ORIGINAL ARTICLE

CHARACTERIZATION OF INDOOR MOLDS WITH ALLERGENIC POTENTIAL AFTER CRYOPRESERVATION AT -20°C FOR ONE YEAR

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| Received November 22, 2020 | | Accepted December 07, 2020 | | Published December 14, 2020 | | ID Article| M'boh–Ref3-ajira221120|

ABSTRACT

Background: The cryopreservation for long period of mold strains of medical interest essential for research is complex. Our objective is to evaluate the impact of conservation with 10% of Glycerol and Brain Heart Infusion (BHI Broth) on the survival of molds of medical interest at -20°C. **Methodology**: This is an analytical study of the cultural and morphological characteristics of 1310 mold strains isolated from environmental samples (air, surface and dust) collected from all azimuths and preserved in 10% Glycerol and BHI Broth at -20°C for one year. **Results**: Among the 1310 isolated strains and revived, colony growth was observed in 1059 strains (80.84%) against 251 (19.16%) which showed no signs of development and 119 strains (9.08%) were contaminated by other mold species or yeasts. Fungal viable strains observed after cryopreservation are: *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus versicolor, Aspergillus niger, Aspergillus terreus, Aspergillus ochraceus, Aspergillus clavatus, Aspergillus sp, Penicillium sp, Fusarium solani, Fusarium sp, Curvularia sp, Mucor sp, Rhizomucor sp, and Trichoderma sp.* **Conclusion**: The ability to resist freezing in suspension medium 10% Glycerol and BHI Broth varied from strain to strain, but with a better cryopreservation for *Aspergillus, Penicillium, Fusarium, Mucor, Rhizomucor and Trichoderma.*

Keywords: *Characterization, Mould, Environnement, Health, cryopreservation, Glycerol, BHI Broth*

1. INTRODUCTION

Molds are eukaryotic, filamentous and multicellular microscopic fungi. They are heterotrophic and immobile thallophytes that live in symbiosis or are parasites of plants and animals including humans [1]. They have important lytic properties (cellulosic, pectinolytic, amylolytic, proteolytic and lipolytic). These properties allow them when the physicochemical conditions are adequate (Temperature ranging from - 05 to over 50°C, Humidity over 50% and pH around 4, 5 to 8) [2], to grow on any type of substrate in indoor environments (wood, paint, plaster, carpet) [2].
They are therefore able to metabolise and assimilate sugars such as glucose, maltose, saccharose and polymers such as cellulose, starch, peptides and proteins [3,4]. Their proliferation in indoor environments poses allergic and/or infectious problems that require their isolation, identification and conservation in medical environments for a better diagnosis and a more elaborate treatment. However, the long-term conservation of molds in culture is difficult, especially since the media in Petri dishes and dry tubes over time without ignoring the concerns of contamination of cultures [5,6]. Cryopreservation is an alternative to the problems of preserving mold strains because this process allows the preservation of fungal cells by cooling them down to very low temperatures in the presence of cryoprotectant such as glycerol. At these extremely low temperatures, all biological activity is suspended, including biological reactions that would cause cell death, thus keeping the fungal cells alive for several years [7,8]. Thus, the objective of this project is to evaluate the effect of cryopreservation at -20°C on the survival of fungi suspended in 10% glycerol and Brain Heart Infusion (BHI Broth).

2. MATERIALS AND METHODS

2.1. Microorganisms samples

Mold strains (1310) were isolated from a potentially allergenic indoor environment in samples (air, surfaces and soil dust) and used for conservation testing.

2.2 Mold Isolation

The strains the molds have been isolated by culturing the samples (air, surfaces and soil dust) on Sabouraud chloramphenicol agar and incubated at 27°C for 5 to 7 days.

2.3 Replicating and purification

Each isolated colony has been revived on a new medium from Sabouraud chloramphenicol agar to obtain a pure strain, with a sufficient growth for characterization and identification. Transplantation has been made by taking a

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colony fragment from the Bunsen burner using a single-use sterile Pasteur pipette. This fragment was placed in the center of a new Petri dish incubated for 5 to 7 days.

