 min at 94 °C, followed by 35 amplification cycles each consisting of 1.5 min denaturation at 94 °C, 1 min annealing at 59 °C, and a 2 min elongation at 72 °C, with a final 5 min elongation at 72 °C. Then the PCR products were sequenced by Eurofins Genomics India Pvt Ltd. Bangalore. The sequences were analysed using advanced BLAST search program on the NCBI Website (http://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic analysis was also performed for further conformation with the default setting of MEGA 4 using the neighbour-joining method.

2.4. Vitrification

Encapsulation-vitrification was executed as described previously with minor modifications [44]. Briefly, algae cell suspension (concentration: ~2 × 10^8 cells/ml) were mixed with 3% (w/v) sodium alginate (HiMedia Chemicals, Mumbai) solution. Eight to 10 μL of this solution was added drop-wise into 0.1 M $CaCl_2$ (HiMedia Chemicals, Mumbai) solution with the help of a micropipette and maintained at room temperature for 30 min to obtain the microbeads. The algae-encapsulated microbeads were exposed to equilibration solution followed by vitrification solution at room temperature (~27 °C) for 30 min and 15 min respectively. Equilibration solution consisted of 15% (w/v) glycerol, 7.5% (w/v) ethylene glycol, 7.5% (w/v) dimethylsulfoxide and vitrification solution consisted of 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethylsulfoxide. The components of the solutions were finally dissolved in Bold Basal Medium that contained 0.4 M sucrose, pH was adjusted to 5.8–6 and the solutions were filter sterilized. In case of open encapsulation vitrification (OEV), the CPA-treated microbeads were transferred to test tubes and directly plunged into LN_2 to allow direct contact between the sample and LN_2. In case of closed encapsulation vitrification (CEV), the CPA-treated microbeads were transferred to cryovials, sealed and then plunged into LN_2 to avoid direct contact of the biological samples with the LN_2. The vitrified samples were stored in LN_2 for at least one week prior to warming and post-viability assessment. To assess the effects of antioxidants, 2-ME (HiMedia Chemicals, Mumbai) or GSH (Sigma–Aldrich, Bangalore) were treated with the equilibration and vitrification solutions at concentration of 10 μM, 50 μM and 100 μM [17].

2.5. Warming and rehydration

After one week of storage in liquid nitrogen, the vitrified samples were transferred to a pre-heated water bath at 40 °C for 3 min. The microbeads were then collected and rehydrated through serial transfer to 2.0 M, 1.5 M and 0.5 M sucrose solutions for 15 min, 10 min and 5 min, respectively. Finally, the algal cells were released by maintaining the microbeads in 3 M sodium citrate solution for 30 min under constant vortexing. Cells in the supernatant were separated carefully by centrifugation at 1000 × g for 5 min [15].

2.6. Assessment of viability

The viability of algae devitrified by warming was assessed by counting the green cells under an optical microscope (Olympus IX71, Japan). The living cells were counted after 0, 7 and 14 days of incubation. The post-warming viability was further confirmed by a 3′ 6′ diacetyl fluorescein diacetate (FDA) assay. This evaluation was based on the esterase enzyme activity and plasma membrane integrity described earlier [16,17]. Briefly, cells were washed in phosphate-buffered saline (PBS) for 1 min followed by incubation with 2.5 μg/ml FDA for 1 min. Stained cells were then washed three times with PBS to remove traces of the dye and were observed under ultraviolet illumination. Alive cells showed green