



Preservation of Indigenous Fungal Cultures by Freeze Drying Technique using Skim Milk and Honey as the Protectants

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Author's contribution

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

COVID-19 pandemic movement restrictions as part of the control measures put in place by countries in Sub-Saharan Africa (SSA) has implications on many sectors which include very high costs of importing materials owing to high exchange rates as well as non-availability of microbial cultures for food/ industrial productions, medical/academic research and applications because of fear of new microbial infections and bio-terrorism. Overcoming these challenges in SSA requires developing better policies and packages to confront the challenges by way of rebooting their strategies and policies for sustainable economic growth through reawakening the potentials they have. The maintenance and production of reliable pure microbial cultures with desirable quality is a key operation and is the first significant stage in the success of fungal applications in any scientific venture where they are used. For many years the process of freeze-drying (lyophilisation) has been employed primarily with the purpose of preserving living materials, including microorganisms, for extended periods of time; this is possible because, upon dehydration, substances no longer change as a consequence of the usual turnover of metabolic reactions characteristic of the living condition. This study assessed lyophilization (freeze drying) of some indigenous filamentous fungal cultures (Spores) using skim milk and honey (supports being maize and cassava flours) as the

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protectants and then critically compared the two protective media for the preservation of active filamentous fungi spores for the first time, and from the data obtained, their applications were optimised. Some fungal isolates (*Aspergillus* sp., *Mucor* sp., *Penicillium* sp., *Fusarium* sp., *Rhizopus* sp., *Cladosporium* sp., *Alternaria* sp. and *Geotricum* sp.) previously purified and identified were obtained from the Products Development Unit, National Biotechnology Development Agency, Abuja, Nigeria and were re-validated where their viability and purity were confirmed. The samples were Freeze Dried in the respective medium and monitored immediately after. After the freeze-drying, the residual water contents were between 0.20-0.32%, 0.39-0.49% and 0.33-0.41%, respectively, with skim milk, Honey with Cassava Starch and honey with Maize Starch. The dried spore viabilities were between 96% (*Aspergillus* spores in Reconstituted Skim Milk) and 21% (*Geotricum* spores in Honey with Cassava Starch). Among the protective agents used, skim milk was found to be the best protectant. 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 fungal species were found viable from both type of lyophilized materials, skim milk and honey and showed the initial colony characteristics and growth rates.

Keywords: Lyophilization; skim milk; honey; freeze-drying; fungi; spore.

1. INTRODUCTION

Fungal spores preservation are important means of recovering the fungal mycellium when they are needed in academic or industrial laboratories as well as in organised microbial (mycology) culture collection centres [1]. For instance, many fungal genera in the mycology kingdom, bears tremendous economic significance in human affairs. It is highly desired to establish the taxonomic position of the isolates due to extensive use of their products in the food and feed industry. They produce extracellular 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. Culture collections are important genetic resources that are becoming increasingly valuable to industry and they must employ techniques which retain both the viability and stability of the organisms [2]. The organisms can then be used with confidence for reference purposes, taxonomic comparison, for future experimental and industrial work and confirmation of results.

Fungal isolates were usually preserved in water at room temperature, 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).

Freeze drying technique 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. As such, long-term maintenance of industrial and medically important fungal species is essential for detailed studies and to avoid wasting time on re-isolation of already isolated and characterised cultures [4].

Although freeze-drying is widely used in production of stable microbial cultures, however, during such a treatment, cells or spores are exposed to freezing and drying processes that subject them to the stresses of high concentration of solutes including extremes of pH, low temperature, the formation of ice crystals and the removal of water from the cell which might cause damage, protein and deoxyribonucleic acid (DNA) denaturation, and resulting in low survival [5]. Survival during freeze-drying process is dependent on many factors, one of which is the protective suspension medium used. Protective agents play an important role in the conservation of viability. A good protectant could be easily dried and provide cryoprotection to the cells during the freezing process and also be a good matrix to allow stability and ease of rehydration. Various groups of substances including polyols, polysaccharides, disaccharides, amino acids, proteins, minerals,

salts of organic acids and vitamins-complex media have been tested for their protective action [6]. However, protection of a given additive depends on the type of organism and the nature of the protectant used.

This study examines some protectants; skim milk and honey as a means of preserving fungi spores, assesses their suitability and effectiveness.

2. MATERIALS AND METHODOLOGY

2.1 Preparation of Fungal Spores

Fungal isolates were isolated, purified and identified on the basis of morphological characteristics following protocols described earlier [7].

The fungal spores were there after prepared by growing fungi under conditions that induced maximum sporulation so that sufficient spores were available for the experiment [8].

The spore suspensions were made by transferring loopful of spores from the sporulated cultures into sterilised normal saline (0.85% NaCl) with Tween80 (to keep spores dispersed) [9] and was resuspended in the same solution in order to obtain a 20× concentration factor. The initial spore concentration was then calculated by determining the optical density at wavelength of 650 nm (this wavelength gives the best correlation between the optical density of the culture and the total number of spores it contains).

