Cryopreservation at -20 and -70 °C of *Pleurotus ostreatus* on Grains

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Abstract Alternative substrates for cryopreservation at -20 °C have been little explored for basidiomycetes and could bring new possibilities of lower cost cryopreservation. Nevertheless, freezing temperatures between -15 and -60 °C are very challenging because they frequently result in cryoinjuries. The objective of this study was to evaluate substrates associated to cryoprotective agents for *Pleurotus ostreatus* cryopreservation at -20 or -70 °C in order to develop alternative techniques for basidiomycete cryopreservation. *P. ostreatus* was grown on potato dextrose agar or whole grains of oat, wheat, rice or millet and transferred to cryovials with cryoprotective solution with 1 % dimethyl sulfoxide, 5 % glycerol, 10 % saccharose, 4 % glucose, 6 % polyethylene glycol-6000 or 5 % malt extract. The mycelium in the cryovials were cryopreserved at -20 or -70 °C and recovered for evaluation of the mycelial growth viability after 1 and 3 years. Both substrates and cryoprotectants affect the viability of the mycelial growth cryopreserved at -20 or -70 °C; wheat grains combined with cryoprotectants such as saccharose or glucose are effective for keeping mycelium viable after cryopreservation at -20 °C for 1 or 3 years; for cryopreservation at -70 °C after 1 or 3 years, any substrate

combined with any cryoprotectant is effective for preserving the mycelium viable, except for millet grains with polyethylene glycol after 3 years; semi-permeable cryoprotective agents such as saccharose and glucose are the most effective for cryopreservation at -20 or -70 °C for at least 3 years.

Keywords *Pleurotus ostreatus* · Cryopreservation · Freezing · Substrate · Cryoprotective agent

Introduction

Pleurotus ostreatus (Jacq. ex Fr.) P. Kumm. is a basidiomycete that produces edible mushrooms that are commercialized all around the world [1]. It is used for bioremediation and mushrooms have medicinal use since they contain statins such as lovastatin which reduces cholesterol [2]. To preserve this fungus, continual sub-culture [3] or storage under mineral oil [4] or water [4, 5] is generally used. These techniques are cost-effective but time consuming, laborious and impractical for large culture collections. In addition, their suitability to longer-term storage protocols is doubtful. Another largely used technique is cryopreservation from -60 to -130 °C in ultra-freezers or at -196 °C with liquid nitrogen [6], both considered the best way to keep cell viability. However, the preservation at -196 °C often requires substantial initial outlay, and an alternative method without the use of costly equipment would provide significant benefits mainly for filamentous fungus maintenance.

Cryopreservation at -20 °C has been little explored and could be a low cost alternative to filamentous fungus preservation. However, freezing temperatures from -15 to -60 °C are a challenge to cell cryopreservation as they often

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result in cryoinjuries caused by ice formation, water migration and/or ion concentration [7]. One of the main cell injuries to overcome in cryopreservation at -20°C is ice formation in the cell interstice [8] which causes mechanical injuries [7]. An alternative to reduce these cryoinjuries is the use of cryoprotective agents [6] that decrease ice formation and/or affect the plasmatic membrane elasticity reducing cellular breaking and increasing mycelial viability [9]. Another alternative to reduce cryoinjuries is the use of different substrates for mycelial growth before freezing. They are little explored but can alter the amount of free water in the mycelium, controlling ice formation inside and around the cell [10]. In addition, mycelium can grow inside of the grain capillaries providing physical protection against cryoinjuries when grains are used as substrate before freezing. Cryopreservation at -70°C is more effective than at -20°C but it requires expensive equipment. Kitamoto et al. [11] report a long-term cryopreservation protocol for some species of basidiomycetes using ultrafreezers at -80°C . However, Ryan et al. [3] reported that no method can be universally applied to all fungi and that while some species are difficult to preserve, others can be preserved by almost any method. Thus, the objective of this study was to evaluate substrates associated to cryoprotective agents for *P. ostreatus* cryopreservation at -20 or -70°C .

Materials and methods

Pleurotus ostreatus U6/8 strain from the Molecular Biology Laboratory culture collection of the *Universidade Paranaense* was used. For inoculum cultivation medium, 39 g/L of potato dextrose agar (PDA) was previously autoclaved at 121°C for 20 min. The mycelial growth was at $25 \pm 1^{\circ}\text{C}$ in the dark for 15 days. Uniform mycelium without sectioning obtained from the mycelial growth edge was used as inoculum.

The substrates used before cryopreservation were whole oat (*Avena strigosa* Schreb.) grains (OG), wheat (*Triticum aestivum* L.) grains (WG), rice (*Oryza sativa* L.) grains without hulls, known as brown rice (RG), millet (*Setaria italica* (L.) P. Beauvois) grains (MG) and PDA (control). Each grain (200 g) was kept at 90°C in water excess during 45 min for protein and starch gelatinization. The water excess was removed and each grain was autoclaved at 121°C for 20 min in flasks with screw caps. Then, they were inoculated with five PDA disks containing mycelium and kept at $25 \pm 1^{\circ}\text{C}$ in the dark.

