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An example for comparison of storage methods of macrofungus cultures: *Schizophyllum commune*

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Abstract: Twelve different methods were compared for storage of a newly isolated mushroom strain, *Schizophyllum commune* Fr. The fungal strain was stored in different media [sterile distilled water (SDW), glycerol (15%) or media including trehalose], in different forms (mycelium-colonised wheat seeds, mycelium-colonised agar plugs) and at different temperatures (+4, +20, –20 °C). The fungal culture was checked periodically for its viability and biological activity for up to 1 year. The mycelial recovery ratio, mycelial growth rate, mycelial biomass weight, and enzyme activity of the recovered cultures were used for comparison of the different methods**.** After 12 months' storage, the vitality of the strain was successfully preserved and the mycelial growth rates and biomass weight were similar for the different methods of storage. The enzymatic activity showed variability for the different storage methods. Our results show that agar plugs stored in sterile water at +4 °C and in 15% (v/v) glycerol at +20 °C are preferable methods for the preservation of *S. commune*. Additionally, the methods of mycelium-colonised wheat seeds in sterile water at +4 °C and agar plugs in sterile water at +20 °C are acceptable alternatives.

Key words: *Schizopyhllum commune*, Basidiomycetes, storage method

Makrofungus kültürlerinin saklama yöntemlerinin karşılaştırılması için bir örnek: *Schizophyllum commune*

Özet: Bu çalışmada, yeni izole edilen bir makrofungus suşunun, *Schizophyllum commune* Fr., on iki farklı yöntem ile saklanması karşılaştırılmıştır. Fungus suşu farklı ortam [steril distile su, gliserol (% 15) ya da trehaloz içeren ortam], farklı saklama formu (misel ile kolonize edilmiş buğday tohumu, misel ile kolonize edilmiş agar diski) ve farklı sıcaklıklarda (+4, +20, –20 °C) saklanmıştır. Fungal kültür bir yıl boyunca periyodik olarak canlılığı ve biyolojik aktivitesi açısından kontrol edilmiştir. Saklama yöntemlerinin karşılaştırılması için, misellerin geri dönüş oranı, geri dönen misellerin misel büyüme oranı, miselyal biyomas ağırlığı ve enzim aktivitesi değerleri kullanılmıştır. On iki aylık saklama periyodu sonrasında farklı saklama yöntemleri ile saklanan suş canlılığını korumuş olup, benzer misel büyüme oranı ve miselyal biyomas ağırlığı değerlerine ulaşılmıştır. Farklı saklama yöntemlerinden elde edilen enzim aktivitesi değerleri, değişkenlik göstermektedir. Sonuçlarımız, +4 °C de steril distile suda ve +20 °C de % 15 (v/v) gliserolde saklanan agar disklerinin *S. commune* kültürlerinin saklanması için daha uygun yöntemler olduğunu göstermektedir. Ayrıca, +4 °C de steril distile suda saklanan misel ile kolonize edilmiş buğday tohumu ve 20 °C de steril distile suda saklanan agar diskleri de iyi saklama alternatifleri olarak kabul edilebilir yöntemlerdir.

Anahtar sözcükler: *Schizopyhllum commune,* Basidiomycetes, saklama yöntemi

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Introduction

The preservation of fungal cultures is very important for systematic, biodiversity, cultivation, and industrial studies. In successful storage, the purity, viability, and genomic integrity of the culture should be maintained and the morphological, physiological, and genetic characteristics of the culture should not change. Therefore, the storage of microbial cultures is difficult and time consuming.

According to January 2011 data, 585 culture collections in 68 countries were registered in the World Database Centre for Microorganisms (WFCC) and the number of fungi in these collections was calculated at 506,333 species. According to the literature, the preservation of nonsporulating Basidiomycetes is rather difficult (Ito & Nakagiri, 1996; Homolka et al., 2001; Singh et al., 2004; Homolka et al., 2006; Psurtseva, 2010). Fungal stock cultures are usually maintained using a subculturing method. Although this method is simple and inexpensive, it is effective for short-term preservation. Because of the disadvantages of this method (such as the need for periodic transfer, risk of contamination, strain deterioration and loss) different methods have been developed for the long-term preservation of Basidiomycetes cultures in culture collections (Nakasone et al., 2004; Eugenia et al., 2009). The best preservation medium for the storage of mushroom mycelia is accepted as liquid nitrogen (Hwang, 1966; Jodon et al., 1982; Smith, 1982; Chvostovà et al., 1995; Ohmasa et al., 1996; Mata & Rodríguez-Estrada, 2005). Nevertheless, the liquid nitrogen must be replenished regularly and this method is rather complex and expensive. Therefore, cost effective and simple methods are a focus of research for mushroom species. The storage of fungi under sterile water, mineral oil, or glycerol has been tried successfully for different fungal species (Smith & Onions, 1994), being simple, rapid, and inexpensive (Burdsall & Dorworth, 1994; Baskarathevan et al., 2009).

