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Revitalization of lyophilized fungi, molecular identification, and evaluation of amylase production

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ABSTRACT. Lyophilization is the leading storage technique for sporulating fungi. The genera *Aspergillus*, *Penicillium*, and *Fusarium* encompass sporulating species, many reported as hydrolase producers. Hydrolases have noteworthy commercial value; amylases account for 30% of all sales of hydrolases due to their use in industrial starch processing. The objectives of this work were to evaluate the surviving ability of filamentous fungal isolates that had remained lyophilized approximately 30 years and to assess their amylases production. The 57 fungal isolates were collected in Southern Brazil as contaminants in plants or petri plates containing primarily a starch-rich culture medium. A total of 34 fungal isolates were recovered, which belonged to the genera *Aspergillus* (9), *Penicillium* (8), *Talaromyces* (2), *Fusarium* (11), *Rhinocladiella* (1), *Cladosporium* (1), and *Trichoderma* (2). These isolates were identified at the species level using DNA barcoding. In the amylase production survey, isolates of the *Aspergillus* genus were the best producers, followed by isolates of the *Penicillium* and *Fusarium* genera. Some of the amylase producer species are well known, but some produce enzymes yet to be purified and characterized, such as *Aspergillus clavatus*, *Penicillium crustosum*, *Talaromyces funiculosus*, and *Fusarium oxysporum*. In conclusion, we demonstrated the survival capability of several fungal species and the usefulness of fungal amylase production screening.

Key words: Freeze-dried fungi survival; Amylase production; *Aspergillus*; *Penicillium; Fusarium*

INTRODUCTION

Fungus diversity is of supreme importance owing to their various roles as advantageous or harmful microorganisms. The revisited fungal diversity in the world is 2.2 to 3.8 million species (Hawksworth and Lücking, 2017). However, only approximately 144,000 have been described (Willis, 2018), mainly in tropical regions. Thus, it is worth emphasizing the importance of generating reliable information on fungal diversity to compose databases, to determine the influence of both spatial and temporal variation.

Lyophilization is the primary fungus preservation technique used in most culture collections and has been used since its description. In this technique, fungi frozen spores (or hyphae), generally in a solution of skimmed milk, are preserved by sublimation-drying, under vacuum. Cultures can be processed for storage in a relatively short period, after which the lyophilized vials can be archived. Lyophilization is effective for almost all conidial fungi, such as Ascomycetes and Basidiomycetes, but it is not helpful for fungi with very watery cells, such as Oomycetes and Zygomycetes (Smith and Onions, 1994). Fungus lyophilization preservation has the following advantages: sample protection against infestations, for example, by mites; very long viability (4 to 40 years); and culture stability (Smith and Onions, 1994). Considering the importance of fungal preservation by lyophilization, knowing which species survive this process is essential.

The global market for industrial enzymes is expected to reach nearly USD 8.7 billion by 2026 (Mesbah, 2022). α-Amylases account for 30% of the industrial enzyme market (Paul et al., 2021). Amylases are used in the textile, chemical, pharmaceutical, beverage, bakery, children's cereal, animal feed, and bioethanol industries (Pandey et al., 2000). Most enzymes marketed today are of microbial origin (Paul et al., 2021). The advantage of microbial enzymes is that they have high substrate selectivity, are comparatively more stable than plant and animal enzymes, have easier production and purification, and are seasonally independent (Singh et al., 2016).

Amylases are classified according to their mechanism of action. Debranching amylases hydrolyze α-1,6 glycosidic bonds (Pandey et al., 2000). Endoamylases, such as α-amylases, can cleave α-1,4 glycosidic bonds within the starch biomolecules, amylose or amylopectin (Paul et al., 2021). Exoamylases, such as glucoamylases and β-amylases, cleave glycosidic bonds at non-reducing ends of amylose and amylopectin, releasing fermentable monosaccharides and disaccharides (Pandey et al., 2000; Kumar and Satyanarayana, 2009).