Skim milk solution was prepared by adding 20g milk to 80ml of sterile distilled water (20% solution) and autoclaved at 116°C for 20 minutes in 10 mL tubes. The milk solution was stored at 4°C until needed [10].

The Honey solutions were prepared with two different flours (maize flour and cassava flour) as supports. They were purchased from local markets in FCT, Abuja. Two grams (2 g) of each flour were mixed with 50 mL distilled water and heated to 70–80°C for 20–30 min under agitation and then cooled to 30–40°C. The final concentrations were made by adding the honey solution and the respective flours in ration 1:1.

Five (5ml) of the suspension were introduced into each freeze drying bottles for freeze-drying. While waiting for further processing, the set-up was refrigerated at 4°C.

2.2 Freeze-drying Protocol

Fungal spores with the protectants were directly lyophilized in the cotton-plugged bottles using a benchtop Freeze dryer ((TFD5505, Ilshin, Korea) under vacuum at ≤ 50 °C). The lyophilizer was allowed to run for 6-8 hours. Samples were removed from vacuum bottles when pressure reached 25 bar. Lyophilized samples were kept at 4°C and room temperature (28°C) [11].

2.3 Recovery

After FD, viability of lyophilized spores was checked on PDA medium. 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.

The bottles were opened aseptically and, using sterile spatula, transfer 1g of the contents of the preparation to approximately 5 mL of sterile normal saline in a test tube. The contents were allowed to rehydrate for at least one hour then one drop was transferred to a sterile agar. The same media and growth conditions were used for isolation, maintenance and recovery process [12].

2.4 Determination of Water Content

The water content was determined using the modified method described earlier [13]. A known weight of foil was made (m_1). The foil was reweighed containing 1g of freeze dried powder (m_2). The foil and sample were dried in the oven at $103 \pm 2^\circ\text{C}$ for 18–20 h, cooled in the desiccators before weighing, re-dried for 1 h, and reweighed until a constant weight was obtained (m_3). Calculation:

$$\text{Water Content (\%)} = \frac{m_3 - m_2}{m_1 - m_2} \times 100$$

2.5 Determination of the Viable Counts of Freeze-Dried Fungal Spores

An 0.1 g sample of freeze dried powder was rehydrated with buffered 0.1% peptone water (BIO-RAD, France) followed by gentle shaking at room temperature for 20 min. The viability was determined using a pour plate method and the spore suspension was then spread onto PDA-Chloramphenicol agar plates. After cultivated at 30°C for 72 h, viable spores were enumerated and compared with the initial count (before

freeze-drying). All experiments were performed in duplicate. The percentage survival after freeze-drying was calculated as follows:

$$\text{Viability} = \frac{N - N_0}{N + N_0} \times 100$$

Where N and N₀ represent the viable counts (spores/g) after freeze-drying and the initial count (spores/g) before freeze-drying, respectively [14].

3. RESULTS

Table 1 shows the viability and recovery percentage among the fungal spore isolates. The highest recovery percentage was found in Aspergillus using skim milk (96%), followed by Honey with maize starch (87%) and lastly, Honey with cassava starch (83%). The lowest recovery percentage was found in Geotricum where only 32% recovery were recorded with skim, 27% with honey with maize starch and 21% with honey with cassava starch.

The moisture content varied from 0.46% (highest) using HCS to 0.20% (lowest) with skim milk.

Fig.1 shows the moisture percentages of the freeze dried spore samples. The moisture content of the samples preserved using honey with cassava starch consistently had high moisture content, followed by honey with maize starch then skim milk had the lowest moisture content.

Fig. 2 shows the recovery pattern of the freeze dried samples. Skim milk fungal samples consistently had the highest recovery percentages followed by honey with maize starch and lastly, those preserved with honey with cassava starch.

4. DISCUSSIONS

The viability and the purity of the lyophilized fungal spores were monitored immediately after

Freeze drying. 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 species were viable after 1 month of storage. The rate of recovery and percentage viability was equally good for fungal isolates recovered from both type of lyophilized material, all the fungi showed complete compatibility with the initial colony characteristics and growth rates.

Several methods have been used for long term preservation of fungus cultures [4]. 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. Viability of lyophilized cultures has also been reported 100% with minimal cultural variations [6].

Modified method of Penicillium spore preservation by lyophilization has been found cost effective in contrast to liquid nitrogen preservation.

The present study has shown that fungal spore lyophilisation using skim milk and honey respectively as protectants 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 spores and storage should be further validated for the reliability of freeze dry preservation. Furthermore, stability of fungal spores may also need to be assessed through molecular techniques. These studies showed that fungal spores survived intracellular ice formation and death correlated with freeze induced shrinkage.