Lyophilisation has been reported as a good alternative method for storage of Basidiomycetes (Raper & Alexander, 1945; Singh et al., 2004; Voyron et al., 2009). The effects of trehalose in lyophilisation have been studied using *Schizophyllum commune* Fr. and *Coprinopsis psychromorbidus* (Redhead & Traquair) Redhead, Vilgalys and Moncalvo (Tan & Stalpers, 1991). Basidiomycete species, particularly mycelial ones, can be lyophilised effectively in the presence of trehalose (Croan et al., 1999). Terashita et al. (2003) demonstrated the positive effects of trehalose on the growth of Basidiomycetes preserved in a refrigerator.

The present study aims to select an easy, economical, and reliable storage method by comparing different storage methods using a sample of newly isolated Basidiomycetes culture, *Schizophyllum commune*.

Materials and methods

Fungal strain

For accurate comparison, a newly isolated fresh Basidiomycetes culture, *Schizophyllum commune*, was preferred; it is a common species for Turkish mycobiota (Solak et al., 1999; Afyon et al., 2004; Doğan & Öztürk, 2006; Solak et al., 2007; Türkoğlu et al., 2007; Kaya et al., 2009; Sesli & Denchev, 2009; Alkan et al., 2010; Demirel et al., 2010). The basidiomata specimen of mushroom was collected from Osmaneli, Bilecik, Turkey. It was identified in accordance with the relevant literature (Breitenbach & Kränzlin, 1986; Ellis & Ellis, 1990). The culture was initially isolated from basidiomata and was coded as OBCC 5027 for the Basidiomycetes Culture Collection of Eskişehir Osmangazi University. The first stock culture was grown on Malt Extract Agar slants and maintained at 4 °C in the Fungiculture Laboratory at the Department of Biology, Eskişehir Osmangazi University.

Storage methods

The *Schizophyllum commune* strain OBCC 5027 was stored using 12 different storage methods. The 12 methods used differed in the origin of the culture (mycelium-colonised wheat seeds, mycelium-colonised agar plugs), the protectant type (glycerol, trehalose), and the preserving temperature (+4, +20, –20 °C) (Table 1). Firstly, the strain was incubated on Malt Extract Agar (MEA) for 7 days. The agar plugs were punched out using a sterile 5-mm stainless steel cork borer. The mycelium-colonised agar plugs were then transferred into sterile glass bottles filled with sterile water and 15% glycerol and then (M3,

Table 1. The methods used for storage of the *Schizophyllum commune* strain OBCC 5027.

Storage temperatures (°C)	Storage codes	Storage methods
+4	M1	Mycelium-colonised wheat seeds
	M2	Mycelium-colonised wheat seeds in sterile water
	M3	Agar plugs in sterile water
	M4	Agar plugs in 15% (v/v) glycerol
	M5	Cultures on malt extract agar
	M6	Lyophilised as mycelium-colonised wheat seeds
–20	M7	Trehalose medium
	M8	Mycelium-colonised wheat seeds in 15% (v/v) glycerol
	M9	Agar plugs in sterile water
	M10	Agar plugs in 15% (v/v) glycerol
+20	M11	Agar plugs in sterile water
	M12	Agar plugs in 15% (v/v) glycerol

M4, M9, M10, M11, and M12) stored at 3 different temperatures. In addition, the punched agar plugs from the cultures grown for 7 days on the MEA and trehalose medium (M5, M7) were stored at + 4 and –20 °C, respectively.

As a different storage method, spawn prepared from wheat seeds was also used. For this purpose, wheat seeds were washed in sterile distilled water, cooked for 20 min, and then drained. The wheat seeds were then mixed with a 1:4 proportion of calcium carbonate and calcium sulphate and then sterilised at 121 °C for 1 h in glass bottles. The spawn was inoculated with 4 mycelium agar discs of the strain and incubated at 28 °C for 15 days to allow mycelial growth to completely cover the wheat seeds. After incubation, the mycelium-colonised wheat seeds were transferred to sterile glass bottles filled with sterile water or 15% glycerol and then stored at +4 °C or –20 °C (M1, M2, and M8). In addition, the mycelium-colonised wheat seeds were lyophilised using a shelf freeze-drying model (Christ Alpha 1-2 LD) and were maintained at +4 °C (M6).

