CRYOPRESERVATION OF *Agaricus blazei* IN LIQUID NITROGEN USING DMSO AS CRYOPROTECTANT

CRIOPRESERVAÇÃO DE Agaricus blazei *EM NITROGÊNIO LÍQUIDO USANDO DMSO COMO CRIOPROTETOR*

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ABSTRACT: The preservation of *Agaricus blazei* is generally done using successive subcultivations that are laborious and are subject to contaminations or genetic degenerations, resulting in loss of biotechnological interest characteristics. An alternative process would be cryopreservation, but there are no reports of methodologies for this basidiomycete in liquid nitrogen. Thus, the objective of this study was to evaluate mycelial viability of *A. blazei* strains after cryopreservation in liquid nitrogen in order to establish the initial parameters of species preservation. Five strains grown on malt extract agar (MEA) were used. Disks of MEA containing *A. blazei* mycelium were transferred for screw-cap cryovials containing the cryoprotectant, 10% dimethyl sulfoxide. Then, they were cooled at 8 ºC for 30 min and kept at -196 ºC with liquid nitrogen. After 1.5 year of cryopreservation, the cryovials were thawed in water bath at 30 ºC for 15 min. The disks with mycelia were transferred to MEA culture media without cryoprotectant and kept at 28 ºC for 30 days. *A. blazei* strains respond differently to the cryopreservation method at -196 ºC by varying mycelial viability recovery. Cryopreservation with liquid nitrogen, using dimethyl sulfoxide as cryoprotectant, is not the most appropriate one for *A. blazei* preservation.

KEYWORDS: *Agaricus brasiliensis*. *Agaricus subrufescens*. Thawing. Freezing. Viability. Preservation.

Agaricus blazei Murrill ss. Heinemann (HEINEMANN, 1993) reclassified as *Agaricus brasiliensis* Wasser et al. by Wasser et al. (2002) and as *Agaricus subrufescens* Peck by Kerrigan (2005) is a Brazilian basidomycete (COLAUTO et al., 2010) known by its therapeutical properties (MOURÃO et al., 2009; MOURÃO et al., 2011). Due to the discussion on its scientific name and the illegitimacy of the proposed names (COLAUTO et al., 2011a), the name *A. blazei* will be used in this study.

The preservation of *A. blazei* is usually done by successively transferring the mycelium to cultivation media which can cause genetic degenerations and loss of original biotechnological characteristics. This method is also subject to contamination by other microorganisms and/or mites and it is a laborious technique to preserve large culture collections because each strain has to be transferred to new media every four months. However, this technique is widely used in Brazil to preserve *A. blazei* mainly because of the difficulty to preserve this fungus under refrigeration temperatures (3 to 8 ºC), mineral oil or distilled water (COLAUTO et al., 2011b). Wasser et al. (2002) and Kerrigan (2005) also reported the survival difficulty of this fungus when exposed to refrigeration temperatures.

Cryopreservation is an alternative method to preserve basidiomycetes. Although broadly used for bacteria, the use of cryopreservation presents limitations to preserve basiodiomycetes (HUBÁLEK, 2003) and still needs to be thoroughly studied in order to ensure the freezing and recovery of viable mycelia (MATA; ESTRADA, 2005).

The technical viability of cryopreservation depends on the fungus species as well as on its strain (MATA; ESTRADA, 2005), which are important parameters for the successful use of cryopreservation. In general, for basidiomycetes, due to its sensitivity to freezing, cryoprotective agents are used and the most used is dimethyl sulfoxide (DMSO) at a concentration that varies widely, from 1% to 32% with median of 10% (HUBÁLEK, 2003). This cryoprotective agent quickly penetrates in the fungus wall and plasmatic membrane and provides good protection to sensitive cells (HUBÁLEK, 2003). Challen and Elliot (1983) reported the use of 10% DMSO for the cryopreservation of basidiomycetes that are extremely sensitive to cold like *Volvariella volvacea*. Although *A. blazei* has therapeutical and commercial importance, there are no reports of preservation methods with liquid nitrogen for this basidiomycete, and, therefore, the development of techniques to preserve the species is necessary. Thus, the objective of this study was to evaluate mycelial viability of *A. blazei* strains after cryopreservation in liquid nitrogen using DMSO as cryoprotectant.

The studied *A. blazei* strains were ABL 97/11, ABL 99/25, ABL 99/26, ABL 99/28, ABL 99/29, from the culture collection of the Edible and Medicinal Mushrooms of the São Paulo State University - UNESP.

Malt extract agar (MEA), 39 g L^{-1}, was autoclaved at 121 ºC for 20 min and poured aseptically on Petri dishes. The mycelial growth was done at 28 ºC in the dark (COLAUTO et al., 2008). Dishes containing mycelium with uniform growth, without sectioning, were used to provide MEA disks with mycelia for cryopreservation.

Four 3 mm-diameter MEA disks containing grown mycelia were transferred to screw-cap cryovials (Nalge Nunc®) with 1 ml of 10% DMSO solution in distilled water, previously autoclaved at 121 ºC for 20 min. The cryovials were screwed at room temperature (25 ^{o}C), agitated for 2 s in Vortex, cooled at 8 ºC for 30 min in a refrigerator, and stored vertically in a vessel with liquid nitrogen.

After 1.5 year of cryopreservation, five cryovials of each strain were thawed in a water bath at 30 ºC for 15 min, according to Mantovani et al. (2008). Next, they were washed at room temperature (25 ^{o}C) with 70% alcohol and 96% alcohol for 30 s. The cryovials were opened, the cryoprotective agent was removed and the disks with mycelia were transferred to MEA medium at 28 ºC for 30 days, in the dark. The strains were considered recovered and viable when 75% or more of the samples showed mycelial growth within 30 days, without visible morphological or physiological changes (HOMOLKA et al., 2006).

