Advances in cryopreservation methods for microorganisms and plants

Abstracts

1. *Ex situ* cryopreservation of microbial biodiversity

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Abstract

Microbial Biological Resource Centres (BRC's) including: service collections, academic research collections, or collections within the biotech and biomedical sectors serve as depositories of viable microbes. These have a diverse range of applications including: research; teaching; as biological, biomedical, ecological and toxicological standards; or for commercial exploitation. A crucial objective of all BRC's is to ensure that they use appropriate conservation strategies, irrespectively of whether they hold 10 or 10,000 strains, to enable them to supply "fit for purpose" biological resources to their user communities. Clearly, the primary goal of any conservation strategy, is to guarantee that the microbial cultures provided are representative of the original isolate, with, as far as possible, no change in the morphology, functionality or genetic integrity of the preserved material (1,2). This is particularly challenging for the Culture Collection of Algae and Protozoa (CCAP), which hold a uniquely diverse range of organisms including eubacteria (Cyanobacteria) and exemplar organisms from most eukaryotic lineages (Fig. 1). For many microbial taxa long-term preservation methods have not yet been developed, for others we know that optimisation of methodology is crucial for reviving full functionality, such as production of secondary metabolites (3). Much more research is required to both understand the fundamental effects of cryogenic temperatures on biological materials and methodological/protocol development. Furthermore post-recovery assessments of functionality, quality control and methodological validation have only having been explored for a minuscule number of microbes for example the green alga *Chlorella vulgaris* (Fig. 2).

Fig. 1 Biodiversity of strains held the CCAP (4,5) exercise (6)

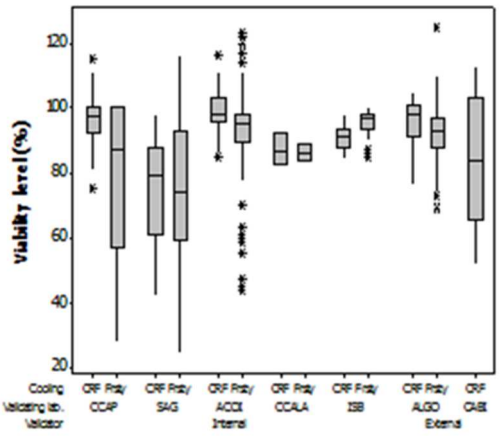

Fig. 2 Methodological validation

This paper will discuss the historical context of the use of cryopreservation to conserve microorganisms, primarily focussing on microalgae. Microalgae are not a simple group of microbes, in reality they have been unilaterally “lumped together” on the basis of their capacity to photosynthesise… They are from a phylogenetic, ecological or functional perspective a hugely diverse range of organisms and as such present significant challenges to those wishing to conserve them using cryopreservation.

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2. Cryopreservation of diatoms

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Abstract

Diatoms are of both ecological and evolutionary importance and hold much value for aquaculture and overall biotechnological potential. In contrast to many other groups of micro-organisms, long-term maintenance of numerous diatom strains by repeated re-inoculation is impossible due to the gradual reduction in cell size of most species, eventually resulting in cell death. Therefore, cryopreservation is the preferred strain preservation method. Cryopreservation success varies strongly with species and differs systematically between marine and freshwater diatoms. Based on previous experiences in the DCG culture collection, 80% of the tested marine species (n=50) could be cryopreserved successfully, whereas for freshwater species only 25% (n=20). Here we formally compare different cryopreservation protocols tested for a panel of model freshwater and marine species used in our laboratory. We will discuss strategies to optimize cryopreservation procedures and possible alternatives for the long-term maintenance of diatom species in culture collections.

3. Cryopreservation of cyanobacteria in the BCCM/ULC collection.

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Cryopreservation is considered as the preferred method for the long-term storage of many micro-organisms, including cyanobacteria. The BCCM/ULC collection is now holding over 240 cyanobacterial strains but only 62 are maintained in a cryopreserved state. The development of improved cryopreservation protocols is therefore required both for the future growth and valorization of the collection.