Experimental parameters

After 2, 4, 6, 9, and 12 months' storage using 12 storage methods, the mycelial recovery ratio, mycelial growth rate, mycelial biomass weight, and enzyme activity of preserved cultures were compared during the preservation period. For this purpose, the agar plugs or mycelium-colonised wheat seeds of the strain were inoculated in the centre of the MEA in a 150-mm-diameter petri dish and incubated at 28 °C for 10 days.

Mycelial recovery ratio

The mycelial recovery ratio was determined from the mycelium-colonised wheat seeds and mycelium-colonised agar plugs incubated on the MEA at 28 °C. After incubation, the mycelial recovery was observed daily by naked eye. The recovery ratio was calculated using 10 parallel applications. The results are given as a percentage.

Mycelial growth rate

The mycelial growth rate was calculated using the method of Bilay et al. (2000). The radius of the fungal colony was measured in 4 different dimensions every day for 10 days. When the increase in the colony diameter (R_2-R_1; mm) was linear in a particular time period (t_2-t_1; day), the mycelia growth rate (mm/day) was calculated for each preserving method and period.

Mycelial biomass weight

To determine the mycelial biomass weight, 10 days after inoculation fungal colonies were harvested following a 1 min microwave exposure to melt the agar. The mycelium was then blotted dry with 3 changes of Whatman paper until no further liquid

was released with only moderate application of hand pressure. Then the colonies were washed 3 times with SDW and were dried to a constant weight at 80 °C and weighed (Blaudez et al., 2000).

Enzyme activity

The enzyme activities of the *Schizophyllum commune* strain OBCC 5027 were investigated with submerged fermentation (SmF) and 2 different solid substrate fermentation methods (SSF1 and SSF2) to determine the optimal method and day.

For the SmF, the medium contained 10 g of glucose, 2 g of NH_4NO_3, 2 g of yeast extract, 0.8 g of KH_2PO_4, 0.75 g of $Na_2HPO_4.7H_2O$, 0.5 g of $MgSO_4.7H_2O$, and distilled water to 1 L. The pH was adjusted to 6.0 with 1 M HCl. The SmF media were inoculated with 4% inoculum and incubated at 28 °C for 20 days on a rotary shaker at 100 rpm. For the SSF, wheat bran was dried in an oven at 60 °C, accurately weighed to 5 g in 250 mL shake flasks, moistened with distilled water (SSF1) or trace element solution (SSF2; in g/L: $ZnSO_4.7H_2O$, 0.002; $FeSO_4.7H_2O$, 0.005; $CaCl_2.2H_2O$, 0.06; $CuSO_4.7H_2O$, 0.02; $MnSO_4.H_2O$, 0.05) and was autoclaved at 121 °C for 40 min. The moisture content of the media was adjusted to 60% by adding sterile distilled water. After cooling, the media were inoculated with 4% inoculum and incubated at 28 °C for 20 days.

The laccase enzyme activities of the cultures were periodically measured over 20 days (Figure 1). Laccase activity was determined using 2,2-azinobis(3-ethylbenzothiazoline 6-sulphonic acid) (ABTS) as a substrate. The assay mixture contained 1 mM ABTS and 100 mM sodium acetate buffer, pH 4.5. Oxidation of the ABTS was followed by an absorbance increase at 420 nm (ε = 36,000 M^{-1} cm^{-1}) (Faraco et al., 2008). After 2, 4, 6, 9, and 12 months of preservation, extracellular enzyme activities were measured on day 11 for laccase activity.

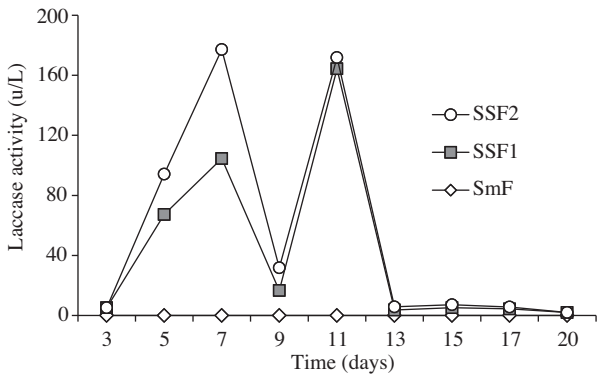

Figure 1. Time dependent laccase activity in SmF, SSF1, and SSF2.

