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Comparison of cryopreservation methods for the long term storage of the marine diatom *Haslea ostrearia* (simonsen) ☆

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ABSTRACT

Long term maintenance of microalgal strains by serial subculturing is often expensive and time-consuming. Alternative methods, such as cryopreservation, present several benefits and thus seem more relevant. Our study aimed at comparing two cryopreservation procedures applied to the marine diatom *Haslea ostrearia* (Simonsen): (1) a two-step freezing method in liquid media using 5%, 10% and 20% MeOH, Me_2SO or Glycerol, and (2) an immobilization-dehydration method consisting in an algal cell entrapped in 0.7 M sucrose dehydrated and air-flow desiccated calcium alginate beads before "direct" or "two-step" freezing. Our results showed that the cryopreservation of *H. ostrearia* was feasible. With the two-step freezing protocol only Me_2SO maintained cell viability without contamination but the low percentage of viability (<10%) prevents its use. Conversely, the immobilization-dehydration methods tested in this study were effective. Average viability of 57% and 77% were obtained with the "direct" and the "two step" cooling assays respectively, ensuring preservation of the genetic traits of *H. ostrearia*.

Introduction

The tychopelagic pennate Bacillariophyceae *Haslea ostrearia,* which synthesizes a hydrosoluble pigment named marennine, is currently used in several applications. This blue-green pigment is responsible for oyster greening thus giving them added-value in farming [42,43]. This pigment is also used as a natural dye in the food and cosmetic industries [29]. Additionally, antioxidant [35] and antiproliferative activities were demonstrated *in vivo* in medical and pharmaceutical applications [7].

Diatoms preserved in the Nantes Culture Collection (NCC – WDCM 856) are usually maintained by serial subculturing. This method is time consuming – strains have to be transplanted every 2 weeks – and expensive. Additionally, the risk of bacterial contamination due to repeated manipulations is high, physiological and even genetic changes of the preserved strains may be observed. The repeated cell sub-culturing indeed quickens the adaptation of the strains to laboratory culture conditions. Eventually, the vegetative cycle of *Haslea ostrearia*, as observed with other diatoms, is characterized by a decrease in cell size at each division, with observable effects on cell morphology such as an increase in the ratio of deformed cells [39] as well as suspected effects on their physiology. Any culture collection would thus aim at having reliable methods for microalgal cell conservation.

Preservation methods for the long-term storage of microalgae are alternatives to mass subculturing, and are becoming increasingly used in culture collections. In the last few years, cryopreservation (usually used in bacteriology), has been the subject of intensive research. Three thousand microalgal species were successfully cryopreserved within the framework of the European project COBRA (COnservation of a vital european scientific and Biotechnological Resource: microAlgae and cyanobacteria – October 2001 to March 2005) [12].

Cryopreservation includes several phases. The first one consists in microalgal cell preparation before freezing where a cryoprotective agent (CPA) can be added to the cell suspension to avoid cellular damage due to ice formation [40,11]. Two categories of CPAs exist: the penetrating and the non-penetrating types. The former pass through cellular membranes and reduce the cytoplasm crystallization rate [40] by decreasing the intracellular water freezing temperature [18]. For that purpose, CPAs reduce the osmotic pressure in cytosol. They are considered more efficient than non-penetrating ones [11]. The three most used CPAs for

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the cryopreservation of marine microalgae are methanol (MeOH), dimethylsulfoxide (Me_2SO) and glycerol (Gly) [24]. The penetration duration depends on the cell type. The second category of CPAs (non-penetrating), such as polyvinylpyrrolidone (PVP) and polyethylene glycol (PEG), less toxic for the cell, are rarely used in cryopreservation. They cause cell dehydration, thus reducing the amount of available water to form intracellular ice. They also have a protective effect towards thylakoid membranes.

The second phase consists in cooling the microalgal cell suspension. The two currently used methods are the direct one and the two-step freezing protocols. Direct freezing (sample plunged directly in liquid nitrogen) is known to increase the risks of cellular injuries due to intracellular ice formation. The two-step process consists in cooling samples slowly until −40 °C is reached and then plunging it directly into liquid nitrogen for preservation [33,2,5,10,8,40,11,38].

The third phase consists in warming the sample either slowly or quickly. A quick thawing of vials, for example by immersing in a 25–40 °C water bath, gives a higher viability rate and decreases contact time with potentially toxic CPAs [2,5,10,8,40,11,38]. Just after being thawed, cells are returned to culture.

The success of cryopreservation is measured by microalgal growth or viability tests [2,8,11].

H. ostrearia is known to be naturally sensitive to certain stresses such as hydrodynamic conditions, light conditions and presence of certain compounds. Its large size is also considered as a handicap with regards to mechanical stress when it is cryopreserved by "classic" methods using CPAs. Another technique, considered as more efficient, consists in immobilizing cells in alginate beads, and possibly dehydrating these beads [15]. Immobilization was previously developed for the cryopreservation of vegetative organs (apex) of plants (e.g. potatoes, fruit trees) [17,45] and was later adapted to marine microalgae cryopreservation. This method consists in entrapping algal cell in calcium alginate beads, followed by an osmotic dehydration before desiccation before being introduced into cryovials. Eventually, the beads are cooled in liquid nitrogen until the preservation temperature (−196 °C) is reached.

