Longevity and physiological characterization of fungi of the genus *Mucor* preserved in sterile distilled water at the UCP Culture Bank

Longevidade e caracterização fisiológica de fungos do gênero Mucor preservados em água destilada esterilizada no Banco de Culturas UCP

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1 INTRODUCTION

The increase in research involving microorganisms has generated concern for the proper conservation of this biological material. Thus, the implementation of collections in research institutions has been improved in the preservation of these microorganisms under appropriate conditions and for long periods (GUIMARÃES et al., 2014; SINGH et al., 2018; DAESCHEL et al., 2019).

The cultivation of fungi, as well as other microorganisms, is an important element for the advancement and development of various research in the field of life sciences. The preservation of microorganisms aims to preserve viable cultures, without morphological, physiological and genetic alterations (DE PAULA et al., 2020; CASTRO-RIOS and BERMEO-ESCOBAR, 2021).

Culture viability and stability are ensured by using methods that reduce or paralyze cell metabolism. Among the most commonly used methods today include:

successive replating, mineral oil, sterile distilled water, freeze-drying and freezing (ODDS, 1991; BORMAN et al., 2006).

The sterilized distilled water method, known as Castellani's method (CASTELLANI, 1939), is a simple and low-cost procedure. It ensures a reasonable storage time and keeps the cultures free from contamination by mites. Many studies have suggested that the Castellani method is the most efficient in maintaining the viability, sporulation and pathogenicity of several fungal isolates (PASSADOR et al., 2001; DIOGO et al., 2005; FIGUEIREDO et al., 2006; DE SOUZA RABELLO et al., 2023).

The UCP Culture Bank of the Catholic University of Pernambuco (UNICAP), located at the Center for Research in Environmental Sciences and Biotechnology (NPCIAMB), currently has about 2,000 cultures of filamentous fungi, yeasts and bacteria that are essential for the development of research and teaching for the Catholic University of Pernambuco and other institutions.

The *Mucor* species preserved in the UCP Culture Bank are resource sources for obtaining biomolecules of wide biotechnological applicability, source of natural chitosan (SOUZA et al., 2020), biosurfactants (DO AMARAL MARQUÊS et al., 2019; DO AMARAL MARQUÊS et al.,2020; DA SILVA CÂNDIDO et al., 2022), lipids (DA COSTA LIMA et al., 2017) and enzymes (NASCIMENTO et al., 2015; DA FRANÇA et al., 2022). However, there are no studies available regarding the viability and stability of this genus of Mucorales in distilled water for long years.

2 OBJECTIVE

To evaluate the viability and enzymatic activity of cultures of fungi of the genus *Mucor* preserved in the UCP Culture Bank for long years in sterilized distilled water.

3 METHODOLOGY

3.1 REACTIVATION AND VIABILITY OF FUNGI

The growth and viability of 46 samples of *Mucor* spp. (Mucorales) preserved in sterilized water were evaluated, 46 samples preserved for 25 years and 19 samples preserved in sterilized water for 6 years. The samples are deposited in the UCP Culture Bank, Catholic University of Pernambuco, Center for Research in Environmental Sciences and Biotechnology, Catholic University of Pernambuco.

Viability determination was performed by transferring blocks with the fungi preserved in Sabouraud liquid medium for up to 15 days for growth observation. After

growth samples were transferred to Petri dishes containing BDA (Potato Dextrose Agar) with the application of a coverslip for microscopic analysis after 72h growth. Morphological analyses were evaluated by observing macroscopic characteristics (coloration and appearance of the colony) and microscopic used for taxonomic authentication of the species, based on the data of the collection catalog.

3.2 ENZYME ACTIVITY

Enzyme activity for amylase, lipase and protease was determinant for physiological characteristics. Only viable and taxonomically authenticated cultures were used. For the amylase production tests, agar -1.5% (w/v); starch-1% (w/v), 0.1M citrate-phosphate buffer; pH 5.0 - 1.0 L, a 0.1 M iodine solution will be used for application on the surface of the medium for 10 minutes. Positive reactions will be identified by the formation of a translucent halo around the colonies. For the determination of proteases, the following ingredients were used: agar -1.5%; colorless gelatin 1%; skimmed milk 1%; citrate-phosphate buffer 0.1M; pH 5.0 - 1.0 L, the enzymatic reaction was detected by the chemical modification of the culture medium, being considered positive when the formation of a translucent or whitish halo was visualized, without the need to add a developer. For determination of lipolytic activity, the medium was composed of: phenol red 0.01% (w/v), Tween 80, 1.0 % (w/v),) CaCl2, 0.1% (w/v) and 2% agar, 1.0 L distilled water, pH 7.4. Lipase activity was indicated by a change in phenol red color. The plates were incubated at 28°C´for 72h.

The enzymatic determination will be expressed as enzymatic index (EI) which will be calculated by the ratio of the mean diameter of the substrate degradation halo and the mean colony diameter, measured using a caliper.