2.4 Identification

The identification of the different strains was done according to cultural (macroscopic) and morphological (microscopic) criteria. Each strain was then subjected to a morphological identification and carried out by microscopic observation between slide and lamina at magnifications 10 and 40 X.

2.5. Cryopreservation of strains

The pure strains characterized were preserved in 10% Glycerol and BHI Broth. 3 to 4 cut of the mycelium were collected and transferred into 2 ml cryotubes containing 1.5 ml of 10% glycerol and BHI Broth from the Bunsen burner. Each strain was stored in triplicate and the cryotubes were subsequently placed in a freezer at -20°C for one year.

2.6 Culture and characterization of cryopreserved isolates after 1 year of storage

A cryotube of each preserved strain was taken out and then left to thaw at +4°C in the refrigerator before to be transferred at the laboratory temperature +18°C for transplantation of the isolates. A square of mycelium was recovered from each cryotube using a sterile single-use Pasteur pipette and then transferred to new agar in a Petri dish from the Bunsen burner. All manipulations were performed in a sterile environment in order not to contaminate our samples and not to falsify our results. Then the Petri dishes were incubated for 7 to 10 or even 14 days at 27°C. The characterization is based on macroscopic criteria (cultural characteristics) and microscopic criteria (morphological characteristics).

3. RESULTS

3.1. Cryopreserved mold strains

The cryopreserved strains have been carefully documented according to their cultural (macroscopic) and morphological (microscopic) characteristics. The specific identification keys have allowed the conservation of the following fungal strains: *Aspergillus fumigatus, Aspergillus flavus, Aspergillus versicolor, Aspergillus niger, Aspergillus terreus, Aspergillus ochraceus, Aspergillus clavatus, Aspergillus sp, Penicillium sp, Fusarium solani, Fusarium sp, Curvularia sp, Mucor sp, Rhizomucor sp, Aureobasidium sp, Scytalidium sp, Trichoderma sp* and *Unknown species* (Table Ia et Ib).

Tableau Ia: cultural and morphological characteristics of cryopreserved *Aspergillus species.*

Replicated species	Effectif	Macroscopic characters	Microscopic characters
		Colour front and back; Texture; Relief; Size; Presence of a diffusing pigment in the agar.	Fructification organs; thallus; colour of hyphae; origin of spores; shape of spores; appearance of spores and other details.
Aspergillus fumigatus	96	Colour front: white, blue-green, green, grey, black; Colour back: colourless, yellow, brown; Texture: powdery; Relief: flat, pleated; Size: small, extensive,	*Conidiophore with a column head; thallus: septate; hyaline; spore: exogenous; subspherical; Amerospores*
Aspergillus flavus	94	Colour front: white, green, green-yellow; Colour back: colourless, yellow, ochre, brown, orange; Texture: powdery with sometimes black grains, woolly; Relief: flat, pleated; Size: extensive	*Conidiophore with a radial head; thallus: septate; hyaline; spores: exogenous; subspherical; Amerospores; rough stipe*
Aspergillus versicolor	35	Colour front: white, green, pink, red, yellow; Colour back: colourless, yellow, brown; Texture: fluffy, relief: cerebriform; Size: small	*Conidiophore with a radial head; septate thallus; hyaline; spores: exogenous; subspherical; Amerospores*
Aspergillus niger	55	Colour front: white, yellow, black, Colour back: colourless, yellow, brown, black; texture: granular; relief: flat, pleated; size: extensive	*Conidiophore with a radial head; thallus: septate; hyaline, melanized; spores: exogenous; subspherical; Amerospores ; smooth stipe*
Aspergillus terreus	12	Colour front: white, yellow, ochre; Colour back: yellow, brown; texture: powdery; relief: flat; size: small, extensive; agar-diffusing pigment for some strains	*Conidiophore with a column head; thallus: septate; hyaline, brown; spore: exogenous; subspherical; Amerospores*
Aspergillus ochraceus	14	Colour front: white, yellow, ochre; Colour back: colourless, yellow, brown; Texture: grainy with sometimes yellow grains; Relief: flat, pleated; Size: extensive	*Conidiophore with a radial head; thallus: septate; hyaline, brown; spores: exogenous; subspherical; Amerospores ;*
Aspergillus clavatus	1	Colour front: white, green; Colour back: yellow; texture: velvety; relief: pleated; size: small	*Conidiophore with a radial head; thallus: septate; hyaline; spores: exogenous; subspherical; Amerospores ;*
Aspergillus sp	177	Colour front: white, green, grey, brown; Colour back: colourless, yellow, brown; Texture: fluffy, flaky; Relief: flat, pleated; Size: small, extensive	*Conidiophore with a radial head; thallus: septate; hyaline; spores: exogenous; subspherical; ellipsoid ; Amerospores*