α-Amylases can be derived from various bacteria, archaea, eukaryotes, yeasts, and fungi, but industrial α-amylases are produced mainly by bacteria of the genus *Bacillus* and fungi of genera *Aspergillus*, *Penicillium*, and *Rhizopus* (Paul et al., 2021). Filamentous fungi are the primary source of glucoamylases, and species of the genera *Aspergillus* and *Rhizopus* are the leading producers of glucoamylases for industrial use (Kumar and Satyanarayana, 2009). Although β-amylases are usually of plant origin, they are also produced by some microorganisms, mainly bacteria and fungi, such as *Rhizopus* sp. (Pandey et al., 2000).

This work aimed to evaluate the survival ability of 57 monosporic fungal isolates lyophilized in glass ampoules. Those isolates were obtained in Southern Brazil as air, plant, and medium contaminants. In addition, this work had the objective of identifying the

revitalized isolates and analyzing the amylase production by them. New amylase producers are always welcome due to the industrial demand for these enzymes.

MATERIAL AND METHODS

Lyophilized isolates

This work used 57 glass ampoules containing lyophilized spores or hyphae of filamentous fungi (Table 1). These ampoules (Figure S1) were prepared and sealed in the years 1992 to 1997 in the laboratory of Dr. C. Kemmelmeier (Emeritus Professor of the Department of Biochemistry at the Universidade Estadual de Maringá), who kindly donated them to us. The lyophilized fungi were originally obtained as contaminants of plants or laboratory cultures or by exposing plates containing Potato Dextrose Agar (PDA) (200 g/L broth of peeled and cooked potatoes, 15 g/L glucose, 20 g/L agar) to the air in buildings I89 (basic biochemistry laboratories) and I90 (clinical microbiology laboratory) of the Universidade Estadual de Maringá (UEM), which is located in the city of Maringá, Paraná State, in Southern Brazil. These isolates belonged to the *Aspergillus*, *Penicillium*, *Fusarium*, or unidentified genera (Table 1).

Table 1. Freeze-dried isolates.

Aspergillus	**Source#**	**Date**	**Other**	**Source#**	**Date**
UEM 01	PDA	20/07/1992	UEM 10*	PDA (air)	09/10/1992
UEM 04	PDA pH 5.0	17/07/1992	UEM 12*		23/05/1997
UEM 05	PDA pH 5.0	20/07/1992	UEM 13*		23/05/1997
UEM 07	LIT	21/07/1992	UEM 14*	PDA	13/10/1992
UEM 11	PDA	22/07/1992	UEM 15*	Gooseberry (fruit)	23/05/1997
UEM 13	PDA	22/07/1992	UEM 16		23/05/1997
UEM 33	PDA	17/07/1992	UEM 17*	Gooseberry (fruit)	23/05/1997
UEM 41	Rusty can	21/07/1992	UEM 18*		23/05/1997
UEM 63	PDA	17/07/1992	UEM 24*		23/05/1997
Penicillium	**Source#**	**Date**	UEM 30*	PDA (air)	02/12/1992
UEM 06	PDA	21/07/1992	UEM 31*		23/05/1997
UEM 12	PDA	22/07/1992	UEM 34*		23/05/1994
UEM 28		23/05/1997	UEM 40*	PDA	27/10/1992
UEM 32		23/05/1997	UEM 41*		23/05/1997
UEM 45	PDA	22/07/1992	UEM 44	PDA	23/05/1997
UEM 50	PDA	21/07/1992	UEM 50*		23/05/1997
UEM 53	Lemmon	17/02/0992	UEM 51*		23/05/1997
UEM 61*	PDA	20/07/1992	UEM 52	PDA	20/07/1992
UEM 68	PDA	22/07/1992	UEM 70	Basidioma	26/11/1992
UEM 101	Trigo	06/11/1992	UEM 77*	PDA	04/12/1992
UEM 106	CMA	26/01/1994	UEM 82*	PDA	22/06/1992
Fusarium	**Source#**	**Date**			
UEM 09	MA	12/05/1992			
UEM 19	PDA	25/05/1992			
UEM 20*	PDA	25/09/1992			
UEM 21-a	PDA	12/05/1992			
UEM 29	Gooseberry (fruit)	12/05/1992			
UEM 57-a*	PDA	12/05/1992			
UEM 59	PDA	12/05/1992			
UEM 60	PDA	12/05/1992			
UEM 65	PDA	27/05/1992			
UEM 67	Clover (leave)	25/05/1992			
UEM 76	PDA	12/05/1992			
UEM 80*	Peach (fruit)	09/07/1992			
UEM 100*	Apple	11/09/1992			
UEM 102	Bean	16/11/1992			
UEM 103*	Bean	16/11/1992			
UEM 104	Wheat	26/11/1992			

