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Is axenicity crucial to cryopreserve microalgae? ☆

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ABSTRACT

Large culture collections of microalgae and cyanobacteria such as the Coimbra Collection of Algae (ACOI) hold unialgal cultures consisting of a population of cells/colonies of a certain species. These cultures are usually non-axenic, as other organisms such as bacteria and microfungi are also present in culture due to co-isolation. Attention has been recently given to partner organisms since studies indicate that some bacteria are important for nutrient uptake of the algal cells, acting as simbionts. Despite this benign effect in the actively growing cultures, when cryopreservation is applied for inactive-stage storage, these organisms may recover faster than the algae, thus affecting their recovery and the viability assessments. In this study, a set of mucilaginous ACOI microalgae were selected, cell features known for their relevance in cryopreservation success were recorded and simple two-step cryopreservation tests were applied. Thawed samples were transferred to fresh culture medium for recovery. Viability was assessed and partner organism proliferation (pop) was recorded. Results were analyzed by *t*-tests. Statistical models allowed us to support the known tendency for small, unicellular algae with no outer structures to be successfully cryopreserved and the negative effect of vacuoles in the cell prior to cryopreservation. On average cryopreservation with MeOH or Me_2SO led to the recovery of nearly half the cells. It was found that the cryoprotection step with MeOH is when pop is triggered and that the use of Me_2SO can prevent this effect. Progress on understanding the cultured consortia will assist the improvement of cryopreservation and research using microalgal cultures.

Introduction

Many large culture collections of microalgae and cyanobacteria keep their holdings in a non-axenic state. Non-axenicity implies the presence in culture of organisms other than algae, even in such low numbers that they are not visible to the naked eye. These partner organisms consist mainly of bacteria and microfungi, whose proliferation is disadvantaged by the use of culture media with algal-specific formulas [29]. The isolation and cultivation of microalgae became a broad activity in 1960–70 with the work of researchers, who established culture collections such as ACOI [29]. Attempts to remove the co-isolates were developed by mechanical methods and also by the use of antibiotics, but the latter was criticized. The reasons were the difficulty of maintaining viable cultures cleaned by antibiotics [11] and the cell damage inflicted by the antibiotic action, together with the observation that sometimes axenicity was not fully achieved [18]. Important progress in understanding the dynamics of nutrient uptake by the microalgae growing in batch culture and chemostat was achieved by M.R. Droop's studies on cobalamin (vitamin B_{12}) uptake by a symbiotic interaction of the algal cells with co-cultivated bacteria. His "cell-quota model" of phytoplankton growth relates the microalgal growth rate to the internal nutrient content of the cell, in this case, the nutrient is vitamin B_{12} ([19] and references therein). This prompted the suggestion that the reason for the failure in cultivating pelagic diatoms without the associated bacteria was a "possible obligatory relationship" [12]. The most common symbiotic interaction shown to occur for B_{12} uptake was observed in at least half of 326 surveyed microalgae from different groups [7]. Nevertheless, other nutrients are also made available by simbiont partners. The study of dinoflagelates in oceanographic dynamics research revealed a singular ability of their symbiotic bacteria to enhance iron uptake [3]. The community composition of co-isolates is starting to be revealed by molecular studies that reinforce the potential of non-axenic cultures as material for studies on algal-bacteria associations [32] that may be useful for the fermented food industries and waste water treatment [34]. The symbiotic interaction between microorganisms and photosynthetic organisms is still unclear and it is not restricted to microalgae. It has also been observed in macroalgae [26] and in land plants, sometimes with a chain of mutualists involved such as the triplet virus-fungus-plant associations in geothermal soils [21].

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Years of observations of algae belonging to a broad range of taxa in the ACOI collection show that partner organisms such as bacteria and fungi are either distributed in the culture medium or embedded in the mucilage produced by the algae. This last case provides a robust scenario for studying the effects of cryopreservation stresses in non-axenic cultures, because partner organisms live very closely to the algal cells, it is also probable that a symbiotic relationship is present. The sterilization of all glassware and the handling of the cultures at all times in the flow chamber prevents the contamination of the culture with organisms not belonging to the isolated microbial consortia. Regarding the culture media, quite often the formulas include the addition of vitamins. The preparation recipes for vitamin solutions are either provided by culture collections in their websites or in published methods and lists [30] and usually there is an indication that stock vitamins should be prepared separately and filter sterilized prior to use. Paradoxically, in most cases, the general recipe of the culture media includes the vitamin stock as part of the stock solutions to be added to the final mixture, later autoclaved at 120 °C. This probably leads to total or partial disintegration of the vitamins, since most of them are heat-sensitive. This is not fully the case of vitamin B_{12}, which is generally considered as heat stable. Nevertheless, like all water-soluble vitamins, some disintegration may occur on heating if they are dissolved in large volumes of water [20]. When included in 1 L of culture medium to be autoclaved, unpredictable loss may occur and cobalamin-auxotrophs developing in such culture medium, may suffer from vitamin deficiency. This aspect reinforces the importance of partners with their role as cobalamin providers.

