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Preservation of basidiomycete strains on perlite using different protocols

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ABSTRACT

For preservation of 31 basidiomycete strains on perlite in cryovials we used five different perlite protocols to compare their applicability in laboratories with different equipment, namely a viability of the controlled freezing device or the electric deep-freezer and liquid nitrogen supply. The viability of the strains, macromorphological characteristics and the production of laccase were tested after 48 h, six months and one year of storage in the respective device. Our results indicated that the different response to the freezing/thawing process is an intrinsic feature of the respective strain. Nevertheless, the highest viability and preservation of laccase production in our tested strains was found when we used pre-freezing to −80 °C at a freezing rate of 1 °C/min in a programmable IceCube 1800 freezer or in freezing container Mr. Frosty before storage in liquid nitrogen or at ultra-low temperature freezer at −80 °C, respectively. The two abovementioned protocols enable all tested strains to survive three successive freezing/thawing cycles without substantial reduction of growth rate. The majority of the strains also do not lose laccase production. Our results showed that direct immersion of the strains into liquid nitrogen or placing them into −80 °C without pre-freezing is not suitable for basidiomycete cryopreservation.

1. Introduction

Various storage methods have been developed in order to eliminate the disadvantages of subculturing (Prescott and Kernkamp 1971; Heckly 1978; Smith 1982; Nakasone et al. 2004 and many others). Besides lyophilization (unsuitable for the majority of basidiomycetes), cryopreservation at low temperatures seems to be a very efficient way to attain this goal. Especially the storage in liquid nitrogen has been considered as the best and most widely applicable preservation technique available for fungi, which seems to surpass all others in the ability to preserve genomic and phenotypic features. It is a safe and reliable method for a long-term maintenance of most fungal species, especially those not amenable to freeze-drying. But neither this cryopreservation method is applicable to preservation of all fungal cultures in the present form. In the development of alternative storage methods it is important to consider their cost and accessibility. Ryan et al. (2000) devised a decision-based key, which facilitates the choice of the most appropriate method for long-term preservation of cultures using questions related to fungal characters and user facilities and economics. Basic

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methods such as continual subculture/serial transfer in agar, storage under mineral or paraffin oil, in sterile water, cool storage in a standard refrigerator at 5–8 °C, storage in sand, grain or soil, or on silica gel, may be suitable in the absence of alternatives.

Besides survival, another principal requirement for successful maintenance of fungal strains is the ability to preserve their features (growth, morphology, production of metabolites etc.) unchanged. The cryopreservation process includes freezing and thawing and the protocol of these procedures plays an important part (Leef and Mazur 1978; Ryan et al. 2001). Different fungal species and even strains within a single species can often exhibit different cryoresistance under similar conditions (Dahmen et al. 1983; Smith 1993; Homolka, unpublished results). Also the growth and many other requirements of fungal cultures can vary from strain to strain. Therefore, there should be developed new or modified preservation protocols with an emphasis on strain-specific criteria in order to reduce the prospects of instability in physiological features and in secondary metabolite production. Recently, some new methods of cryopreservation using different carriers of fungal mycelium (perlite, polystyrene or ceramic beads, modified soil, sand, plant seeds etc.) have been developed and successfully verified on a large number of fungal strains from a broad spectrum of taxonomical groups of fungi. An ideal method enabling preservation of all cultures, keeping their specific characteristics, protecting them from contamination and at the same time not being expensive and time-consuming has not been created up to now. Therefore it is highly desirable to develop new preservation methods or to improve the current ones, combining advantages and eliminating disadvantages of individual techniques.

As mentioned above, storage in liquid nitrogen (LN) has been considered the best and most widely applicable preservation technique available for filamentous fungi (Smith 1998). Agar blocks immersed in an appropriate cryoprotectant were originally used as carriers of fungal mycelium for the cryopreservation process (Hwang and Howell, 1968). Then a useful "straw technique" for the preservation of fungi in LN using agar miniblocks in polypropylene straws was developed by Elliott (1976), improved by Stalpers et al. (1987) and modified by other authors (Hoffmann 1991; Homolka et al. 2003 – straw protocol SP).