*Non-viable isolates. #PDA – Potato Dextrose Agar. LIT - Liver Infusion Tryptose. CMA – Coconut Milk Agar. MA – Malt Agar. All isolates were kindly donated by Dr. C. Kemmelmeir.

Reactivation and culture maintenance

After superficial disinfection with 70% ethanol, the ampoule´s necks were broken (Figure S1), and 1.0 mL of sterile distilled water was added to each ampoule. After brief hydration (5 min), the obtained suspension was applied into a Petri dish (Ø= 9 cm) containing PDA medium supplemented with 350,000 U/L of penicillin and 145 U/L of streptomycin. The plates were incubated at 25°C, with a photoperiod of 12 h, for 3-5 days. The revitalized fungi were transferred to tubes containing slanted PDA and cultured similarly. When contamination occurred, monosporic isolation was performed to obtain pure cultures. For this, a 1 cm^3 colony fragment on the plate was stirred in 20 mL of distilled water. A 100 µL aliquot of the obtained spore suspension was spread on the surface of a Petri dish (Ø= 9 cm) containing 2% water agar. After 24-48 h of incubation at 25°C, a germinating spore or hypha was transferred to a tube containing slanted PDA. After growth in the same conditions, the culture tubes were stored at room temperature. Duplicates of cultures in tubes with PDA slants were covered with sterile mineral oil for long-term maintenance (Smith and Onions, 1994) and stored at room temperature.

Morphological analysis of isolates

Macro-morphological characteristics (e.g., colony color on PDA medium) were observed with the naked eye or under a stereoscopic microscope. In addition, micro-morphological characteristics (e.g., size and shape of spores and conidiophores, and presence of microstructures, such as chlamydospores and others) were observed under a binocular optical microscope. The macro- and micro-morphological characteristics were analyzed using traditional keys for the initial classification of the genus and study of the isolates (Smith and Onions, 1994; Leslie and Summerell, 2006).

DNA extraction and molecular identification of isolates

The inoculum consisted of 1.0 mL of a spore suspension made by stirring 1 cm^3 of the culture in PDA slant in 20 mL of sterile distilled water in a blue-capped 100 mL bottle; it was used to inoculate 125 mL Erlenmeyer containing 25 mL of liquid potato dextrose medium. Stationary cultivation was carried out for 2-3 days at 25°C, with a 12-hour photoperiod. The mycelium was collected by filtration of the culture in sterile nylon and macerated with liquid nitrogen in a porcelain mortar for genomic DNA extraction with the PureLink™ Genomic Plant DNA Purification kit (Thermo Fisher Scientific, USA). DNA concentration was estimated by fluorometry with the Qubit™ dsDNA HS Assay kit and Qubit™ fluorimeter (Thermo Fisher Scientific, USA).

Amplification reactions of the ITS-5.8S-rDNA region were performed in PCR tubes containing 25 µL of the following reaction mixture: 20 mM Tris buffer, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 1.5 U of Platinum *Taq* DNA polymerase (Thermo Fisher Scientific, USA), 0.2 mM of each dNTP, 25 pmol of primers ITS4/5 (White et al., 1990) and 2-5 ng of genomic DNA. The PCR reaction consisted of 30 cycles of 1 min and 30 sec at 94°C, 1 min and 30 sec at 50°C, and 2 min at 72°C. Before the cycles, the samples were heated for 5 min at 94°C and, after the cycles, for 10 min at 72°C. After evaluating the amplification on an agarose gel, the amplified DNA was purified with the PureLink™ Quick Gel Extraction and

PCR Purification Combo Kit (Thermo Scientific, USA) and sent for sequencing (Sanger) at the Center for Human Genome Studies at the University of São Paulo, São Paulo, SP, or at GoGenetic in Curitiba, PR. The obtained sequences were trimmed, deposited in GenBank, and used for BLAST search. The classification of isolates was carried out by at least 99% identity with sequences of species cataloged in databases.