4 DEVELOPMENT

The results showed that only 04 (8.69%) of the 25-year-old samples were viable after the preservation methodology. However, the fungi from 06 years presented a percentage of 47.36% of viable fungi (Table 1). The fungi considered viable presented sporulated and abundant cultures.

Table 1: Number of preserved cultures (P) of *Mucor* spp. and viable cultures (V).

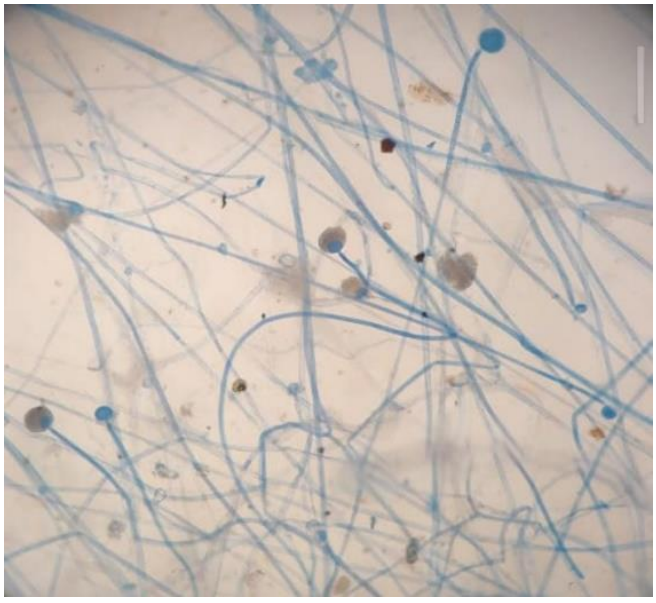
Species	06 years		25 years	
	P	**V**	**P**	**V**
M. circinelloides	09	05	19	02
M. hiemalis	07	02	16	01
M. racemosus	03	02	06	01
M. genevensis	01	0	03	0
M. piriformis	0	0	02	0
Total	**19**	**09**	**46**	**04**

Source: Authors (2023)

The Castellani method has been shown to be efficient to preserve several groups of fungi: Mycotoxigenic fungi (Guimarães et al., 2014), pathogenic fungi (DE CAPRILES et al., 1993; MENDOZA et al., 2005; BORMAN et al., 2006; ANDREU et al., 2013; UNDANETA & DA SILVA LACAZ; MCGINNIS et al., 1974; RODRIGUES et al. 1992), phytopathogens (QIANGQIANG et al., 1998;SAKR, 2018, SAKR, 2020; FINATTI & APARECIDO, 2021).

Studies evaluating the viability of Mucorales fungi preserved in distilled water are scarce. Bueno & Gallardo (1998) evaluated the viability of 26 isolates of filamentous fungi, including mucorales fungi preserved in sterilized distilled water for 2 years. And they obtained a viability of 100%, without morphological variations and contamination by mites and other fungi. In our study, regardless of time, the viable samples did not show pleomorphism (Figure 1). Only 04 samples showed contamination by other fungi.

Figure 1. Sample of *Mucor racemosus* UCP 0004 preserved for 25 years with reproductive and vegetative structures.



Source: Authors (2023).

In the study of Torres (2008), 86 cultures of zygomycetes fungi, among them 10 samples of *Mucor* spp. preserved for 06 to 20, only 30% had viability years did not have viability. However, all isolates of the genus *Mucor*, did not grow.

De Capriles' study is referenced for recommending the method of preservation in sterilized water, since, they were able to recover 90% of the 19 fungal samples preserved in sterilized distilled water for 20 years. However, the fungi belong to genera such as *Cephalosporium, Diplorhinothricum, Endomycopsis, Fonsecaea, Madurella, Phialophora, Exophiala, Trichosporon, Trichophyton, Wangiella, Cladosporium* and *Xylophypha*, genera represented by ascomycetes of medical importance. No fungi of the order Mucorales were analyzed.

Rodrigues et al., (1992) tested the viability of some fungi preserved in sterilized distilled water, including some mucorales: *Cunninghamella (*2), *Mucor (*2), *Syncephalastrum (*4) and *Rhizopus* (1). All samples tested were found to be viable, including the samples from the genus *Mucor*. Only one sample of the genus *Cunninghamella* showed no growth with only 1 year of preservation. The preservation period evaluated in this study was between 6 and 24 months, shorter periods compared to our study.

The method of preservation in sterilized water also does not seem to be suitable for isolates of Basidiomycota for long years. In the study by Johnson and Martin (1992), only 26% of the samples of this group of fungi survived preservation in sterilized distilled water for 10 years. However, when preserved in mineral oil they had a higher survival rate (98%).

Regarding the physiological characteristics, the enzyme production of the viable cultures is shown in Table 2.