The decision of keeping the co-isolates is based on these arguments and also on the concern that the use of detergents, antibiotics or other chemicals for their removal may cause selection of resistant microalgal populations and genetic erosion. Despite the reasons for keeping non-axenic cultures, the presence of bacteria and fungi may become problematic when cryopreservation stress is imposed on the cultures. Algal cells may require more time to recover than their partner organisms and this may result in a higher risk of their proliferation after thawing [9]. One month after recovering from cryopreservation procedures, some thawed samples show a great level of opacity denoting pop, which spreads throughout the culture [1]. One of the causes for this exacerbation is the use of sugar-based cryoprotectants, a preferential carbon source for heterotrophic partners. An extra source of nutrients may also be the release of cellular content as a result of cell lysis caused by cryoinjury [5].

The 'cell vigor' of the microalgal culture prior to cryopreservation is considered as a key-factor for its success [6]. It is generally accepted that cryopreserved cultures in the actively growing phase show better viability after thawing than those that were in the stationary phase, or growing under stress conditions before being cryopreserved [9]. Also, previous reports indicate that strains displaying specific cell characteristics are usually highly viable after thawing. These features include microalgae which are unicellular, small, spherical, with no spines and without vacuoles at the time of cryopreservation (e.g. [27,1]).

Assessments of the dynamics and effects of pop in cryopreserved cultures are complex since they must take into consideration the importance of the cell characteristics in relation to cryopreservation stress. It is desirable that these aspects be studied, together with the occurrence of pop in a comprehensive approach to determine when and how pop impacts take place.

The two-step cryopreservation method developed by Morris [23] has been broadly applied to cryopreserve microalgal cultures except for large, sensitive or fragile species. In the case here, the microalgae are usually cryopreserved by encapsulation/dehydration, a method developed by Hirata et al. for higher plant material [16], which now is quite often used to overcome recalcitrance in microalgae, although it promotes high proliferation of partner organisms due to the use of sugar-based cryoprotectants [5].

For collections with large holdings waiting to be cryopreserved, including species with no previous cryopreservation history published in the literature, it is useful to develop standard approaches. This is done at ACOI, where sets of microalgal cultures are cryopreserved using a step-by-step approach designed to draw the best possible protocol for each strain (Fig. 1). After the sequence of tests is applied but no suitable protocol for permanent storage is found for a strain, changes in the culture conditions are considered. This is also done if good viability is achieved but the culture shows too lower cell density to consider permanent storage. The cell density of the culture may be enhanced for example by raising the pH

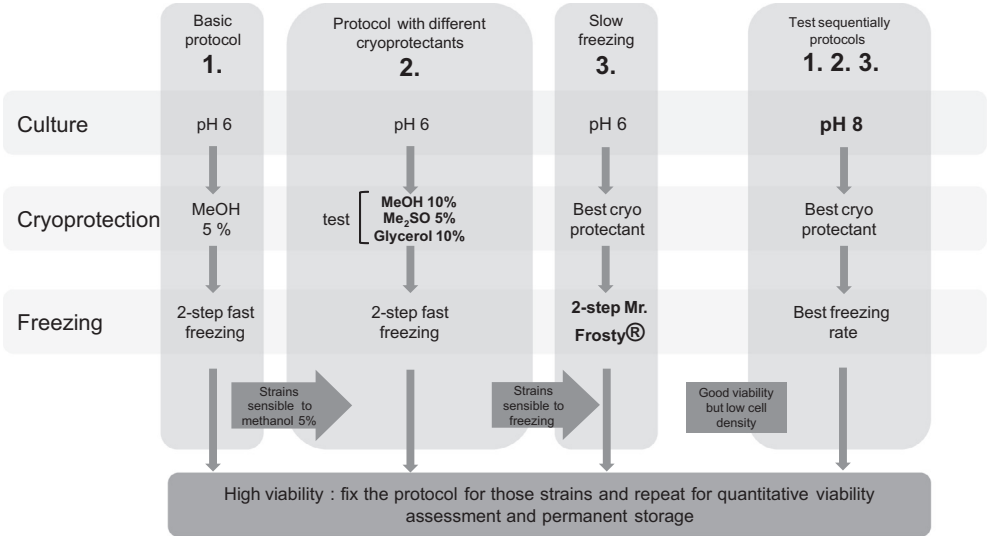

Fig. 1. Schematic view of the 'step-by-step approach' designed to draw the best protocol for cryopreservation of the ACOI strains. A basic two-step protocol with MeOH 5% as a cryoprotectant and a fast freezing is applied to a set of different strains. (1) If low viability is achieved, each critical point of the two-step method is assessed, starting with a test of different cryoprotectants. (2) If a better result is achieved with a specific cryoprotectant but viability is still low, the next step is to replace fast freezing by slow freezing. (3) When good viability is achieved with the tested protocol but the culture shows low density, changes in the culture conditions are considered and the protocol is applied to a denser culture. When high viability (>50%) is achieved with one of the tested protocols, permanent storage is possible.