Tableau Ib: Cultural and morphological characteristics of other species and unknown species cryopreserved.

Replicated species	Effectif	Macroscopic characteristics	Microscopic characteristics
		Colour front and back; Texture; Relief; Size; Presence of a diffusing pigment in the agar.	Fructification organs; thallus; colour of hyphae; origin of spores; shape of spores; appearance of spores and other details.
Penicillium sp	267	Colour front: white, blue-green, green, grey; Colour back: colourless, yellow, green, red, brown; Texture: fluffy, powdery; Relief: flat, pleated, cerebriform; Size: small, extensive; Agar diffusing pigment for some strains	*Brush-branched conidiophore (monoverticulate, biverticulate, triverticulate, tetraverticulate); thallus: septate; hyaline; spore: exogenous; subspheric, ovoid; Amerospores*
Fusarium solani	5	Colour front: white to cream, beige; Colour back: colourless, yellow; Texture: fluffy; Relief: flat; Size: extensive,	Single and/or branched conidiophore; septate thallus; hyaline; spore: exogenous; spindle-shaped, cylindrical; *amérospore ; scolecospore* the presence of chlamydospores
Fusarium sp	157	Colour front: white, beige, yellow, orange, red, pink, violet, green; Colour back: colourless, yellow, orange, red, pink, violet, brown; texture: fluffy; relief: flat; spread, agar-diffusing pigment for some strains	*Branched conidiophore with sporodochia; thallus: septate; hyaline; spore: exogenous; subspherical, cylindrical; Amerospores ; the presence of chlamydospores in some strains*
Curvularia sp	60	Colour front: white, brown, light brown, black; Colour back: dark brown, black; Texture: velvety, fluffy; Relief: flat; extensive,	*Simple and/or branched conidiophore; thallus: septate; brown; spore: exogenous; cylindrical ; phragmospore,*
Mucor sp	17	Colour front: white, light brown; Colour back: colourless; Texture: woolly; Relief: flat; Size: extensive	*Branched sporangiophore with an absence of rhizoids; thallus: siphon; hyaline; spores: endogenous; ovoid; amerospore; absence of apophysis; the presence of chlamydospores*
Rhizomucor sp	28	Colour front: white, brown, black; Colour back: colourless; Texture: cottony; Relief: flat; Size: pervasive	*Branched sporangiosphore with presence of rhiziodes; thallus: siphonate; brown; spores: endogenous; ovoid; amerospore; absence of apophysis*
Aureobasidium sp	2	Colour front: brown; Colour back: brown; Texture: mucoid; Relief: flat; Size: extensive	*Simple and/or branched conidiophore; septate thallus; melanized; spores: exogenous; cylindrical; amerospore; didymospore; presence of chlamydospores*
Scytalidium sp	1	Colour front: black; Colour back: black; texture: fluffy; relief: flat; size: extensive	*Conidiophore absent; thallus: septate; melanized; spores obtained by fragmentation; cylindrical; amerospore*
Trichoderma sp	1	Colour front: white, green; Colour back: colourless; Texture: flaky; Relief: flat; Size: extensive	*Simple and/or branched conidiophore; thallus: septate; hyaline; spores: exogenous; subspherical; amerospore*
Unknown species	288	Colour front: white, green, grey, black, brown, salmon; Colour back: colourless, yellow, black, brown; Texture: flaky, woolly, fluffy, fluffy, cottony; Texture: powdery, granular; Relief: flat; pleated; Size: small, extensive, pervasive	*Conidiophore absent, simple and/or branched conidiophore; thallus: septate, siphonate; hyaline, melanized; spores: exogenous; obtained by fragmentation; subspherical, ellipsoid, cylindrical, pyriform; amerospore; phragmospore,*

