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SHORT COMMUNICATION

Viability of *Agaricus blazei* after long-term cryopreservation

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Abstract *Agaricus blazei* is a basidiomycete of medicinal and gastronomic importance, but few publications have appeared on preservation. We have evaluated *A. blazei* cryopreservation at −70°C using different cryoprotectants and freezing protocols. Malt extract agar disks containing grown mycelia were transferred to cryotubes containing different cryoprotective solutions. Freezing protocols were from 25°C to: (1) 4°C for 30 min and then to −70°C or (2) directly to −70°C. The results demonstrate that it is the cryoprotective agent—and not the freezing protocol—which was the most important variable for maintaining mycelial viability after cryopreservation. Long-term cryopreservation (4 years) was effective when saccharose or glucose was used as cryoprotectant regardless of the freezing protocol, dimethyl sulfoxide was effective when a slow freezing protocol was used, and glycerol, polyethylene glycol, and malt extract were ineffective as cryoprotectants regardless of the freezing protocol. All of the cryoprotectants tested were effective for the short-term cryopreservation (1 year) of *A. blazei*, with the exception of malt extract.

Keywords Cryoprotective agent · Cryopreservation · Freezing · *Agaricus subrufescens* · *Agaricus brasiliensis*

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Introduction

Agaricus blazei Murrill ss. Heinemann (Heinemann 1993) is a basidiomycete native to Brazil (Colauto et al. 2010a, b, c) that has been reclassified by Wasser et al. (2002) as *Agaricus brasiliensis* Wasser et al. and also denoted by Kerrigan (2005) as *Agaricus subrufescens* Peck. However, Colauto et al. (2011b) reported that those last names had already been used and therefore illegitimate. Consequently, in this study, this basidiomycete is referred as *A. blazei*. This fungus has received considerable attention from researchers due to its sensorial characteristics (Escouto et al. 2005) and antitumor (Mourão et al. 2009), immunomodulating, antidiabetic, anti-infectious, anticarcinogenic, antimetastatic, antimutagenic (Firenzuoli et al. 2008), anti-inflammatory (Mourão et al. 2011a), antioxidant (Mourão et al. 2011b), and laccase producing (D'Agostini et al. 2011) activities.

The traditional method used to preserve this fungus is continuous mycelial subculturing with the aim of preventing contamination and the appearance of genetic mutations. Although this method is relatively inexpensive, it requires periodic maintenance, time, and physical space, becoming impractical for large-scale culture collections (Monaghan et al. 1999). Well-defined freezing protocols have been developed for the cryopreservation of mammalian cells, such as the Slow Programmable Freezing (Vutyavanich et al. 2010), but this latter procedure requires an expensive biological freezer and the process is time-consuming. A fast alternative method, without the use of costly equipment, would provide significant benefits for fungus maintenance.

Cryoprotectants are widely used to facilitate cell cryopreservation. In general, these compounds are highly

hydrophilic, and the permeability of the cell determines the mechanisms by which they exert their protective effects. Permeable cryoprotectants, such as dimethyl sulfoxide and glycerol, can make the cell membrane more plastic and can bind to intracellular water, which prevents excessive dehydration, reduces ion toxicity, and prevents the formation of ice within the cell. Semi-permeable cryoprotectants, such as saccharose and glucose, induce the partial dehydration of cells prior to freezing. They accumulate between the cell membrane and the cell wall where they function as a buffer layer against the growing ice crystals and thus mechanically protect the cell membrane. Non-permeable cryoprotectants, such as polyethylene glycol and malt extract, do not interact directly with the cell membrane or wall, rather they cause partial efflux of water from the cell, inhibit the growth of ice crystals by increasing solution viscosity, and prevent an amorphous ice structure from entering the cell (Hubálek 2003). Although cryoprotectants are versatile, their action can be affected by innumerous physical and chemical factors, demanding the adaptation of protocols for each kind of microorganism (Dumont et al. 2004).

Thus, due to the limited amount of information on *A. blazei* cryopreservation, the importance of this fungus, and the need to develop low-cost freezing protocols, we have evaluated *A. blazei* cryopreservation at −70°C using different cryoprotectants and freezing protocols.

Materials and methods

Microorganism

Agaricus blazei (U2/1; ABL97/11) was obtained from the fungus collection of the Molecular Biology Laboratory of the Universidade Paranaense. For the inoculum growth, malt extract agar medium (MEA; 48 g/L; Merck, Darmstadt, Germany) autoclaved at 121°C for 20 min was used. The mycelia were first grown at 28°C in the dark for 15 days (Colauto et al. 2008), following which uniform mycelia from the mycelial growth edge, without sectoring, were selected as inoculum.