Phylogenetic analysis

Phylogenetic analyses were performed using the neighbor-joining method (Saitou and Nei, 1987) in the MEGA 11 program (Tamura et al., 2021). Branch formation confidence limits were evaluated using Bootstrap analyses with 1000 heuristic replicated trees (Felsenstein, 1985). Phylogenetic clustering between sequences was considered reliable when values were greater than 70% in this test. Finally, evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004).

Amylase production in liquid medium

Cultivation of each fungus was carried out in three 125 mL Erlenmeyer flasks containing 25 mL of the following liquid medium: 3 g/L yeast extract, 5 g/L peptone, 21 mM NaH_2PO_4, 29 mM $Na_2HPO_4 \cdot 7H_2O$, pH 7.0, and 2 g/L of soluble starch (Lago et al., 2021). The inoculum consisted of 1.0 mL of a homogenate made as follows. A 1 cm^3 fragment of a slanted PDA culture was transferred and chopped into a 125 mL Erlenmeyer flask containing 25 mL of the medium described above. After stationary growth for four days at 25°C, with a photoperiod of 12 hours, the culture was homogenized in a stainless-steel sieve and passed through a sterile needle (18G) attached to a sterile syringe. After inoculation in 25 mL of the same medium, the test flasks were incubated for 72 h at 25°C with orbital agitation (100 rpm). The obtained cultures were filtered through filter paper, and the enzymatic activity was evaluated in the filtrate. The mycelium was dried at 50°C for 24 h, and the weight of the dried mycelium was used to assess the growth of the microorganism.

Amylase enzyme assay

The used method was based on the starch-iodine complex decolorization (Palanivelu, 2001). A 500 µL aliquot of the pure or diluted filtrate was added to 1.0 mL of 100 mM phosphate buffer, pH 7.0, and 500 µL of 0.1% (w/v) soluble starch, prepared in the same buffer. The reaction was incubated for 10 min at 50°C. Then, 500 µL of 100 mM HCl and 500 µL of iodine solution (2% KI; 0.2% I_2) were added. Absorbance was read at 690 nm. The blank was prepared with 2.0 mL of 100 mM phosphate buffer, pH 7.0. This reaction was done in duplicate for each culture flask. The initial starch concentration was evaluated in a reaction with 500 µL of 0.1% starch solution and 1.5 mL of 100 mM phosphate buffer, pH 7.0. Residual starch in the cultures was evaluated in reactions with 500 µL of the filtrate and 1.5 mL of 100 mM sodium phosphate buffer, pH 7.0. Before absorbance reading, the blank and control reactions were added with 500 µL of 100 mM HCl and 500 µL of iodine reagent. The absorbance of the sample was subtracted from the absorbance of the residual starch control in the filtrate. One unit of the enzyme was defined

as the amount of enzyme that causes a 20% decrease in absorbance per minute under the assay conditions.

RESULTS

Revitalized isolates

The recovered isolates are listed in Table 2. The revitalized fungi consist of nine isolates of the genus *Aspergillus*, (Figure 2S) eight of the genus *Penicillium* (Figure 2S), two of the genus *Talaromyces* (Figure 2S), and 11 of the genus *Fusarium* (Figure 3S). In addition, one isolate from the genus *Rhinocladiella*, one isolate from the genus *Cladosporium*, and two isolates from the genus *Trichoderma* were also obtained (Figure 3S).

Table 2. Identified isolates.