3.2 Characteristics of strains replicated after 1 year of storage

Of the 1310 strains revived, colony growth was observed in 1059 strains (80.84%), however, 251 strains (19.16%) showed no signs of development and other mold species or yeasts contaminated 119 strains (9.08%). The cultural and morphological characteristics of the colonies of the viable strains were identical to those before conservation

(Tables II and III). However, some strains of *Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger, Aspergillus terreus, Aspergillus ochraceus* and *Aspergillus sp* with powdery or granular textures produced very few spores up to 10-14 days of culture after cryopreservation and had a downy texture and whitish coloration that varied very slightly from the colour of the original strain. All cultures of the strains *Aspergillus, Penicillium, Fusarium, Trichoderma, Mucor* and *Rhizomucor* developed after one year of cryopreservation in 10% glycerol and BHI Broth at -20 °C. The strains, which showed no signs of development, were mostly melanized fluffy strains belonging to the genera: *Scytalidium, Curvularia* and *Unknown species* (Table IV).

Among the viable and non-viable strains, a few strains are contaminated during the year of conservation; these are respectively in a decreasing way the unknown species (3.59%), and other species such as *Aspergillus* (3.43%), *Fusarium* (1.60%), *Rhizomucor* (0.38%) and *Curvularia* (0.08%).

Table II: Macroscopic characteristics of some fungal strains revived after cryopreservation at -20°C for 1 year.

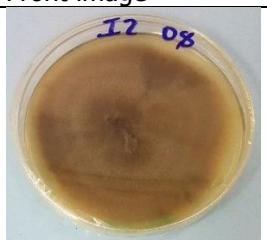
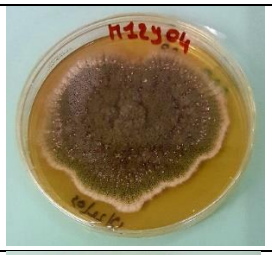
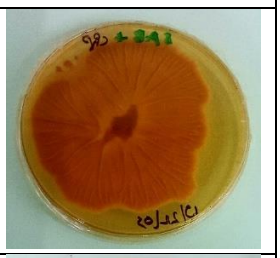
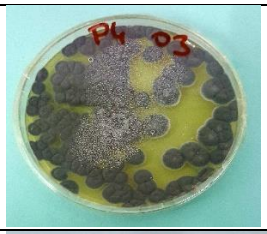
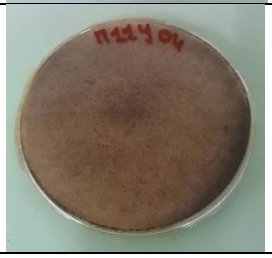
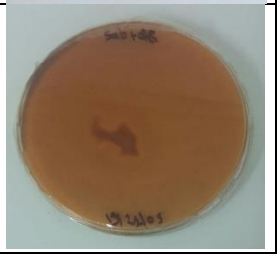
Species / Macroscopic characteristics strains	Culture photography	
	Front image	Back Image
Species: *Curvularia sp* Macroscopic characteristics: Light brown colony on the reverse side and dark brown on the reverse side, fluffy, flat and extensive.		
Species: *Aspergillus flavus* Macroscopic characters: Yellowish green colony with white outline on the front, ochre to brown on the back, powdery, pleated and extensive.		
Species: *Aspergillus niger* Macroscopic characters: Colony black on the front and ochre on the back, granular, pleated and extensive.		
Species: *Penicillium sp* Macroscopic characters: Dark green colony with white outline on the front and yellow on the back, downy, flat and small.		
Species: *Fusarium sp* Macroscopic characteristics: Colony is pale pink and white on the front and orange-yellow on the back, downy, flat and extensive.		
Species: Rhizomucor sp Macroscopic characters: Colony grey to black on the front and colourless on the back, cottony, flat and pervasive.		