Table 1. Viability and moisture contents of FD (Spore) Products

Spore Samples	Protectant	Moisture Content (%)	Recovery (%)
Aspergillus	RSM	0.32	96
	HMS	0.41	87
	HCS	0.46	83
Mucor	RSM	0.28	91
	HMS	0.35	76
	HCS	0.40	65
Pencillium	RSM	0.25	94
	HMS	0.39	68
	HCS	0.44	64

Spore Samples	Protectant	Moisture Content (%)	Recovery (%)
Fusarium	RSM	0.24	85
	HMS	0.36	83
	HCS	0.39	78
Rhizopus	RSM	0.20	96
	HMS	0.37	54
	HCS	0.49	36
Cladosporium	RSM	0.22	78
	HMS	0.33	56
	HCS	0.39	49
Geotricum	RSM	0.26	32
	HMS	0.38	27
	HCS	0.41	21

Key: RSM= Reconstituted Skim Milk; HMS= Honey+Maize Starch; HCS=Honey+Cassava Starch

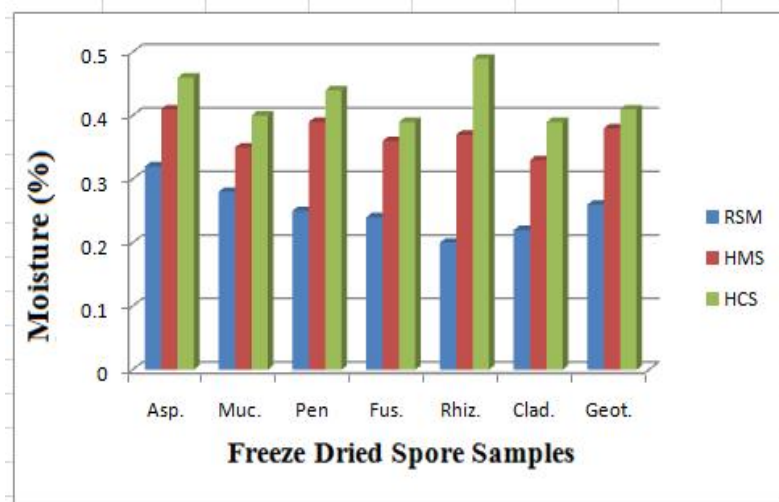


Fig. 1. Moisture (%) of FD spores using the protectants

KEY: RSM=Reconstituted Skim Milk; HMS= Honey+Maize Starch; HCS= Honey+Cassava Starch

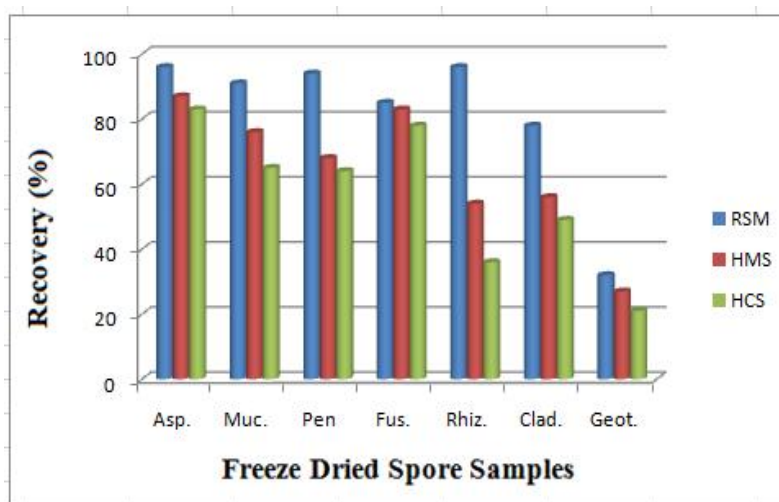


Fig. 2. Recovery (%) of FD spores using the protectants

KEY: RSM=Reconstituted Skim Milk; HMS= Honey+Maize Starch; HCS= Honey+Cassava Starch

I was found that the final residual water content must be between 1 and 5 for most microorganisms to retain viability. This was achieved in this study. However, there still remain isolates of fungi that can be frozen successfully but do not survive dehydration.

5. CONCLUSIONS

It was concluded based on the knowledge acquired in the study that lyophilization is simple, inexpensive, reliable and effective method for the long term preservation of many fungal isolates using their spores and appropriate protectants such as skim milk and honey.

Although, there are a large number of different techniques, mainly due to the large number of variables participating in the process, such as velocities of freezing, lowest temperature attained, type of protective colloids, suspending medium, storage temperature, storage atmosphere, residual moisture, reactivation conditions, etc., however, if the procedures have been correctly handled, the final result is that the water content of the samples under treatment will be only a fraction of a percent of the original preparation.

As opposed to the destructive action of drying from the liquid state, the removal of water from material previously frozen under proper conditions, allows the maintenance of three major characteristics of the specimen: morphology, solubility and chemical integrity. These were also achieved in this study.

The maintenance of vigour and genetic characteristics of a pure strain in the form of a culture is the main objective of any microbial culture preservation including those of fungi. Therefore, the present study was found suitable because it is simple, inexpensive and reliable method for the preservation of fungal cultures on large scale for longer period of time.

6. RECOMMENDATIONS

Time was not enough time for an extended length of storage of the freeze dried spores, therefore, more storage times up to six months is recommended for the studies on the effects of time on the stability of freeze dried spores.

Also, there is need to further study the specific effects of honey and the respective carriers on

the protective roles played in the freeze drying processes

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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