Isolate	Genus and species	GenBank	GenBank of similar sequences (percentage of identity)
UEM 01	*Aspergillus ruber*	OP329178	MH858702.1 (100%), MH854663.1 (100%)
UEM 04	*Aspergillus clavatus*	OP329179	HQ026749.1 (100%), NR_121482.1 (100%)
UEM 05	*Aspergillus flavus*	OP329180	OL711682.1 (100%), MZ158654.1 (100%)
UEM 07	*Aspergillus terreus*	OP329181	MT530046.1 (100%), KF624770.1 (100%)
UEM 11	*Aspergillus flavus*	OP329182	OL711682.1 (100%), MZ158654.1 (100%)
UEM 13	*Aspergillus sydowii*	OP329183	MH854859.1 (99.8%), MT312735.1 (99.8%)
UEM 33	*Aspergillus parasiticus*	OP329184	AY373859.1 (100%), KY937934.1 (100%)
UEM 41	*Aspergillus niger*	OP329185	MN267569.1 (100%), KP131604.1 (100%)
UEM 63	*Aspergillus fumigatus*	OP329186	MG991618.1 (100%), KU296267.1 (100%)
UEM 06	*Penicillium citrinum*	OP329187	MT597828.1 (100%), LC514694.1 (100%)
UEM 12	*Penicillium brevicompactum*	OP329188	MH863910.1 (100%), MN636211.1 (100%)
UEM 28	*Penicillium expansum*	OP329189	NR_077154.1 (100%), FJ463031.1 (100%)
UEM 32	*Penicillium olsonii*	OP329190	MH860601.1(100%), AY373925.1 (100%)
UEM 45	*Penicillium crustosum*	OP329191	MH862985.1(100%), MN511336.1 (100%)
UEM 50	*Penicillium citrinum*	OP329192	MG554246.1 (100%), KY921947.1 (100%)
UEM 101	*Penicillium brasilianum*	OP329195	MH865219.1 (100%), MH911419.1 (100%)
UEM 106	*Penicillium citrinum*	OP329196	MH858380.1 (99.6%), NR_121224.1 (99.6%)
UEM 53	*Talaromyces variabilis*	OP329193	MG957181.1 (100%), KU216713.1 (100%)
UEM 68	*Talaromyces funiculosus*	OP329194	MH860684.1 (100%), GU183120.1 (100%)
UEM 09	*Fusarium proliferatum*	OP329197	MT466521.1 (100%), OL873221.1 (100%)
UEM 19	*Fusarium equiseti*	OP329198	MH578585.1 (100%), KR364597.1 (100%)
UEM 21-a	*Fusarium verticillioides*	OP329199	MT505436.1 (100%), OM955992.1 (100%)
UEM 29	*Fusarium proliferatum*	OP329200	MT466521.1 (100%), OL873221.1 (100%)
UEM 59	*Fusarium lateritium*	OP329201	MH861014.1 (96.9%), AF310982.1 (96.9%)
UEM 60	*Fusarium chlamydosporum*	OP329202	MH859028.1 (100%), MG250449.1 (100%)
UEM 65	*Fusarium equiseti*	OP329203	MH578585.1 (100%), KR364597.1 (100%)
UEM 67	*Fusarium equiseti*	OP329204	MH857953.1 (100%), JX045847.1 (100%)
UEM 76	*Fusarium proliferatum*	OP329205	MT466521.1 (100%), OL873221.1 (100%)
UEM 102	*Fusarium oxysporum*	OP329206	MH865884.1 (100%), KX385043.1 (100%)
UEM 104	*Fusarium equiseti*	OP329207	MH857953.1 (100%), JX045847.1 (100%)
UEM 16	*Rhinocladiella similis*	OQ511500	MH864632.1 (100%), NR_166008.1 (100%)
UEM 44	*Cladosporium tenuissimum*	OQ511501	OL579743.1 (100%), MF469070.1 (100%)
UEM 52	*Trichoderma longibrachiatum*	OQ511502	MT520608.1 (100%), MN749761.1 (100%)
UEM 70	*Trichoderma harzianum*	OQ511503	MK784067.1 (100%), MH153633.1 (100%)

Molecular identification of isolates and phylogenetic analysis

All isolates were identified by DNA barcoding with high identity between the obtained sequences and those deposited in databases (Table 2). The *Aspergillus* genus was represented by eight different species; while the *Penicillium* and *Fusarium* genera were represented by six different species each; *Talaromyces* and *Trichoderma* genera had two species each; and the *Rhinocladiella* and *Cladosporium* genera had only one species. The phylogenetic analysis showed the separation between genera and species (Figure 1). The species sequences of different genera were grouped in separate clades. The *Rhinocladiella* and *Cladosporium* species did not form clades because each genus had only one isolate sequence.

