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PRESERVATION OF *PENICILLIUM* SPECIES BY LYOPHILIZATION

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ABSTRACT

The maintenance and production of reliable pure cultures with desirable quality is a key operation and the first significant stage in the success of fungal identification. This study assessed spawn preservation and lyophilization (freeze drying) of cultures, for a long time. The sampling was done during the years 2008-2010. Samples were processed in fungal research laboratory of First Fungal Culture Bank of Pakistan (FCBP), Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan. Lyophilization has been used to stabilize living cells and fungal isolates, to dehydrate vast range of materials, including foodstuffs, pharmaceuticals, biotechnology products, vaccines, and diagnostic and biological materials, to improve the storage and the quality of DNA after extraction. Long-term maintenance of *Penicillium* species is essential for detailed studies. Fifty *Penicillium* species were isolated from different sources by direct and dilution method, and then preserved by spawn and lyophilization technique. Fungal isolates were purified and identified based on their morphology. The viability and purity of the fungal species by fungal spawn and lyophilized material (fungal spawn and fungal mat) were monitored immediately after storage at 1, 2 and 3 months at 4 ^{o}C. Each fungal isolate was considered viable if the rate of growth present was the same as that of the original culture and if the morphology of the colony matched the fungal identification documented for each species. All lyophilized *Penicillium* species were found viable at 1-3 months of storage recovered each time from both type of lyophilized material, fungal spawn and fungal mat and showed the initial colony characteristics and growth rates. Neither stabilizer nor skimmed milk was used in fungal material to protect it from sticking with the walls of the container. Modified method of *Penicillium* spawn preservation by lyoplilization has been found cost effective in contrast to liquid nitrogen preservation. It was concluded that lyophilization is simple, inexpensive, reliable and effective method for the long term preservation of *Penicillium* isolates.

Key words: Lyophilization, spawn, *Penicillium*, freeze-drying

INTRODUCTION

Genus *Penicillium* bears tremendous economic significance in human affairs. It is highly desired to establish the taxonomic position of *Penicillium* isolates due to extensive use of their products in the food and feed industry. It produces extra cellular enzymes, isozymes, acids, metabolites and other commercial products. *Penicillium* is one of the commonest moulds causing multifarious infections worldwide. The maintenance and production of reliable pure cultures with desirable quality is the key operation and the first significant stage in the success of any fungal identification.

Long-term preservation of *Penicillium* species is essential for further detailed research. However, both the viability and the stability of living cells should be ensured during the preservation period. *Penicillium* belongs to the phylum *Ascomycota*, while its taxonomic characterization is still a matter of discussion [1] and the difficulties in identifying most *Penicillium* species requires multidisciplinary approaches. Clarification of species concepts in the genus *Penicillium* is supported mainly by morphological characteristics.

Fungal isolates were usually preserved in water at room temperature [2], an easy and economical procedure introduced for fungi [3]. However, the stability of fungal cells is not ensured by this simple procedure. Other methods have been suggested, such as preservation in soil or on oil or in culture slants, cryopreservation either in liquid nitrogen or at low temperature (-20 and -70°C) and lyophilization (the freeze-drying procedure)[2,3,4,5,6,7,8,9,10,11,12,13].

Cryopreservation in liquid nitrogen and lyophilization are recommended and used by the American Type Culture Collection [12]. Lyophilization is used for dehydrating vast range of materials, including foodstuffs, pharmaceuticals, biotechnology products, vaccines, diagnostics and biological materials. Lyophilization (also known as Freeze-drying, or cryodesiccation) is a dehydration process typically used to preserve a perishable material or make the material more convenient for transport.

Freeze-drying works by freezing the material and then reducing the surrounding pressure to allow the frozen water in the material to sublime directly from the solid phase to the gas phase. Since World War II (WWII) lyophilization has been used to stabilize living cells and fungal isolates. Lyophilized mycelia increase the storage and the quality of DNA extracted for molecular studies [14]. Maintenance of vigor and genetic characteristics of a pure strain in the form of a culture is the main objective of *Penicillium* culture preservation. Therefore, the present study was conducted to assess the suitability of lyophilization (freeze drying) as simple, inexpensive and reliable method to preserve fungal cultures on large scale for longer period of time.