All the enzyme activities were measured and expressed in International Units (IU), where one unit of enzyme activity is defined as the amount of enzyme that oxidises one μmole of substrate in 1 min.

Statistical analysis

All of the experiments were conducted at least 3 times each. The data are expressed as mean ± standard deviation (S.D.) and one-way analysis of variance (ANOVA) and Duncan's multiple-range tests, and correlations were performed using SPSS to compare the experimental groups. The P values < 0.05 were considered to indicate statistical significance.

Results and discussion

As a highly specialised fungus group, Basidiomycetes are known for many properties, such as biological, pharmaceutical, nutritional, and industrial. Therefore, preservation of these organisms as pure, viable, and stable cultures is of critical importance. The preservation of biological and genetic properties of fungi plays an important role in culture collections and biological source centres. The mycelial recovery ratio, mycelial growth rate, and biomass weight are important criteria in storage methods of fungal strains. In this study, these parameters and fungal enzyme activities were compared during the preservation period for the *Schizophyllum commune* strain OBCC 5027.

The principal criterion for a successful recovery of fungal strains was the ability to survive the preservation process. After a 1-year storage period, no contamination was detected in any of the preservation methods. There was no report of any loss of vitality or recovery ratio (100%) for 10 of the storage methods for the *S. commune* strain OBCC 5027. However, low vitality was recorded when agar plugs were stored in 15% (v/v) glycerol at –20 °C (M10) as from month 4 of preservation (Figure 2).

The concentration and type of the cryoprotectant, and the cooling rates are known to be important

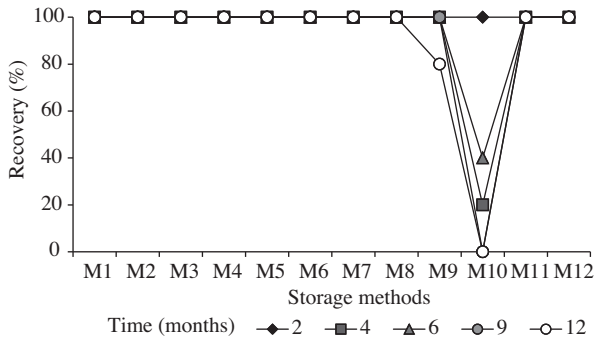

Figure 2. Viability (%) of the strain during preservation duration with different storage methods.

factors that affect the viability of cultures after storage. Previous studies (Mata & Pérez-Merlo, 2003; Mata & Rodríguez-Estrada, 2005) show that treating spawn stocks with 10% glycerol as a cryoprotectant results in 100% recovery of the studied edible mushrooms. Kitamoto et al. (2002) demonstrated a positive effect of 10% glycerol as a cryoprotectant on the growth of Basidiomycota cultures preserved in the sawdust-freezing method. In the present work, the cultures represent a negative effect of 15% glycerol in M10 from 4 months with a positive effect of trehalose in M7. In addition, we show a positive effect of 15% glycerol in the methods of M4 and M12.

The preservation temperature is an important factor in the viability of fungal cultures. Agar plugs of the strain stored in the M10 form did not grow after 9 months of storage. When agar plugs were stored in sterile water at –20 °C (M9) for the *S. commune* strain OBCC 5027, the recovery ratio decreased to 80% after 12 months' preservation. Richter (2008) report that mycorrhizal fungi demonstrated a lower viability than saprotrophic fungi in sterile cold water. The viability of fungal cultures stored for many years has been reported by various authors (Table 2).

In the present work, the radial growth rates vary according to different storage methods (Table 3). After 12 months' storage, malt extract agar cultures stored at +4 °C (M5), mycelium-colonised wheat seeds in sterile water stored at +4 °C (M1), and agar plugs in glycerol stored at +20 °C (M12) had the greatest mycelial growth rates of 9.62 ± 0.14, 9.18 ± 1.05, and 9.02 ± 1.35 mm/day, respectively. In addition, the other groups, M2, M3, and M11, had a high mycelial growth rate after 12 months. According to statistical analysis of mycelial growth rate data, the M1, M2, M3, M5, M11, and M12 methods were determined in the same statistical group and were accepted as the best preservation methods (Table 3). Some authors have reported that radial growth decreased after preservation in various storage methods (Dahmen et al., 1983; Burdsall & Dorworth, 1994; Stoychev et al., 1998; Elliot, 2005).