Table 2. Detection of enzymes by *Mucor* spp. preserved in sterilized distilled water in the UCP Culture Bank**.**

Isolated (06 years)		**Protease**	**Amylase**	**Lipase**			
		Hydrolysis zone (cm)*	**Production efficiency****	Hydrolysis zone (cm)*	**Production efficiency****	Hydrolysis zone (cm)*	**Production efficiency****
UCP 0001	*M. circinelloides*	-	-	8,2	**1,15**	4,6	**1,12**
UCP 0003	*M. racemosus*	4,6	**1,02**	8,6	**1,22**	-	
UCP 0004	*M. racemosus*	-		8,6	**1,13**	4,0	**1,17**
UCP 0006	*M. circinelloides*	-		-	-	6,6	**1,65**

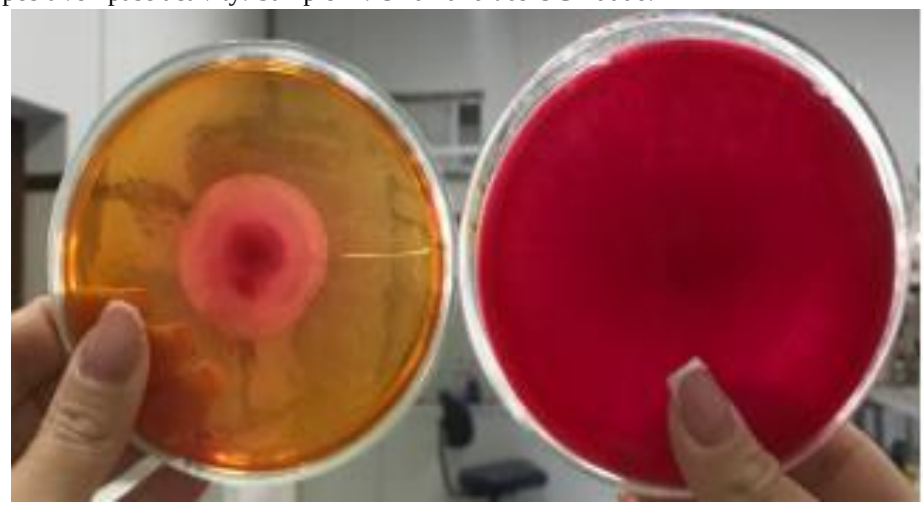
UCP 0008	*M. circinelloides*	-		8,2	**1,04**	-	
UCP 0013	*M. hiemalis*	-		-	-	-	
UCP 0016	*M. hiemalis*	4,4	**1,08**	-	-	6,5	**1,58**
UCP 0017	*M. circinelloides*	-		7,5	**1,13**	-	
UCP 0018	*M. circinelloides*	-		7,7	**1,09**	5,3	**1,20**

Isolated (25 years)		**Protease**	**Amylase**	**Lipase**			
		Hydrolysis zone (cm)*	**Production efficiency****	Hydrolysis zone (cm)* **Production efficiency****		Hydrolysis zone (cm)*	**Production efficiency****
UCP 0001	*M. circinelloides*	-	-	-		8,6	**1,22**
UCP 0004	*M. racemosus*	-	-	-	-	8,5	**1,18**
UCP 0005	*M. hiemalis*	-	-	8,7	**1,16**	+	
UCP 0045	*M. circinelloides*	-	-	8,8	**1,16**	-	-

*Hydrolysis zone diameter = colony+halo
**Production efficiency = hydrolysis zone/colony
Source: Authors (2023).

Most isolates produced amylase (08), accompanied by lipolytic activity which was recorded in 07 samples. Highlighted samples of 25 years that did not lose amylolytic and lipolytic activity. However, none showed protease production. Only samples of *M. racemosus* UCP 0003 and *M. hiemalis* UCP0016 preserved for 06 years showed proteolytic activity. Figure 1 shows the highlighted isolate with the highest lipase production efficiency.

Figure 2. Lipase activity indicated by change in color of phenol red. Conversion from red to yellow color indicates positive lipase activity. Sample *M. Circinelloides* UCP 0006.



Source: Authors (2023).

Species of the genus *Mucor* are recognized for producing various enzymes such as amylases (MOHAPATRA et al., 1998; ANUPMA et al., 2020), lipases (Abbas et al., 2002; MOENTAMARIA et al., 2019DA SILVA FRANÇA et al., 2022), and proteases (Nascimento et al. 2020; QASIM et al...), 2022)In the study by Alves et al., (2002), of the 56 isolates of *Mucor* spp. investigated for the production of amylase, protease and especially lipase, in which the majority 84% of the isolates produced amylase, 82% protease and 66% showed lipolytic activity.

The genus *Mucor* is recognized in the literature as a good producer of proteases. Alves et al., (2005), were able to detect extracellular protease production in all *Mucor* isolates at different pH ranges. In the study of Nascimento et al., (2015) *M. subtillisimus* UCP 1262 produced a new fibrinolytic protease with potential in medical application for thrombolytic diseases.

5 FINAL CONSIDERATIONS

The low number of fungi that did not survive for long years may be due to the inoculum time making the spores non-viable or even lack of balance in osmotic pressure. Therefore, this study suggests, inoculation of monospore and young culture and evaluation of buffered solution to balance the osmotic pressure. Adaptations that may contribute to this preservation method.

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