Supplementation of cryoprotectant to cultivation medium

The main characteristic of a cryoprotective agent is the high capacity to link to water (Hubálek 2003), resulting in an increase in the osmotic force in the culture medium and in the energy demand. Thus, it is important to determine the highest cryoprotector concentrations that do not paralyze the mycelium after thawing because the cryoprotective solution remains over all of the mycelia, functioning as a barrier to be overcome before the culture medium can be reached. Therefore, the addition of cryoprotectant to the culture medium has the purpose of assisting in the selection of less toxic concentrations of cryoprotectants to be used for mycelial growth after cryopreservation. The cryoprotectants dimethyl sulfoxide (DMSO) at 1, 2.5, or 5%, glycerol (GLY) at 5, 10, or 30%, saccharose (SAC) at 5, 15, or 30%, glucose (GLU) at 2, 4, or 10%, polyethylene glycol-6000 (PEG) at 3, 6, or 8%, and malt extract (MEX) at 4, 5, or 13% were added to the MEA and autoclaved at 121°C for 20 min, with the except of DMSO which was filtered (0.22-μm filter). After inoculation, the culture media were kept in the dark at 28°C; after 14 days the radial mycelial growth was measured with a caliper. The highest cryoprotectant concentrations that did not alter the mycelial morphological characteristics of the fungus were used in the cryopreservation experiments. All treatments were carried out in quadruplicate.

Cryopreservation

For the cryopreservation experiments, polypropylene plastic tubes (juice straws, 0.4 × 4.5 cm) were thermo sealed at one end and autoclaved at 121°C for 20 min (Challen and Elliot 1986; Mantovani et al. 2008) so that they could be used as cryotubes. Each cryotube received 300 μl of cryoprotective solution, previously autoclaved at 121°C for 20 min (except for the DMSO solution which was filtered through a 0.22-μm filter), and five MEA disks (diameter 3.5 mm) containing mycelium. After the other end of the tube had been thermo sealed, the tubes were subjected to two freezing protocols: (1) from 25°C to 4°C for 30 min and then kept at −70°C or (2) from 25°C directly to −70°C, without the cooling step. For each treatment, a cryotube contained five disks with mycelial growth, and each disk was considered a replication.

Mineral oil preservation

Mycelial preservation by immersion in mineral oil in glass tubes (Perrin 1979) was used as the control. Mineral oil (2 mL) that had previously been subjected to dry heat on a stove (150°C for 150 min) was added to glass tubes containing mycelium grown on MEA. Half of the glass tubes were kept in the dark at 4°C, and the other half were kept at 20°C. For each treatment, a glass tube was considered a replication.

Mycelial viability after preservation

After 1 and 4 years of cryopreservation, cryotubes were thawed in a water bath at 30°C for 15 min (Lara-Herrera et al. 1998) and cleaned with 70 and 92.5% alcohol. One of the ends of the cryotube was cut, and the cryoprotective solution was removed. Similarly, the glass tubes containing mycelia preserved in mineral oil were first kept at 25°C for

30 min and then cleaned with 70 and 92.5% alcohol before the mineral oil was removed.

The mycelia were then transferred to MEA (from cryotubes or glass tubes) and kept at 25°C in the dark. The mycelia in each treatment was considered to have recovered and be viable when at least 75% of the disks showed mycelial growth, without sectoring, within 30 days (Homolka et al. 2006).

Results

The average mycelial growth of *A. blazei* in MEA with different cryoprotectant concentrations is shown in Table 1. Overall, most mycelial growth inhibition occurred at the highest cryoprotectant concentrations, with the cryoprotective agents DMSO and GLY demonstrating the highest mycelial growth inhibition (DMSO totally inhibited >2.5% of mycelial disks). SAC, GLU, PEG, and MEX showed low mycelial growth inhibition. For the mycelial cryopreservation assessments, we selected the higher cryoprotectant concentration that presented lower or slight lower mycelial growth inhibition, such as 1% DMSO, 6% GLY, 15% SAC, 4% GLU, 8% PEG, and 5% MEX (Table 1). The selected cryoprotectants caused no mycelium atypical features, such as sectoring, aerial or cottony mycelial growth, or darkening of the cultivation medium.