The preservation of *H. ostrearia,* immobilized in calcium alginate beads and then preserved at 4 °C under low irradiance, showeds that stored cells were viable for several months [26,20] but cannot withstand longer term storage. The present study aimed at answering a recent initiative of the NCC microalgal resources in supplying long term preservation methods for new species of microalgae by cryopreservation. *H. ostrearia* was subject to two compared processes: free cell cryopreservation using different CPAs and the immobilization-dehydration procedure.

Materials and methods

Cell culture conditions

This study was carried out by using the (NCC-Jμ1) strain of *H. ostrearia* isolated from oyster ponds of Bourgneuf Bay (France). This clone was characterized by an average modal length of 68.5 ± 0.5 μm (95% Confidence Interval).

Experimental cultures were grown in 250 mL flasks filled with 150 mL F/2 medium [21] and inoculated with exponential growth phase-stock cultures. Cultures were incubated in a culture chamber at 16 ± 1 °C under 35 μmol photon m^{-2} s^{-1} light intensity and 14:10 h light:dark regime.

Two step free cell freezing procedure

The cryopreservation procedure was adapted from the method developed by Day and Brand [11]. The CPAs, i.e., Me_2SO, MeOH and glycerol, were tested at 5%, 10% and 15% concentrations (v/v in media). The control consisted in F/2 medium instead of CPA. Stock solutions of CPAs were prepared by using F/2 medium as the diluting agent. These solutions were sterilized by filtration at 0.2 μm (Osmonics MicronSep™ Mixed Esters of Cellulose). Cultures were centrifuged at 4000*g* (5 min, 4 °C) to obtain a minimal cell concentration of 2.10^6 cells mL^{-1} and controlled by counting cells with a Nageotte haematimetric cell. Then, 1 mL of this concentrated cell suspension was introduced into a 2 mL cryovial and gently mixed with 1 mL of the CPA solution to attain a final culture density of 1.10^6 cells mL^{-1}. The mixture was incubated for 2–3 min at room temperature. After incubation, each cryovial was cooled immediately using a two step procedure: cryovials were placed in a prechilled freezing container (NALGENE® "Mr. Frosty") for 1 h in a −80 °C freezer. Then, they were quickly transferred into a liquid nitrogen storage container. Each experiment was performed in triplicate.

After 48 h, the cultures were thawed by plunging the cryovials into a 35 °C water bath until all ice crystals had melted. Thawed cultures were transferred into 250 mL flasks containing 150 mL F/2 fresh medium and kept in low light for 12 h. Flasks were then placed under normal culture conditions as described before.

Although protective effects of cryoprotectants have been demonstrated on cells, they may also cause lethal effects which were estimated in this study. Tests consisted in CPAs mixed with algal cells at three concentrations, i.e., 5%, 10% and 15%. Two milliliters of the resulting frozen algal suspension was then added to 150 mL fresh medium – the final CPA concentration in the medium ranged from 0.07% to 0.20%. Algal cell growth was monitored by counting cells using a Nageotte haematimetric cell.

Immobilization-dehydration protocol

The following solutions were prepared and autoclaved (120 °C, 20 min) separately. Sucrose (0.5 M) and sodium chloride (28 $g.L^{-1}$), used respectively as a dehydration agent and for the maintenance of the culture medium salinity, were mixed under stirring to a 4%-sodium alginate solution prepared with distilled water. Microalgal cultures in exponential growth phase were centrifuged at 4000*g* (5 min, 4 °C) and mixed with the resulting solution yielding a final concentration of 10^6 cells.mL^{-1}. The mixture was then dropped into a 100 mM $CaCl_2$ solution using a 50 mL syringe. Beads of about 5 mm in diameter polymerized progressively at the contact of the $CaCl_2$ solution where they remained for no more than 20 min.

Beads were then transferred into a 0.7 M sterile solution of sucrose prepared with F/2 medium and left in this solution for 24 h to finish cell dehydration. Beads were removed with a sterile sieve, and left for 2 h in a sterile open Petri dish, under a laminar air-flow hood for desiccation. Finally, they were divided into 2 mL cryovials. Cryovials were plunged directly into liquid nitrogen or maintained for 1 h at −80 °C in a pre-chilled freezing container (NALGENE ® "Mr. Frosty") before being introduced into liquid nitrogen. All these assays were performed in triplicate.

Tubes were maintained for at least 48 h in liquid nitrogen before being thawed in a 35 °C water bath [22] during 2–3 min. Beads were then transferred into 150 mL-fresh medium under usual culture conditions.

Viability Assessment

Algal cells were colored by adding 10 μL of acridine orange ($C_{17}H_{19}N_3HClZnCl_2$) diluted solution for 1 mL algal solution. Microscopic observations were carried out just after staining. A minimum of 300 cells were counted on each glass slide in order to calculate the viability rate. This method was chosen as a fast and simple way to evaluate microalgae survival rate. Viable cells