The mycelial biomass weight produced from the recovered cultures of the strain showed apparent differences using different storage methods. After 12 months of storage, higher mycelial biomass weights were obtained for M3 (70.66 ± 5.02 mg/cm^2) and M12 (61.25 ± 0.97 mg/cm^2). According to a statistical analysis of our results, the biomass weights obtained from the methods M3 and M12 were significantly different from those obtained from the other storage methods (Table 4). This means that the mycelial intensity of these organisms' colonies was denser than the others and they had healthier colonies.

The *S. commune* strain OBCC 5027 was tested for its ability to maintain the production of laccase enzyme after preservation with different storage methods (M1-M12). The results of time-dependent laccase activity of the *S. commune* strain OBCC 5027 are shown in Figure 3. Although the results were

Table 2. Recovery of fungal cultures stored by different storage methods.

Storage method	Duration	Recovery (%)	Reference
Sterile water, 4 °C	7 years	94	Burdsall and Dorworth (1994)
Sterile water, 4 °C	20 years	88	Richter (2008)
Sterile water, –80 °C	50 years	88.4	Ito and Nakagiri (1996)
Silica gel, –140 °C	25 years	64	Sharma and Smith (1999)
Perlite in liquid nitrogen	10 years	100	Homolka et al. (2010)
Spawn, –80 °C	2 weeks	100	Mata and Pérez-Merlo (2003); Mata & Rodríguez-Estrada (2005)

Table 3. The radial growth rates (mm/day) of the strain during preservation duration with different storage methods.

	Duration (months)				
	2	4	6	9	12
Codes					
M1	10.11 ± 0.12 abd	9.34 ± 0.24 ad	9.59 ± 0.06 acd	8.34 ± 0.17 ae	9.18 ± 1.05 ab
M2	9.59 ± 0.13 bd	9.55 ± 0.11 ab	9.04 ± 0.06 ae	9.16 ± 0.10 b	8.73 ± 1.06 abc
M3	10.68 ± 0.28 a	9.63 ± 0.19 abc	9.15 ± 0.12 ace	8.77 ± 0.30 ab	8.13 ± 0.15 abc
M4	9.61 ± 0.16 bd	9.15 ± 0.25 ad	8.43 ± 0.22 b	6.69 ± 0.12 c	7.33 ± 0.52 bc
M5	10.01 ± 0.35 bcd	9.06 ± 0.05 ad	9.29 ± 0.05 ace	8.67 ± 0.31 abd	9.62 ± 0.14 a
M6	10.86 ± 0.24 a	10.06 ± 0.43 bc	9.62 ± 0.11 acd	8.23 ± 0.06 ad	7.32 ± 0.18 bc
M7	10.40 ± 0.08 acd	10.05 ± 0.12 bc	9.65 ± 0.06 cd	7.56 ± 0.45 eg	7.87 ± 0.11 bc
M8	10.71 ± 0.21 a	10.16 ± 0.25 c	10.16 ± 0.17 d	8.63 ± 0.03 ab	7.18 ± 0.40 c
M9	10.44 ± 0.19 acd	9.41 ± 0.05 ad	9.15 ± 0.25 ac	8.75 ± 0.14 ab	7.66 ± 0.12 bc
M10	9.99 ± 0.19 d	9. 38 ± 0.26 ad	8.76 ± 0.42 eg	0.00 ± 0.00 f	0.00 ± 0.00 d
M11	9.66 ± 0.06 b	8.84 ± 0.17 d	8.95 ± 0.17 eg	8.26 ± 0.39 ae	8.33 ± 0.86 abc
M12	9.64 ± 0.34 b	9.33 ± 0.20 ad	9.08 ± 0.35 ac	8.50 ± 0.48ab	9.02 ± 1.35 abc

Table 4. Biomass weight (mg/cm^2) of the strain during preservation duration with different storage methods.