MATERIALS AND METHODS

Microorganisms

Fifty isolates of *Penicillium* species were isolated from different sources (Table 1) directly and by serial dilution method. The soil samples from specific sources were thoroughly mixed and 25 g of each sample was suspended in 225 ml of sterilized distilled water (SDW) in 1:10 ratio and 10 ml of this suspension were added to 990 ml of SDW (1:1000).

These suspensions were stirred for 20 min before making 7-fold falling dilutions then; 1 ml of the desired suspensions (10^{-4}) was plated in triplicate on petri dishes containing 2% malt extract agar (MEA) composed of malt extract (20g) and agar (15g) in 1 L of distilled H_2O (pH 7.2). The suspension was evenly spread on the surface of medium by a sterile glass rod while rotating the petri dish. Soil plates were prepared in triplicate by uniformly distributing 0.5 g of soil directly by sprinkle method on the surface of 2% MEA medium. The plates were incubated at 26±2°C for six days before counting the growing fungal colonies. Individual fungal colonies were further isolated by sub-culturing onto new MEA plates.

Fungal isolates were purified and identified on the basis of morphological characteristics following protocols described by Pitt [15], and Domsch *et al*. [16]. Fungal isolates were collected in Punjab province from 2008 to 2010.

Preparation of the isolates for preservation

Spawn preparation: For the spawn preparation *Pennisetum glaucum* (Millet, Bajra) and *Triticum aestivum* (wheat) seeds were selected, washed and soaked in water overnight (18 hours). After removing excess water by spreading seeds on paper towel fifty grams of soaked seeds were sealed in each plastic bag and autoclaved. Sterilized seed bags were inoculated by fungal discs (8 mm) from 7 days old culture. Inoculated bags were kept at 25±2° C for 15 days for spawn formation and were preserved at 4° C for further studies (Fig 1a, b).

Figure 1: (a) & (b): Spawn formation on wheat grains

Lyophilization: Efficacy of lyophilization was evaluated by direct lyophilization of the fungal mat and lyophilization of the fungal spawn. For direct method 7-10 days old fungal colony mat (5-6 cm diameter) was used. Fungal mat was prepared in 2% Malt Extract (ME) broth. Each fungal mat was washed three times with distilled sterile water and dried in paper towel for two minutes. Fungal mat was transferred into sterile falcon tube for lyophilization. Neither stabilizer nor skimmed milk was used in fungal material to protect it from sticking with the walls of the container. Nylon mesh (200µm) was used in order to close the mouth of falcon tube. Falcon tubes were placed in the vacuum bottles. Fungal spawn was directly lyophilized by placing spawn bags in vacuum bottles. Material was lyophilized in freeze dryer (TFD5505, Ilshin, Korea) under vacuum at ≤ 50 °C (Fig. 2).

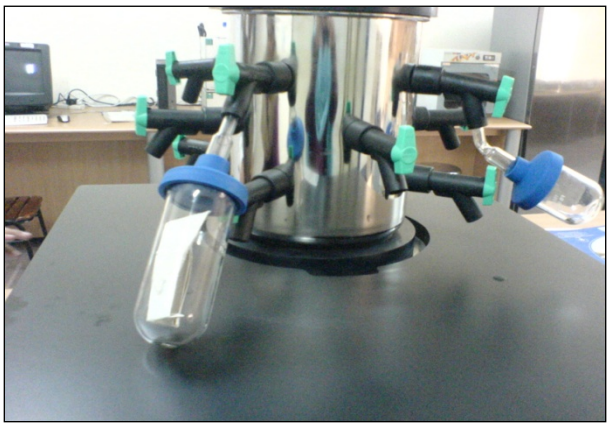

Figure 2: Lyophilization

The lyophilizer was allowed to run for 6-8 hours. Samples were removed from vacuum bottles when pressure reached at 25 bar. Lyophilized samples were kept at 4 ^{o}C and room temperature (28 ^{o}C). After 1, 2, and 3 months, viability of lyophilized and spawn cultures were checked on MEA medium (Fig. 3). Each fungal isolate was considered viable if the rate of growth present was the same as that of the original culture and if the morphology of the colony matched the fungal identification documented for each species.