Table III: Microscopic characteristics of some fungal strains revived after cryopreservation at -20°C for 1 year.

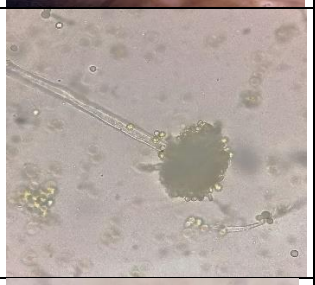
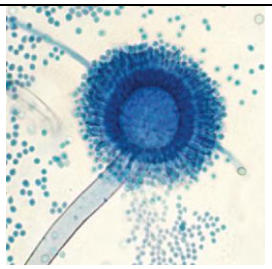
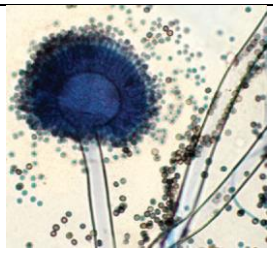
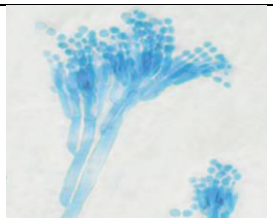
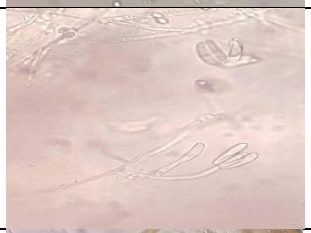
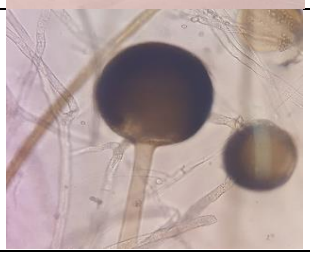
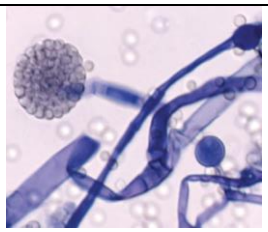
Species / Microscopic characteristics strains	Our Fungal Species aspect pictures (optical microscopy 40X)	Reference Picture
Species: *Curvularia sp* *simple and/or branched conidiophore; septate thallus; melanized; exogenous spores; cylindrical; phragmospore*		 (9)
Species: *Aspergillus flavus* *conidiophore with radial head; septate, rough, hyaline stipe; spore exogenous, subspherical, amerospore*		 **(9)**
Species: *Aspergillus niger* *conidiophore with a radial head; septate, smooth, thick-walled, hyaline stipe; spore exogenous, subspherical, amerospore*		 **(9)**
Species: *Penicillium sp* *biverticulate brush-branched conidiophore; septate thallus; hyaline; exogenous; subspherical spores; amerospore*		 (9)
Species: *Fusarium sp* *branched conidiophore with sporodochia; septate thallus; hyaline; exogenous spores; cylindrical; amerospore*		 (9)
Species: *Rhizomucor sp* *branched sporangiosphore with presence of rhiziodes; siphonated thallus; melanized; endogenous spores; ovoid; amerospore; absence of apophysis*		 (9)

Table IV: Analysis of strains revived after cryopreservation

Mold strains	Viable strains (%)	Non-viable strains (%)
Aspergillus species	36.95	0
Penicillium species	20.38	0
Fusarium species	12.37	0
Curvularia species	0.30	4.27
Mucor species	1.29	0
Rhizomucor species	2.14	0
Aureobasidium species	0	0.15
scytalidium species	0	0.08
Trichoderma species	0.08	0
Unknown species	7.33	14.66
TOTAL	**80.84**	**19.16**