Table 2 shows the mycelial viability of *A. blazei* after cryopreservation at −70°C for 1 or 4 years with different freezing protocols and cryoprotective solutions. After 1 year of cryopreservation, the freezing protocol had only a minimal effect on mycelial viability. However, after 4 years, the protocol with the cooling step was more effective than that without the cooling step. 15% SAC and 4% GLU cryoprotective solutions were clearly more effective than the other cryoprotectants, independent of the freezing protocol or the cryopreservation period, whereas 1% DMSO and 6% GLY, which are considered to be standard cyroprotectants for basidiomycetes, were effective only for the first cryopreservation year. 8% PEG and 5% MEX presented similar ineffective results after 4 years of cryopreservation (Table 2).

Mycelia immersed in mineral oil at 4°C were not viable after 1 or 4 years. These results corroborate a previous observation in our laboratory in which *A. blazei* did not survive at refrigeration temperatures (Colauto et al. 2011a). However, when preserved under mineral oil at 20°C, the mycelia showed 100% viability after 1 year, although it showed 0% viability after 4 years of preservation.

The visualization of mycelia after 4 years of cryopreservation varies from 9 to 17 days. On average, mycelial visualization was 9 days for treatments with cryoprotective agents SAC and GLU, 12 days with MEX and GLY, and 13 days with DMSO and PEG.

Table 1 Mycelial growth of *Agaricus blazei* in malt extract agar medium supplemented with different cryoprotectants

Cryoprotectant[a]	Concentration (%)	Mycelial growth (mm)	Mycelial growth/ 4% MEX × 100 (%)
DMSO	1	12	27
	2.5	0	0
	5	0	0
GLY	6	24	54
	10	11	25
	30	4	9
SAC	5	52	118
	15	43	98
	30	38	86
GLU	2	44	100
	4	36	82
	10	20	45
PEG	3	44	100
	6	43	98
	8	44	100
MEX	4	44	100
	5	47	107
	13	26	59

[a] DMSO, Dimethyl sulfoxide; GLY, glycerol; SAC, saccharose; GLU, glucose; PEG, polyethylene glycol-6000; MEX, malt extract

Table 2 Mycelial viability of *A. blazei* cryopreserved at −70°C with different cryoprotective solutions for 1 and 4 years

Cryoprotective solution[a]	Viability after 1 year (%)		Viability after 4 years (%)	
	Freezing protocol		Freezing protocol	
	from 25 to 4 to −70°C	from 25 to −70°C	from 25 to 4 to −70°C	from 25 to −70°C
DMSO 1%	96	100	80	20
GLY 6%	95	95	73	27
SAC 15%	100	100	100	100
GLU 4%	100	100	100	100
PEG 8%	100	90	46	67
MEX 5%	76	60	60	27

Discussion

The cryoprotectants 8% PEG and 5% MEX were efficient only for the 1-year cryopreservation and MEX was efficient only when the cooling step was included in the procedure. The low cryoprotectant capacity of these compounds is associated with their molar mass. PEG and MEX are high molar mass polymers, do not penetrate the cell wall or plasma membrane, and act only externally to the cell by preventing ice formation and facilitating cell dehydration (Hubálek 2003). *A. blazei* contains a large amount of water and favors environments with a humidity of >90% (Braga et al. 1998), which demand high water diffusion through the membrane and cellular wall. Thus, for *A. blazei* cryopreservation, it is difficult to avoid damage caused by intracellular ice formation using non-penetrating cryoprotectants.

In our study, the cyroprotectants 1% DMSO and 6% GLY were not very effective for long-term cryopreservation (Table 2), although they are considered to be the most effective and commonly cryoprotectants for fungi (Jong and Birmingham 2001; Chetverikova 2009). Both are able to penetrate the cell wall and plasma membrane, thereby preventing intracellular ice formation and altering the elasticity of the plasma membrane to allow a better accommodation of the cellular volume expansion during freezing (Dumont et al. 2004). However, DMSO and GLY can also affect cellular metabolism due to its toxicity (Hubálek 2003). In the case of *A. blazei*, the presence of DMSO or GLY in the cultivation medium caused a high degree of mycelial growth inhibition (Table 1). This inhibition can be an indication that although these substances help against cryo-damage, they may hinder mycelial recovery due to their toxicity.

In tests carried out in our laboratory, cryopreservation in liquid nitrogen with DMSO was not effective for all of the *A. blazei* strains assessed after 1.5 years, with the strains showing a large variation in mycelial viability (data not published). Challen and Elliot (1986) reported the use of glycerol instead of DMSO for the cryopreservation of basidiomycetes sensitive to freezing, such as *Volvariella volvacea*. However, Colauto et al. (2011a) reported no recovery of *A. blazei* strains cryopreserved at −80°C with 10% glycerol for 1 year depending on the freezing protocol. Ito and Nakagiri (1996) reported that 77% of a collection with 427 basidiomycetes was effectively cryopreserved at −80°C with 10% glycerol for 15 years. However 12% of the strains did not survive even the first year of cryopreservation. These results reinforce the statement of Ryan et al. (2000) that is important to adapt the protocols for cryopreservation for each strain, even those of the same species, because there is no universal method for storing fungal cultures (Jong and Birmingham 2001).