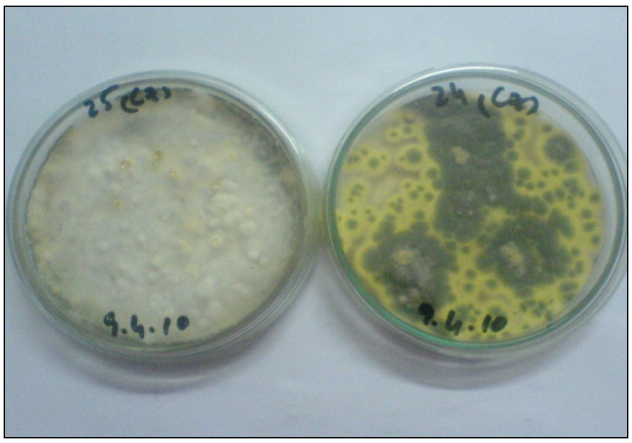

Figure 3: Revival of lyophilized *Penicillium* spp.

RESULTS

Thirty two *Penicillium* species were isolated from 25 variable soil and other samples of different types of plants parts, growing in ten districts of Punjab, Pakistan. Three species belong to subgenus *Aspergilloides*, twelve of *Furcatum*, fourteen species of *Penicillium* and three species belong to subgenus *Biverticillium*. Overall prevalence showed that *Penicillium oxalicum* was the most frequent species with six isolates followed by *P. simplicissimum* (4 isolates), *P. implicatum* and *P. melinii* (3 isolates each). Whereas *P. canescens*, *P. italicum*, *P. atrovenetum*, *P. granulatum P. expansum* and *P. chrysogenum* were there with two isolates, respectively. On the other hand the rest of the species were isolated once from different samples amongst the genus *Penicillium*.

The viability and the purity of the fungal species by fungal spawn and lyophilized fungal mat were monitored immediately after storage, at 1, 2 and 3 months at 4 ^{o}C. The fungal isolates were compared in terms of rate of growth, colony morphology and sporulation after rejuvenation. Each of the isolates was also matched with the parent cultures for the above said features. The cultures isolates were subcultured for verification of their viability. It was recorded that all lyophilized *Penicillium* species were viable after 1-3 months of storage. The rate of recovery and percentage viability was equally good for fungal isolates recovered from both type of lyophilized material, fungal spawn and fungal mat showing complete compatibility with the initial colony characteristics and growth rates.

DISCUSSION

The genus *Penicillium* is considered as a group of "opportunistic fungi" being found everywhere; it is unavoidable in life. The purpose of present study was to isolate species from climatically diverse zones of Punjab and long term preservation of the species of *Penicillium* so that they may be used for teaching, research and industry.

Storage procedures and recovery methods exhibit considerable differences in response to stresses by different taxonomic groups and even strains within a given species [17]. Important parameters such as rate of cooling, size of propagules, and thickness of cell wall [18, 19, 20] are not considered in this study and they may explain intra and interspecific variability of fungal cultures after different storage periods.

Several methods have been used for long term preservation of fungus cultures [5, 21, 22]. The appropriate selection and success of preservation method varies with the fungal species being preserved. Studies confirm that lyophilization is much simpler, effective and convenient for preservation of fungal cultures in the laboratory at large scale as compared to conventional freeze-drying methods [23, 24, 25]. Viability of lyophilized cultures has also been reported 100% with minimal cultural variations [26]. Modified method of *Penicillium* spawn preservation by lyoplilization has been found cost effective in contrast to liquid nitrogen preservation.

CONCLUSION

The present study has shown that fungal spawn lyophilization is much easier, convenient, economical, and effective tool for the long term preservation of fungal isolates. In case of other microscopic and macroscopic fungi, longer monitoring of preserved isolates and storage will be further validated for the reliability of freeze dry preservation. Furthermore, stability of fungal cells will also be assessed through molecular parameters.