For cryopreservation, the used cryoprotective solutions were 1 % dimethyl sulfoxide (DMSO) or 5 % glycerol (GO) that penetrates the cellular wall and the plasmatic membrane; 10 % saccharose (SA) or 4 % glucose (GU) that penetrates only the cellular wall; 6 % polyethylene

glycol-6000 (PEG) or 5 % malt extract (ME) that does not penetrate the wall or the plasmatic membrane [10]. Cryotubes were made of polypropylene plastic tubes (0.3×7.0 cm juice straws) that initially had one end thermo sealed [12]. The cryoprotective solutions and the cryotubes were autoclaved separately at 121°C for 20 min, except for DMSO solution which was filtrated through sterile $0.22\text{ }\mu\text{m}$ pore membranes. Each cryotube received 800 μL of cryoprotective solution, and then five PDA disks or grains containing mycelium grown were added. Cryotubes had the other end thermo sealed and were frozen from room temperature (23°C) to -20 or to -70°C in mechanical ultrafreezers. Each disk or grain was considered a replication. For each treatment, fifteen replications were recovered from three cryotubes.

After cryopreservation for 1 or 3 years, the cryotubes were thawed in water bath at 30°C for 15 min [10], washed with 70 % alcohol and then with 96 % alcohol. One of the cryotube ends was cut, the cryoprotective solution removed, grains or disks were transferred to PDA cultivation medium and kept at $25 \pm 1^{\circ}\text{C}$ in the dark. Fungus was considered viable when at least 75 % of the grains or disks had homogenous mycelial growth, without sectioning, within 30 days [13].

Results

Cryopreservation at -20°C showed reduced mycelial viability after 1 year and lower viability after 3 years (Table 1). Treatments with WG were 100 % effective regardless the cryoprotectant used after 1-year cryopreservation (Table 1). Mycelium viability of the substrate/cryoprotectant combination for OG/SA, RG/GU, MG/SA or MG/ME was effective and varied from 80 to 92 %. Nevertheless, after 3 years, only two treatments were effective with 87 % of mycelial viability when the combination of WG/GU or WG/SA was used (Table 1). It shows that *P. ostreatus* cryopreservation at -20°C is effective even in adverse freezing conditions when there is the proper combination of substrate and cryoprotectant.

Cryopreservation at -70°C was effective with grains and had high mycelial viability after 1 or 3 years (Table 2) because 100 % of mycelial viability was obtained from every combination of substrate and cryoprotectant, except for MG/PEG and PDA. The treatment with PDA disks (control) did not present mycelial viability when cryopreserved for 1 or 3 years at -20°C and lower viability at -70°C only after 1 year of cryopreservation with DMSO or SA (Tables 1, 2). PDA disks are broadly used for fungus cryopreservation [14] but the results in this study indicate that wheat grains were more promising for cryopreservation of this fungus.

Table 1 *Pleurotus ostreatus* mycelial viability (survival percentage) after cryopreservation at -20°C for 1 and 3 years

Cryoprotectant	After 1 year of cryopreservation (%)						After 3 years of cryopreservation (%)					
	Cultivation medium or substrates					Grain substrate average	Cultivation medium or substrates					Grain substrate average
	PDA	OG	WG	RG	MG		PDA	OG	WG	RG	MG	
GO	0	33	100	12	0	36	0	0	53	0	0	13
DMSO	0	53	100	30	60	61	0	0	60	0	0	12
GU	0	70	100	83	40	73	0	13	87	0	0	25
SA	0	80	100	66	92	84	0	13	87	13	47	40
ME	0	73	100	25	83	70	0	6	67	0	0	18
PEG	0	60	100	40	52	63	0	0	47	0	0	19
Cryoprotectant average	0	62	100	43	54	–	0	5	67	2	8	–

Cultivation medium and substrates with mycelium: potato dextrose agar disks (PDA); whole oat grains (OG); whole wheat grains (WG), whole rice grains without hulls, known as brown rice (RG) and whole millet grains (MG). Cryoprotective agents: 1 % dimethyl sulfoxide (DMSO); 5 % glycerol (GO); 10 % saccharose (SA); 4 % glucose (GU); 6 % polyethylene glycol-6000 (PEG) and 5 % malt extract (ME)

Table 2 *Pleurotus ostreatus* mycelial viability (survival percentage) after cryopreservation at -70°C for 1 and 3 years

Cryoprotectant	After 1 year of cryopreservation (%)						After 3 years of cryopreservation (%)					
	Cultivation medium or substrates					Grain substrate average	Cultivation medium or substrates					Grain substrate average
	PDA	OG	WG	RG	MG		PDA	OG	WG	RG	MG	
GO	56	100	100	100	100	100	0	93	100	87	100	95
DMSO	93	100	100	100	93	98	54	87	100	75	80	86
GU	18	100	100	100	100	100	0	100	93	93	100	96
SA	83	100	100	100	100	100	40	100	100	100	100	100
ME	23	100	100	100	100	100	0	100	87	93	93	93
PEG	0	100	100	100	100	100	0	100	80	80	73	83
Cryoprotectant average	46	100	100	100	99	–	16	97	93	88	91	–