Table 1 shows the mycelial growth recovery of cryopreserved *A. blazei* strains. Differences on the mycelial recovery can be observed according to the cryopreserved strains. ABL 99/29, ABL 99/25 and ABL 28 did not show satisfactory mycelial recovery whereas ABL 97/11 and ABL 99/26 were recovered. In addition ABL 97/11 had a better response to the cryopreservation process than ABL 99/26 (Table 1).

Table 1. Viability of *A. blazei* strains grown on malt extract agar disks and cryopreserved at -196 ^{o}C for 1.5 year in cryovials with the cryoprotectant 10% dimethyl sulfoxide solution.

Strain	Viability (%)
ABL 97/11	100
ABL 99/25	25
ABL 99/26	75
ABL 99/28	60
ABL 99/29	0

Colauto et al. (2002) genetically characterized these strains by RAPD analysis with 20 primers and showed that ABL 97/11, ABL 99/25 and ABL 99/29 strains did not present genetic divergence. However, similarly, Tomizawa et al. (2007), using 108 primers, verified, by RAPD analysis, a maximum genetic distances of 6% among the strains ABL 97/11, ABL 99/25 and ABL 99/29 showing few genetic differences among these strains. These small genetic differences may be related to the fungus capacity to keep the cellular integrity during cryopreservation. During the freezing, cryoinjuries may occur because of cellular dehydration, formation of ice crystals and/or volume expansion (HUBÁLEK, 2003; RYAN et al., 2000). Therefore, genetic characteristics like cellular elasticity, thickness of cellular wall, the composition and the concentration of solids, and the interaction capacity with the cryoprotective agent are important factors for the fungus survival by ultra freezing method. The results obtained in this work show that, even with very close genetic distance among strains, it is important to adapt the protocols of cryopreservation for each strain of the same species, as Ryan et al. (2000) also reported.

The recovery variation of the mycelial growth after freezing at -196 ^{o}C (Table 1) indicates that *A. blazei* is sensitive to the formation of intracellular ice crystals or even to the quick expansion of the cellular volume. In this case, DMSO was not efficient to avoid cryoinjuries, as reported by Challen and Elliot (1986), for basidiomycetes such as *Volvariella volvacea* that are extremely sensitive to cryopreservation. Colauto et al. (2011b) reported strain recovery variation with a protocol that freezes at -196 ºC before cryopreserving at -80 ºC. Probably the determining factor for the reduced viability of cryopreservation at -196 ^{o}C of *A. blazei* was the moist content over 90% (BRAGA et al., 1998). Cells with high moist content generally need slow freezing to dehydrate and reduce the formation of ice crystals and the volume expansion, the main responsible factors for the rupture and loss of cellular viability

(HUBÁLEK, 2003). Therefore, *A. blazei* has low adaptation capacity to fast freezing that may have caused instantaneous cell expansion without the possibility of water loss to the medium. It indicates that slower freezing protocols are probably more recommended as a cryopreservation method for this fungus. It was reported by Colauto et al. (2012) that DMSO was only effective to cryopreserve *A. blazei* in the long run at -70 ^{o}C when a slow freezing protocol was used.

The use of mycelial growth in disks and immersed in cryoprotective solution is reported as the most used and efficient method for fungus cryopreservation (HUBÁLEK, 2003). However, in our study, the cryoprotective agent DMSO presented reduced viability for cryopreservation at -196 ºC of *A. blazei* (Table 1). Similarly, Ryan et al. (2000) reported that cryopreservation must be used carefully for more sensitive fungi, especially those that have big vacuoles or high water activity in the cell, or that, due to the response variation for the preservation of each species, there is the need to develop specific protocols even for strains of the same species.

It was concluded that *A. blazei* strains respond differently to cryopreservation with DMSO, having a recovery of mycelial viability of two out of five strains. The fast freezing of *A. blazei* mycelia in liquid nitrogen with DMSO is not the most appropriate method to preserve this species. It establishes the first freezing limits for cryopreservation in liquid nitrogen of this species.

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RESUMO: A preservação de *Agaricus blazei* ocorre usualmente por subculturas sucessivas que são laboriosas e estão sujeitas a contaminações ou degenerações genéticas, com eventual perda das características de interesse biotecnológico. Uma alternativa a este processo é a criopreservação, no entanto não existem relatos de metodologias para este basidiomiceto com uso de nitrogênio líquido. Desta forma, o objetivo deste trabalho foi avaliar a criopreservação em nitrogênio líquido de linhagens de *A. blazei*, visando estabelecer os parâmetros iniciais de criopreservação para a espécie. Foram utilizadas cinco linhagens do fungo crescidas em meio de ágar-extrato de malte (MEA). Os discos de MEA contendo o micélio crescido foram transferidos em temperatura ambiente (25 ºC) para criotubos com rosca contendo o agente crioprotetor dimetilsulfóxido a 10%. Em seguida foram resfriados a 8 ºC por 30 min e mantidos a -196 ºC com nitrogênio líquido. Após 1,5 anos de criopreservação os criotubos foram descongelados por imersão em água a 30 ºC por 15 min. Os discos com micélio foram transferidos para meio de cultura MEA, sem o agente crioprotetor, e mantidos por 30 dias a 28 ºC. As linhagens de *A. blazei* respondem de forma diferente ao processo de criopreservação a -196 ºC com variação na recuperação da viabilidade do micélio. A criopreservação em nitrogênio líquido de *A. blazei*, com dimetilsulfóxido como crioprotetor, não é a forma mais adequada para a preservação desta espécie.

PALAVRAS-CHAVE: *Agaricus brasiliensis*. *Agaricus subrufescens*. Descongelamento. Congelamento. Viabilidade. Preservação.

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