	Duration (months)				
	2	**4**	**6**	**9**	**12**
Codes					
M1	101.44 ± 5.10 a	69.27 ± 4.44 ac	60.45 ± 1.02 a	53.28 ± 1.97 ac	56.76 ± 6.43 ace
M2	80.86 ± 1.53 b	74.89 ± 5.03 ac	72.44 ± 0.14 b	67.05 ± 2.00 bdf	58.73 ± 5.40 ae
M3	66.15 ± 1.43 c	69.04 ± 0.68 ac	63.84 ± 5.60 ad	68.24 ± 4.53 bf	70.66 ± 5.02 b
M4	77.10 ± 0.41 bc	64.79 ± 1.50 ab	60.91 ± 2.24 a	44.94 ± 1.31 c	54.21 ± 2.24 ace
M5	72.28 ± 1.24 bc	64.35 ± 3.99 ab	59.08 ± 3.68 a	62.31 ± 0.61 ab	50.30 ± 1.55 ac
M6	70.14 ± 7.82 bc	56.66 ± 3.13 b	45.05 ± 0.49 c	60.99 ± 7.84 ab	47.02 ± 1.75 c
M7	69.57 ± 4.17 bc	65.23 ± 1.46 ab	65.94 ± 4.33 ab	61.17 ± 1.51 ab	56.28 ± 0.13 ace
M8	75.01 ± 2.91 bc	65.36 ± 10.02 ab	59.47 ± 1.44 a	59.21 ± 5.98 ab	46.89 ± 1.02 c
M9	65.28 ± 3.25 c	54.45 ± 1.31 b	46.18 ± 1.12 c	57.38 ± 2.41 ad	47.04 ± 4.02 c
M10	63.12 ± 1.59 c	53.48 ± 0.86 b	46.18 ± 1.12 c	0.00 ± 0.00 e	0.00 ± 0.00 d
M11	81.21 ± 10.64 b	74.14 ± 1.68 c	71.64 ± 0.99 bd	74.22 ± 0.60 f	48.76 ± 4.80 ac
M12	74.09 ± 6.28 bc	65.56 ± 1.72 ab	58.54 ± 3.98 a	56.91 ± 3.42 ad	61.25 ± 0.97 be

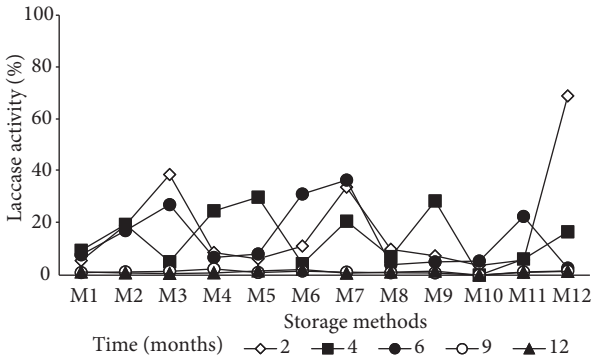

Figure 3. Time-dependent laccase activity of the *Schizophyllum commune* strain OBCC 5027.

variable, it was observed that laccase activity started to decrease from month 4 of storage and had stopped by month 12 in all the storage methods tested. After preservation, the percentage of the *S. commune* strain OBCC 5027 that displayed significant differences of laccase activity ranged between 0.6% (M3) and 1.4% (M12).

Enzyme activity of fungal strains is very important for biotechnological studies, but there is no general consensus on the effect of the preservation methods on enzyme activity (stimulation or inhibition). Voyron et al. (2009) reported that tested enzymatic activities are not correlated to storage methods as demonstrated by the fact that isolates belonging to the same species could react in different ways.

Homolka et al. (2001) demonstrated that some basidiomycete strains lost their ability to produce laccase after freezing. Similarly, Stoychev et al. (1998) reported that laccase activity is low in all white rot basidiomycete strains after recovery from cryopreservation in liquid nitrogen. Results show that laccase activity decreases more in our storage methods after a storage period compared with methods in the literature (Voyron et al., 2009). On the other hand, the ligninolytic enzyme activities of 30 different Basidiomycetes strains were slightly affected after 10-year cryopreservation on perlite in liquid nitrogen (Homolka et al., 2010). According to the key proposed by Ryan et al. (2000), M2, M3, M11, and M12 can be accepted as successful methods for the preservation of fungi.

In conclusion, based on statistical analysis of the results of the recovery ratio, growth rate, biomass weight, and enzyme activities of the preserved cultures, we can argue that, of all the methods tested, M2, M3, M11, and M12 may be preferred for the storage of tested basidiomycete culture. The presented results can be accepted as encouraging for the preservation of mushroom cultures. Such preservation methods must be considered as a fruitful approach in the development of mushroom biotechnology. Further investigations on a large number of mushroom species may provide a better source to evaluate the suitability of the protocols.

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