Cultivation medium and substrates with mycelium: potato dextrose agar disks (PDA); whole oat grains (OG); whole wheat grains (WG), whole rice grains without hulls, known as brown rice (RG) and whole millet grains (MG). Cryoprotective agents: 1 % dimethyl sulfoxide (DMSO); 5 % glycerol (GO); 10 % saccharose (SA); 4 % glucose (GU); 6 % polyethylene glycol-6000 (PEG) and 5 % malt extract (ME)

Cryoprotectants in general were effective for keeping viable mycelium in grains after 1- or 3-year cryopreservation at -70°C (Table 2). However, when the cryopreservation was at -20°C for 3 years, mycelial viability was effective only when a combination of WG/SA or WG/GU were used (Table 1). It indicates that substrate/cryoprotectant combination positively affects the micelial viability of cryopreserved fungus probably because it promotes the survival of a higher number of mycelial cells in the freezing and/or thawing process.

Discussion

Mycelial viability was in general higher for those cryopreserved at -70°C than at -20°C (Tables 1, 2). Slower

freezing at -20°C has probably formed long ice crystals causing cellular membrane injuries. Besides the loss of cellular structure, slow freezing of external water causes cell dehydration and increase of cytoplasmic ion concentration. This process reduces the freezing temperature of the cytoplasm turning it in a highly viscous concentration mixture with low metabolic activity but with irreversible damages to cell and/or cell proteins [15].

Our results showed that wheat grains were effective to keep fungus viable after cryopreservation at -20°C for 1 year regardless the cryoprotectant or 3 years when combined with glucose or saccharose. It is possible that WG substrate effects on cryopreservation are related to carbohydrates and proteins capable of binding water to the grain [16], and consequently reducing free water and intercellular crystal formation. Moreover, the increase of

linked intracellular water reduces the differences between intra and intercellular freezing temperatures [15] and controls the cellular dehydration at slow freezing temperatures. Another possibility is that the wheat grain structure is composed of a capillary net [16], which provides physical protection to mycelium. Thus, chemical and physical factors of the grain could have contributed to reduce cryoinjuries, keeping mycelial cell viable even after frozen at -20°C for 3 years. This result is an important component to understand cryopreservation process and indicates new pathways for cryopreservation at -20°C .

For a long term cryopreservation at -20 and -70°C the best cryoprotectant was saccharose or glucose, although other cryoprotectants were effective. Saccharose and glucose are semi-permeable cryoprotectants concentrating between the plasmatic membrane and the cellular wall. Their main characteristics are linking water to the interstice, accelerating cell dehydration and avoiding both ice formation and cryoinjuries to the plasmatic membrane [6]. Besides the cryoprotective action, saccharose and glucose can be used as a carbon source for mycelial growth recovery after cryopreservation.

DMSO and GO cryoprotectants were not so effective on cryopreservation at -20°C in this research, although they are widely used [17] and considered the most effective cryoprotectants to fungi [18] in cryopreservation. Both penetrate the cell wall and plasmatic membrane, avoiding the intracellular ice formation and altering the plasmatic membrane elasticity, allowing a better accommodation of the cellular volume expansion during water freezing [15]. However, DMSO and GO can also affect the cellular metabolism due to their toxicity [6]. This indicates that these substances can protect against cryoinjuries, but can also make it difficult to recover mycelium after cryopreservation.

In general, for 1- or 3-year cryopreservation at -70°C , any substrate combined with any cryoprotectant was effective for preserving the mycelium viable, except for millet grains with polyethylene glycol-6000 and PDA with any cryoprotectant after 3 years. Thus, our analysis was focused on the most adverse environment to the fungus, cryopreservation at -20°C , in order to select the most likely combination of substrate and cryoprotectant for other future studies on long-term cryopreservation at -20 or -70°C .

The results found in this study allow a new approach for cryopreservation at -20°C with other basidiomycetes besides *P. ostreatus*. The mycelial viability obtained after 3 year cryopreservation at -20°C indicates forms to improve longer-term storage systems that are usually harmful to filamentous fungi. However, other studies should be developed in order to expand knowledge of cryopreservation at lower costs.

It was concluded that combination of substrate and cryoprotectant affects mycelial viability after cryopreservation at -20°C or at -70°C . Wheat grains combined with any cryoprotectant are effective for keeping the mycelium viable after 1-year cryopreservation at -20°C , and when combined with saccharose or glucose, they are effective after 3 years. For cryopreservation at -70°C after 1 or 3 years, any substrate combined with any cryoprotectant is effective for preserving the mycelium viable, except for millet grains with polyethylene glycol-6000 after 3 years. Potato dextrose agar is ineffective for cryopreservation at -20°C or -70°C for 1 or 3 years, except when combined with dimethyl sulfoxide or saccharose for 1 year at -70°C . The semi-permeable cryoprotectants saccharose and glucose are the most effective ones for cryopreservation at -20 or -70°C for 1 or 3 years.

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