put breaks on the decline of the vulture population. However, care should be taken while replacing Diclofenac with other NSAIDs, because the replacement drugs may not be environmentally safe either, and their impacts are unknown. Research into the ecological impact of all major therapeutics, especially the common and widely used ones, should be undertaken urgently to facilitate the policy makers to regulate these compounds with potentially hazardous environmental impacts. The exact physiological mechanism through which Diclofenac acts in three species of only a single genus (*Gyps*), appears mysterious and highly intriguing. The metabolic pathways through which Diclofenac poisoning causes renal complications, gout and consequent mortality in only *Gyps* vultures is yet to be elucidated. As of now, it appears premature to conclude that the Diclofenac residue is the universal causative agent behind the decline of vulture population.

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Development of a novel lyophilization protocol for preservation of mushroom mycelial cultures

The maintenance and production of reliable pure culture spawn with desirable quality is the key operation and the first critical stage in the success of mushroom cultivation. Maintenance of vigour and genetic characteristics of a pure strain in the form of a culture is the main objective of culture preservation. Besides this, strain improvement of cultivated mushrooms demands a well-planned system of maintenance, preservation and availability of genetic diversity[1]. Mushroom culture repositories/gene banks play a vital role in supply of pure and authentic cultures to most of the mushroom spawn-producing units. There are various methods of maintenance and preservation of mushroom cultures and a good culture collection section adopts more than one method to preserve them. Mushrooms might be of academic, medicinal or horticultural importance. Mushroom strains having industrial importance are patented and preserved, although availability of such strains becomes restricted[2]. If no degenerative changes were to take place during preparation or maintenance of mush-

room cultures or spawn, then it would be a relatively simple, routine process. Unfortunately, this is not true. Degeneration of culture spawn refers to the loss of desired traits leading to slow development, poor rate of survival and low productivity[1,3].

Mushrooms are invariably stored as mycelial cultures because spores of heterothallic and secondary homothallic species are produced through a sexual process and have genetic differences, which may not eventually result in fruiting[4]. In the absence of hardy structures like double-walled spores or sclerotia, microbial cultures become susceptible to sudden change in temperature and pressure[5]. Maintenance of mushroom stock cultures under liquid nitrogen has been reported stable with little or no genetic change[6]. Singh *et al.*[7] modified the cryopreservation protocol using mycelium multiplied on wheat grain instead of mycelial disk and experimentally demonstrated genetic stability of mushroom stock cultures in liquid nitrogen.

Cryopreservation under liquid nitrogen gives high survival rates and is universally acceptable. Nevertheless, methods using liquid nitrogen are rather expensive, and regular supply of liquid nitrogen is not always guaranteed. Lyophilization is a good alternative. It employs the preservation of fungi by drying under vacuum from the frozen state by sublimation of ice. Glass ampoules can be stored easily in compact packing without any special requirement. Cultures need not be revived on agar slants prior to dispatch. The product is light, inactive and dry, enabling easy distribution by mail. Fungal cultures containing conidia and spores can be freeze-dried successfully; but despite the early success of the method, many strains of fungi failed to survive, particularly isolates of Mastigomycotina and Basidiomycotina[8]. However, the survival rates of frozen fungal cells increased considerably when cells were cooled[9,10] at the rate of – 1°C/min.

Earlier reports on freeze-drying of fungal hyphae used aqueous solution of culture suspension in Trehalose, skimmed milk, bovine serum albumin, sucrose, glycerol, etc.[11–13]. The survival rate of freeze-dried fungal hyphae of a number of ascomycetes was recorded to be better than two basidiomycetes, *Schizophyllum commune* and *Coronus psychromorbidus*[14]. Deriving inspiration from low rate of survival of filamentous fungi in general, and basidiomycetes in particular, using available freeze-drying protocols, an attempt has been made to improve lyophilization protocols for satisfactory preservation of mushroom mycelial stock cultures.

The pure mycelial cultures of 11 edible mushroom strains, namely *Agaricus bisporus* (S-11, U-3), *A. bitorquis* (NCB-13), *Pleurotus sajor-caju* (Pl-10 A), *P. ostreatus* (Pl-20), *P. sapidus* (Pl.-40), *P. flabellatus* (Pl.-50) *Auricularia polytricha* (OE-4), *Lentinula edodes* (OE-9), *Morchella esculenta* (ME-1) and *Volvariella volvacea* (OE-12) were procured from the National Mushroom Repository maintained at National Research Centre for Mushroom (NRCM), Solan. All the stock cultures were subcultured on wheat extract agar (WEA) culture medium in test tubes[15].

Each mushroom mycelial culture was multiplied on pearl millet (*Pennisetum typhoides*) grains. The pearl millet grains were thoroughly washed with sufficient water to remove debris, straw particles, undesirable seeds of grasses, etc. Washed grains were soaked in sufficient water for 30 min and then boiled for 20 min. Excess water was drained by sieving through a muslin cloth and dried in shade for 4 h to allow evaporation of any excess water. The boiled grains were mixed with 2% gypsum (calcium sulphate, $CaSO_4$) and 0.5% calcium carbonate ($CaCO_3$) to maintain pH around 7.0. Subsequently, the treated grains were filled in wide-mouth test tubes up to two-thirds of their capacity and plugged with non-absorbent cotton. These grain-containing tubes were autoclaved at 22 lb psi at 126°C for 2 h. After autoclaving, the grain-containing test tubes were allowed to cool overnight to evaporate the moisture on the inner walls of test tubes to avoid contamination. These test tubes were then exposed to ultraviolet light in a laminar flow for 30 min before inoculation. Each mycelial mushroom culture was inoculated in three such test tubes by transferring aseptically growing mycelium from freshly subcultured stocks maintained on WEA. All inoculated test tubes were then incubated at 25°C for 2 weeks, except *V. volvacea* which was incubated at 32°C for 10 days. Mycelial cultures multiplied on pearl millet grain were subjected to lyophilization and also used for pre-lyophilization count.