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Table 1: Isolation of *Penicillium* species from different substrates

Sr.No.	FCBP NO.	*Penicillium* species	Substrate
01	FCBP-IK-1003	*Penicillium* sp.	*Malus domestica*
02	FCBP-IK-1010	*Penicillium oxalicum*	Rhizosphere of *Hibiscus esculenta*
03	FCBP-IK-1028	*Penicillium canescens*	Rhizosphere of *Eucalyptus camaldulensis*
04	FCBP-IK-1029	*Penicillium simplicissimum*	Rhizosphere of *Psidium guajava*
05	FCBP-IK-1030	*Penicillium italicum*	Rhizosphere of *Pennisetum americanum*
06	FCBP-IK-1037	*Penicillium canescens*	Rhizosphere of *Dalbergia sissoo*
07	FCBP-IK-1038	*Penicillium oxalicum*	Effluent of Amar Fabrics
08	FCBP-IK-1039	*Penicillium* sp.	Rhizosphere of *Vitis vinifera*
09	FCBP-IK-1040	*Penicillium atrovenetum*	Rhizosphere of *Eucalyptus camaldulensis*
10	FCBP-IK-1041	*Penicillium verrucosum*	Effluent of Amar Fabrics
11	FCBP-IK-1050	*Penicillium steckii*	Effluent of Amar Fabrics
12	FCBP-IK-1052	*Penicillium verrucosum v cyclopium*	Effluent of Textile industry
13	FCBP-IK-1057	*Penicillium lanosum*	Effluent of Textile industry
14	FCBP-IK-1062	*Penicillium sp.*	Effluent of Textile industry
15	FCBP-IK-1066	*Penicillium implicatum*	Effluent of Textile industry
16	FCBP-IK-1067	*Penicillium granulatum*	Effluent of Pak polyester
17	FCBP-IK-1069	*Penicillium sp.*	Bread
18	FCBP-IK-1075	*Penicillium oxalicum*	Rhizosphere of *Dalbergia sissoo*
19	FCBP-IK-1080	*Penicillium granulatum*	*Malus domestica*
20	FCBP-IK-1082	*Penicillium digitatum*	Effluent of Amar Fabrics
21	FCBP-IK-1083	*Penicillium rubrum*	*Punica granatum*
22	FCBP-IK-1100	*Penicillium citrinum*	Bread
23	FCBP-IK-1101	*Penicillium simplicissimum*	*Allium cepa*
24	FCBP-IK-1102	*Penicillium expansum*	*Allium sativum*
25	FCBP-IK-1106	*Penicillium bilaii*	*Punica granatum*
26	FCBP-IK-1107	*Penicillium rolfsii*	Effluent of Amar Fabrics
27	FCBP-IK-1108	*Penicillium italicum*	Lemon
28	FCBP-IK-1109	*Penicillium implicatum*	*Punica granatum*
29	FCBP-IK-1110	*Penicillium oxalicum*	Rhizosphere of *Vitis vinifera*
30	FCBP-IK-1111	*Penicillium expansum*	Pods of *Albizia procera*
31	FCBP-IK-1112	*Penicillium simplicissimum*	*Vitis vinifera*
32	FCBP-IK-1113	*Penicillium atrovenetum*	Canal water
33	FCBP-IK-1114	*Penicillium simplicissimum*	*Zingiber officinale*
34	FCBP-IK-1115	*Penicillium rugulosum*	Effluent of Glass industry
35	FCBP-IK-1116	*Penicillium oxalicum*	Air
36	FCBP-IK-015	*Penicillium oxalicum*	Soil
37	FCBP-IK-016	*Penicillium claviforme*	*Citrus sinensis*
38	FCBP-IK-017	*Penicillium miczynskii*	*Citrus sinensis*
39	FCBP-IK-018	*Penicillium implicatum*	*Allium cepa*
40	FCBP-IK-019	*Penicillium melinii*	*Allium cepa*
41	FCBP-IK-022	*Penicillium chrysogenum*	*Allium cepa*
42	FCBP-IK-024	*Penicillium viridicatum*	Bread
43	FCBP-IK-025	*Penicillium melinii*	Bread
44	FCBP-IK-026	*Penicillium chrysogenum*	Orange
45	FCBP-IK-027	*Penicillium griseofulvum*	Bread
46	FCBP-IK-028	*Penicillium spinulosum*	*Punica granatum*
47	FCBP-IK-029	*Penicillium janthinellum*	Rhizosphere of *Citrus sinensis*
48	FCBP-IK-030	*Penicillium melinii*	Rhizosphere of *Citrus sinensis*
49	FCBP-IK-031	*Penicillium waskmanii*	Rhizosphere of *Citrus sinensis*
50	FCBP-IK-032	*Penicillium velutinum*	Rhizosphere of *Citrus sinensis*

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