There are many methods of cryopreservation of fungi used in different laboratories. Fungi grown on various organic substrates, such as cereal grains, agar strips, plant parts, and filter paper, and then dried can also be frozen using specific protocols (Nakasone et al. 2004). In many collections, e.g. IFO (Institute for Fermentation Osaka), non-sporulating cultures of basidiomycetes are stored by cryopreservation at −80 °C in electric freezers (Ito 1996). Recent studies of cryopreservation of basidiomycetes are not very frequent and most of them deal with only one or a limited number of species (e.g., Maekawa et al. 1988, 1990; Smith 1998; Croan et al. 1999; Ryan et al. 2001; Kitamoto et al. 2002; Singh et al. 2004).

A new method of cryopreservation using perlite as a carrier of fungal mycelium (perlite protocol, PP) was developed in our laboratory originally for 5 basidiomycete strains (Homolka et al. 2001) and then successfully verified on 442 basidiomycete strains (Homolka et al. 2006) and on 45 strains of *Ascomycota* (including 17 yeasts), 20 strains of *Zygomycota* and on three strains of *Basidiomycota* counted among yeasts (Homolka et al. 2007a). In the further study we tested the ability to survive three successive cycles of freezing and thawing (using PP or SP) of 50 mycelial basidiomycete cultures on perlite in cryovials and the possibility of reusing the stored cryovials for successive inoculations (Homolka et al. 2007b).

The aim of this work is to compare the following 5 different protocols used for preservation of 31 basidiomycete strains on perlite in cryovials: (*i*) cultures were directly immersed in LN; (*ii*) cultures were placed in LN after controlled pre-freezing in a programmable IceCube 1800 freezer to −80 °C at freezing rate 1 °C per minute; (*iii*) cultures placed in LN after pre-freezing using NALGENE Cryo 1 °C Freezing Container (Mr. Frosty); (*iv*) cultures directly placed to −80 °C without pre-freezing or (*v*) after pre-freezing to −80 °C using Mr. Frosty. Using these protocols we studied the viability and laccase production of the strains after their cryopreservation and we attempted to find out, which protocol is the best for our strains.

In addition, the possibility of repeated freezing to save the material was tested; three successive cycles of freezing/thawing were performed and than the viability and laccase production were tested again.

2. Material and methods

2.1. *Strains*

Thirty-one fungal cultures used in this study are listed in Table 1 along with their physiological characteristics. They were obtained from the Culture Collection of Basidiomycetes (CCBAS) at the Institute of Microbiology of the ASCR, v.v.i., Prague. The cultures were maintained by serial transfers every 3 mo and kept on wort agar slants (wort was purchased from Malthouse Bruntál, Bruntál, Czech Republic, and diluted to a density of 4° Balling scale; 1.5% agar was purchased from Difco, Lawrence, KS, USA) at 4 °C.

2.2. *Culture preparation and freeze-thawing protocols*

Our previous perlite cryopreservation protocol (Homolka et al. 2001) was used with minor modifications. Fungal cultures were grown directly in sterile plastic 1.8-ml Nunc CryoTube Vials (Nalgene/Nunc, Rochester, USA) containing 200 mg of agricultural-grade perlite (Agroperlit, GrowMarket s.r.o., Prague, Czech Republic) moistened with 1 ml of wort (4° Balling) enriched with 5% glycerol as a cryoprotectant. The cultures were inoculated with a 6-mm-diameter agar plug cut from the actively growing region of a colony on a Petri dish containing wort agar and then incubated for 14 d at 24 °C. All culture media were sterilized by autoclaving for 20 min at 121 °C. The vials containing perlite overgrown with mycelia were used for the cryopreservation experiments.

Protocol (*i*): Cultures were directly immersed in LN.

Protocol (*ii*): Cultures were pre-frozen in a programmable IceCube 1800 freezer (Sy-Lab Geraete GmbH., Neupurkersdorf, Austria) to −80 °C at a freezing rate of 1 °C/min. The vials were then placed in LN in a Harsco TW-5K container (Harsco, Camp Hill, USA).