For lyophilization, glass ampoules were first sterilized in a hot-air oven at 180°C for 2½ h and allowed to cool to ambient temperature. The mycelium that multiplied on pearl millet grains was filled in each sterilized ampoule with the help of sterilized forceps. Each ampoule containing 50–60 grains was plugged with sterilized non-absorbent cotton. The cotton plugs were pushed inside up to the neck of the ampoules. The constrictions were made from above the cotton region in each ampoule and pre-cooled in a deep freezer to – 40°C. When the shelf temperature of the freeze chamber reached –62°C, the ampoules with frozen samples were attached to the lyophilizer (CHRiST, ALPHA 1–2) and vacuum created. The lyophilizer was allowed to run overnight. Next day, when the pressure reached 0.05 mbar, the ampoules were sealed at the point of constriction with the help of a cross-flamed burner connected to oxygen and liquid petroleum gas (LPG). The vacuum was tested with the help of a vacuum-tester. A purple coloured light spark that glows inside the sealed ampoule, verified proper sealing. Six ampoules per strain of edible mushroom were prepared in this way and stored at room temperature.

The survival test of each edible mushroom culture was carried out on malt extract glucose agar (MGA) culture medium (malt extract 10 g; glucose 5 g; agar 20 g; water l^{-1}). For each test strain, 21 grains (mycelium containing pearl millet seeds) were placed in three pre-sterilized petri plates containing MGA culture medium. Survival counts were made before lyophilization, and one month and three months after lyophilization. Post-lyophilization survival counts were made by breaking three ampoules and plating seven seeds per ampoule in pre-sterilized petri plates containing MGA. Inoculated petri plates were incubated at 25°C for 10 days for all the strains tested, except *V. volvacea* which was incubated at 32°C in a separate BOD incubator. Per cent survival of different mushroom strains before and after lyophilization was counted on the basis of number of colonies retrieved out of the total number of grains tested.

The results of pre- and post-lyophilization of 11 edible mushroom mycelial cultures are presented in Table 1. All the 11 mushroom strains were recorded with 100% survival before lyophilization; however a slight variation in survival rate of different edible mushroom strains was not statistically significant. The result exhibited that there was no decline in per cent survival of all the strains after one

Table 1. Effect of lyophilization on survival of 11 edible mushroom strains

Strain	Per cent recovery		
	Before lyophilization	After one month	After three months
Agaricus bisporus (S-11)	100.0	95.2	95.2
A. bisporus (U-3)	95.2	95.2	95.2
A. bitorquis (NCB-13)	95.2	90.5	90.5
Pleurotus sajor-caju (Pl 10 A)	100.0	95.2	95.2
Pleurotus ostreatus (Pl-20)	100.0	100.0	100.0
P. sapidus (Pl-40)	100.0	100.0	100.0
P. flabellatus (Pl-50)	100.0	95.2	95.2
Auricularia polytricha (OE-4)	95.2	90.5	90.5
Lentinula edodes (OE-9)	95.2	90.5	90.5
Morchella esculenta (ME-1)	100.0	100.0	100.0
Volvariella volvacea (OE-12)	100.0	100.0	100.0
CD at 5%	8.5	10.5	10.5

month to three months of storage. *P. sapidus*, *M. esculenta* and *V. volvacea* showed absolute survival before lyophilization and after three months of storage of mycelial cultures in the lyophilized state. *A. bisporus*, *A. bitorquis*, *P. sajor-caju*, and *P. flabellatus* were recorded with a marginal decline in survival counts from before lyophilization to after three months of storage of mycelial cultures in the lyophilized state. Nevertheless, this meagre decline in survival was not statistically significant. *M. esculenta* and *V. volvacea* are low-temperature-sensitive mushroom species[12,16,17]. In the present study, no decline in the survival of any of the 11 mushroom mycelial cultures was due to improvement in the freeze-drying protocols and perfect lyophilization.

Filamentous, non-sporulating fungi which include mushrooms, are considered to be predominantly non-lyophilizable[18,19]. Smith and Onions[8] compiled lyophilization status and stated that only 62 out of 121 species of Basidiomycetes could survive freeze-drying and many mushroom species failed centrifugal freeze-drying at the Common Wealth Mycological Institute. Tan *et al.*[20] freeze-dried fungal hyphae of basidiomycetes *C. psychromorbidus*, *Lepista nuda*, *Perennipora subsida*, *S. commune* and a number of ascomycetes. They reported that programmed slow cooling of hyphae of ascomycetes as well as basidiomycetes survived freeze-drying, although generally better results were obtained with ascomycetes. Morris *et al.*[12] recorded 38% recovery of *V. volvacea* using glycerol at optimum cooling rate of −1°C per min.

The improvement in the preservation protocol was that the preservation as mycelial culture suspension was replaced by multiplication of mushroom mycelium on pearl millet grains. The modified protocol must have given protection to the soft and tender mycelium, concealed inside the grain and thus could have sustained cooling pressure[5,7,10]. The novel protocols developed shall open new vistas to improve available lyophilization technology for long-term conservation of filamentous fungi in general, and mushroom germplasm in particular.

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