4. DISCUSSION

Experiments were carried out on 1310 strains of molds belonging to the genera: *Aspergillus, Penicillium, Fusarium, Curvularia, Mucor, Rhizomucor, Aureobasidium, Scytalidium, Trichoderma* and other unidentified species to evaluate their viability under cold pressure at a temperature of -20°C in the presence of a cryoprotectant (10% glycerol and BHI Broth). Cryopreservation has the action to lower the temperature of a living organism to extremely low temperatures and then maintain it for conservation purposes. Storage at low temperature suspends the biological activity of microorganisms and their development, which would allow longevity of several years to the cryopreserved cells [10,8]. However, at -20°C, not all biological activities of the conserved strains are suspended. After replicating of the 1310 strains, 1059 species belonging to the genera (*Aspergillus, Penicillium, Fusarium, Curvularia, Mucor, Rhizomucor, Trichoderma* and *unknown species*) remained viable with almost total conservation of their cultural and morphological characteristics. This viability is ensured by the synergistic action of glycerol and BHI Broth, which allowed these strains to retain their cultural and morphological characters during the shelf life. Indeed, glycerol acts as a cryoprotectant with its antifreeze effect, which increases the viscosity of the fluid and prevents the crystallization of cellular compounds during cooling. This property allows glycerol to lower the freezing point and thus increase the chances of cell tracking [11]. Brain Heart Infusion (BHI) Broth is a buffered nutrient medium that provides nutrients for the cellular activities of microorganisms and maintains the pH of the neutral medium necessary for the life of the strains [12]. However, 251 mostly fluffy and melanized strains (*Cuvularia, Scytalidium* and *Unknown species*) showed no signs of development in culture following the cryopreservation process, due to the long shelf life and the effect of freezing. Some fungal cultures lost their regenerative properties. This degeneration would be due to certain phenomena that can damage the cells during the cryopreservation process such as dehydration, cryoconcentration, ice formation outside the cells leading to crushing and perforation effects, but also ice formation inside the cells [8]. These strains did not survive under these conditions because the cryoprotective agents (glycerol, DMSO, ethylene glycol etc.) did not completely protect the preserved cultures [13-15, 6, 16]. However, many researchers have used glycerol for cryopreservation techniques and have suggested that glycerol is the most appropriate preservative for a long-term preservation of fungi in liquid nitrogen [17, 13, 14, 18].

Works also showed that glycerol is effective for the preservation of fungi for up to 90 days at - 70°C [16]. In this study, the Sabouraud chloramphenicol medium was used for mushroom cultivation, it was easy to prepare from commercially available dehydrated powder. It is a commonly used medium in the laboratory.

5. CONCLUSION

The long-term preservation and maintenance of mushrooms is a fundamental need for research. The cryopreservation technique is mainly used for a long-term preservation; in this work, 10% glycerol added to the Brain Heart Infusion Broth was used as a preservative for a period of 1 year at a low temperature of -20°C. Viability was 80.84% for all strains and 100% for *Aspergillus, Penicillium, Fusarium, Trichoderma, Mucor* and *Rhizomucor*. However, 9% of the strains were contaminated. 10% glycerol and Brain Heart Infusion Broth remains a better medium for the cryopreservation of many strains.

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Cite this article: M'boh Epi Reine Elisabeth N'gou, Chiaye Claire Antoinette Yapo-Crezoit, Ama Valérie Bonouman, Diane Kouao, Toure offianan, and Allico Joseph Djaman. CHARACTERIZATION OF INDOOR MOLDS WITH ALLERGENIC POTENTIAL AFTER CRYOPRESERVATION AT -20°C FOR ONE YEAR. *Am. J. innov. res. appl. sci.* 2020; 11(6): 175-181.