We have evaluated two methods as potential long-term preservation techniques: (i) the two step cooling method, the common freezing procedure of algae, and (ii) the encapsulation-dehydration method, often considered as a promising alternative to the traditional cryopreservation method for recalcitrant microalgal strains.

The effects of several factors on the viability of four representative cyanobacterial strains for both methods were first considered to determine which ones are the most important for a successful cryopreservation. For the two step method, these factors include the cryoprotectant choice, the sample preparation methods (e.g. direct growth inside the cryovials) and the growing periods of the cultures tested. For the encapsulation-dehydration method, several cryoprotectants, evaporative drying methods and dissolution solutions of the alginate beads were considered. We compared the storage at -70°C and in liquid nitrogen with both methods. A vital staining method allowing the rapid evaluation of the post-cryopreservation viability was also assessed.

Based on the results for the four strains, an optimized cryopreservation protocol was developed for both methods and tested on another set of 26 cyanobacterial strains. Most of the strains displayed high survival rates using the two-step cryopreservation protocol but only a few survived the encapsulation-dehydration process. Our results demonstrated that cryopreservation by the traditional two-step cooling procedures was effective for cyanobacterial strains having various morphologies and origins.

4. Cryopreservation of microalgae at SAG Culture Collection of Algae: Detection of genetic differences and epigenetics stability using molecular fingerprinting

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The Culture Collection of Algae at Göttingen University (SAG) is one of the largest service culture collections for living algae and cyanobacteria in the world (www.epsag.uni-goettingen.de). Currently 2278 strains (from 521 genera and 1202 identified species) of all major groups of microscopic algae and cyanobacteria are maintained in SAGs open collections. About 1/5 of these strains are defined as authentic material linked to the types. Today 29% of all SAGs strains (40% of authentic strains) are successfully cryopreserved using a standard two-step protocol with MeOH or DMSO as cryoprotectant (CPA). However, many other strains tested showed very low survival rates (163 strains) or did not regrow after thawing (127 strains). For these and many other recalcitrant strains alternative cryoprotocols need to be developed. Promising approaches like alginate bead encapsulation dehydration successfully cryopreserved so far recalcitrant euglenophytes. But they usually need individual optimization, are labour intensive and so far limited to axenic cultures. Beside this, very little is known about the effects of cryopreservation on cyanobacteria and microalgae.

To better understand effects and mechanisms of cryopreservation on these microorganisms, SAG is participating in the KAlT-project (Cryostress - mechanisms of cellular adaptation to extremely low temperatures). This project focuses on so-far unknown key physiological responses towards cryostress and their molecular basis. Selected bacteria, algae, fungi as well as human and plant cell lines and shoot tips are investigated in a comparative approach.

The aim of the study presented here was to investigate the influence of cryopreservation on genetic differences (mutations) and epigenetic stability of different green algae using two different methods of Amplified Fragment Length Polymorphism (standard and methylation-sensitive AFLP). Also, the genetic and epigenetic stability of strains under different

cryoprotectants was analyzed. Nine strains of coccoid green algal genera, such as *Chlorella* spp. and *Micractinium* spp., isolated from different habitats were examined as model organisms. In addition, the green flagellate *Chlamydomonas reinhardtii* was included in this study because of its known sensitivity against cryopreservation. The physiological status of the algae was tested by FDA staining and growth rates before and after cryopreservation. The standard AFLP analyses were performed using two pairs of enzymes (EcoRI+MseI and EcoRI+PstI) with 3 primer combinations to reveal a large genomic diversity below species level, i.e. to discriminate between strains. The methylation rate during cryopreservation was discovered by MS-AFLP using two pairs of enzymes (EcoRI+MspI and EcoRI+HpaII). Eight out of ten investigated strains were characterized by a high survival rate (more than 50%) after cryopreservation. Only two of them are sensitive and showed lower survival rates. Cryopreservation and the relatively toxic cryoprotectants DMSO (5%) and MeOH (3%) had only little effect on the methylation rates, but showed a high variability on the MS-AFLP pattern. Interestingly, the robust strains showed more changes in the pattern than the sensitive strains. Cryopreservation had no effect on the growth rates of the investigated strains.

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