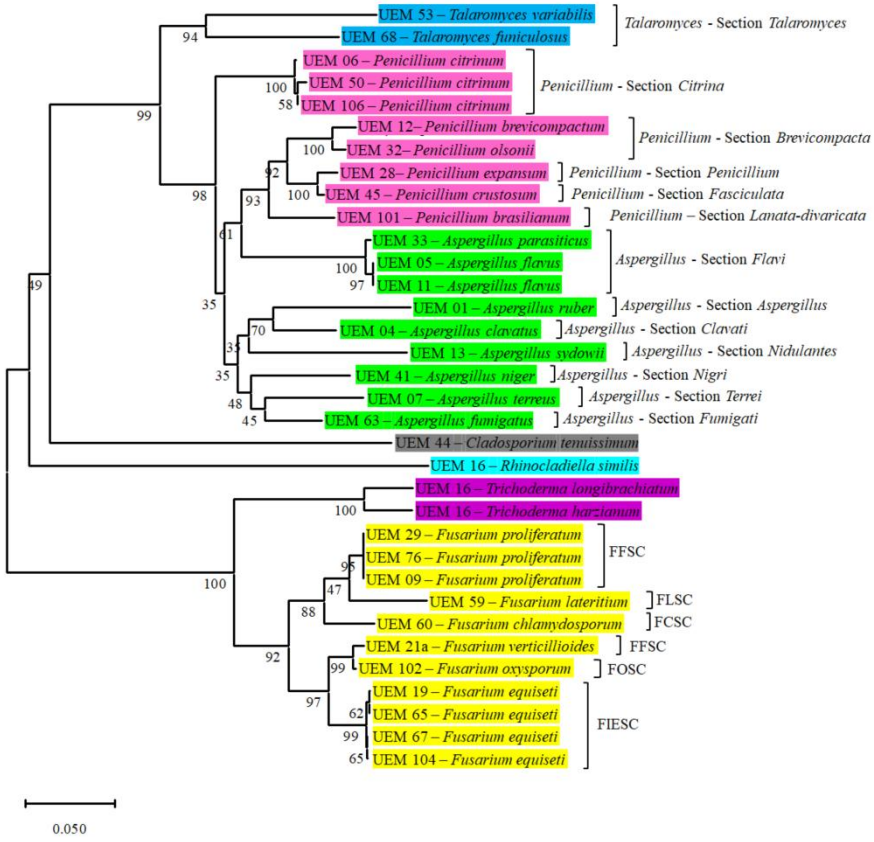

Figure 1. Phylogenetic analysis of the fungal isolates. The bootstrap test results (%) are shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances are in units of the number of base substitutions per site. This analysis involved 34 nucleotide sequences. There were 657 positions in the final dataset. Sequences from the genus *Aspergillus* are indicated in green, *Penicillium* in pink, *Talaromyces* in blue, *Fusarium* in yellow, *Rhinocladiella* in cian, *Cladosporium* in grey, and *Trichoderma* in purpure. FFSC – *Fusarium fujikuroi* species complex. FLSC – *Fusarium lateritium* species complex. FOSC – *Fusarium oxysporum* species complex. FIESC – *Fusarium incarnatum-equiseti* species complex. FCSC – *Fusarium chlamydosporum* species complex.

Amylase production in liquid medium

The results of the amylase production in liquid medium analyses are presented in Figure 2. The data show that the highest production occurred with species of the genera *Aspergillus* and *Penicillium*.