Our results show that 1% DMSO and 6% GLY were effective for the short-term but not for the long-term cryopreservation of *A. blazei* and that only 15% SAC and 4% GLU were very effective, with 100% recovery, after 4 years of cryopreservation (Table 2). SAC and GLU are semi-permeable cryoprotectants that induce the partial dehydration of cells prior to freezing. They have a high capacity to link water, thereby accelerating cell dehydration. They have a tendency to accumulate between the plasma membrane and cell wall, forming a buffer layer against the growing ice crystals, limiting ice formation and mechanically protecting the cell membrane (Hubálek 2003). In addition to the high cryoprotective capacity of these sugars, the fungus can also use them as a carbon source during the growth recovery period; they therefore also provide a favorable environment for mycelial viability. Despite the good results with SAC and GLU as cryoprotective agents it is important to note that they demand greater microbiological control to avoid contamination. Therefore, for *A. blazei*, the use of cryoprotectants that penetrate only the cellular wall as mono- and disaccharides, amino acids, low molar mass polymers, such as PEG-1000, are potential candidates for future studies on the cryopreservation of this fungus and other basidiomycetes.

The highest recovery rates were reached by the protocol which included the cooling step (Table 2). This cooling step probably allowed a better cell adaptation to the low temperatures as well as a better contact time with the cryoprotective solution. The contact time is an important variable because it allows cryoprotectant diffusion with consequent cell dehydration and reduction of intracellular ice formation. Although the

best results were obtained when the cooling step was included in the protocol, the differences between protocols were small, indicating that it was the choice of cryoprotective agent that was an important variable in determining the efficiency of mycelial cryopreservation.

Oliveira et al. (2011) tested many different protocols and methods and found that *Beauveria bassiana* could only be preserved by subculturing. Colauto et al. (2011a) reported that cryopreservation for all strains of *A. blazei* tested was more effective after 1 year of cryopreservation when the strains were grown on rice grains with husk and cryopreserved at −80°C that when they were grown on malt extract discs and cryopreserved on liquid nitrogen. Alternative substrate for mycelial growth, such as perlite (Homolka et al. 2010), sawdust (Kitamoto et al. 2002), and grains or seeds (Mata et al. 2000; Mata and Estrada 2005; Mata and Pérez-Merlo 2003; Mantovani et al. 2008), have been found to be effective for the cryopreservation of basidiomycetes.

Mycelia preserved by immersion in mineral oil at 4°C did not present mycelial growth viability. This result corroborates that of a previous experiment in our laboratory in which *A. blazei* did not survive cooling temperatures (4°C) and also those reported by Wasser et al. (2002) and Kerrigan (2005). Mycelia preserved in mineral oil at 20°C presented 100% of viability after 1 year. However, mycelia preserved for 4 years showed no recovery. It is likely that the cellular death of the mycelia is related to the accumulation of secondary metabolites produced during anaerobiosis; as such oil immersion is not an appropriate method for the long-term preservation of basidiomycetes.

It took 9–17 days to visualize the recovery mycelia after thawing. On average, mycelia preserved in the SAC and GLU cryoprotective agents presented faster growth, with mycelium visualization at 9 days of growth. Mycelia preserved in MEX and GLY took 12 days to visualize mycelial growth recovery, whereas for those preserved in DMSO and PEG, it took 13 days. Therefore, SAC and GLU showed the shortest recovery time, improving the recovery percentage of the mycelial growth and demonstrating that they were the most efficient cryoprotective agents for *A. blazei*. Despite the good results obtained for *A. blazei* cryopreservation in our experiments, more studies are needed to improve the cryopreservation of this fungus specifically and basidiomycetes and fungi in general.

Conclusions

Based on the results of our study, we conclude that it is the cryoprotective agent and not the freezing protocol that is the most important variable for maintaining *A. blazei* viability after cryopreservation at −70°C. Long-term cryopreservation (4 years) is effective when saccharose or glucose is used as cryoprotectant regardless of the freezing protocol, dimethyl sulfoxide is effective when a slow freezing protocol is used, and glycerol, polyethylene glycol, and malt extract were ineffective regardless of the freezing protocol. All of the cryoprotectants tested are effective for the short-term cryopreservation of *A. blazei* (1 year), with the exception of malt extract, which is only effective in a slow freezing protocol.

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