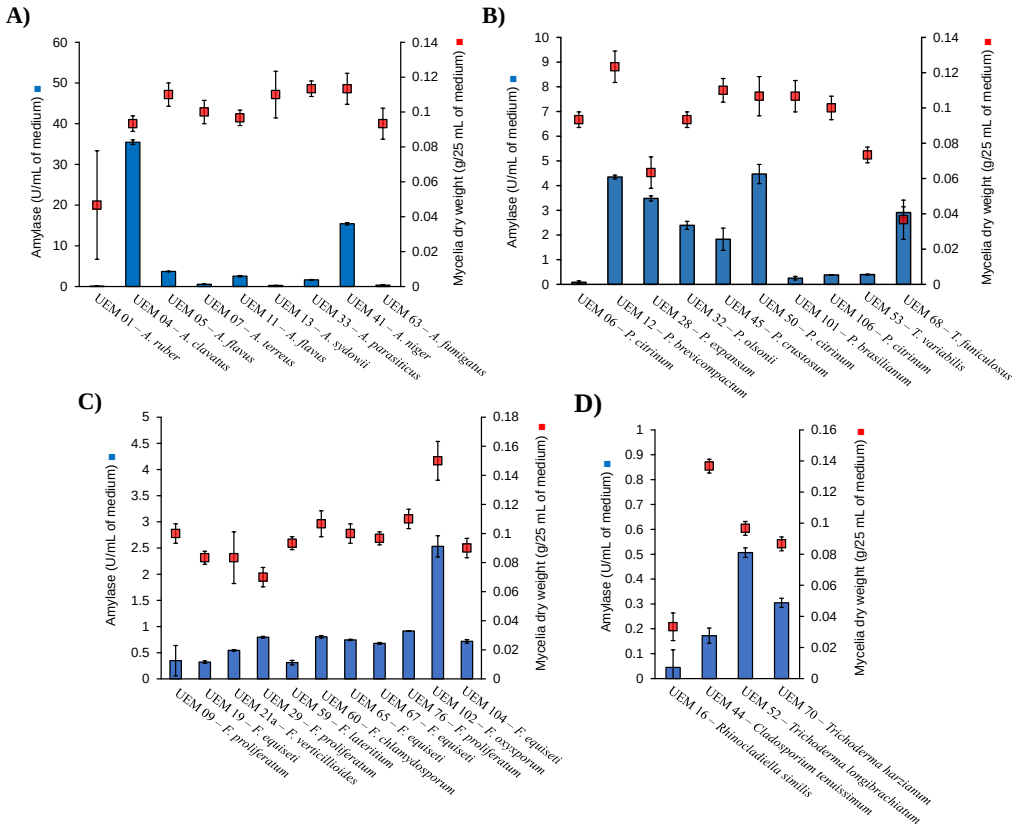

Figure 2. Amylase production of the fungal isolates in liquid medium. A) *Aspergillus* spp. B) *Penicillium* spp., and *Talaromyces* spp. C) *Fusarium* spp. D) *Rhinocladiella* sp., *Cladosporium* sp., and *Trichoderma* spp. Results are the mean and standard deviation of the production obtained from three culture flasks.

DISCUSSION

As previously reported (Smith and Onions, 1994), only highly sporulating fungi, such as isolates from the genus *Aspergillus*, *Penicillium*, *Fusarium*, *Rhinocladiella*, *Cladosporium*, and *Trichoderma*, were able to survive the years of storage by lyophilization (Table 1). However, some isolates within the genus *Fusarium* did not survive, which was expected since not all species produce large amounts of spores (Leslie and Summerell, 2006).

Among the identified isolates, species from seven sections of the genus *Aspergillus* were obtained, namely *Nigri*, *Flavi*, *Terrei*, *Clavati*, *Fumigati*, *Nidulans*, and *Aspergillus*

(Samson et al., 2014), as shown in the phylogenetic tree (Figure 1). Also, species were obtained from five sections of the *Penicillium* genus: *Citrina*, *Lanata-divaricata*, *Brevicompacta*, *Penicillium*, and *Fasciculata* (Houbraken et al., 2020) (Figure 1).

The genus *Talaromyces* was dismembered from the genus *Penicillium* to accommodate species of sexual reproduction (Yilmaz et al., 2014). The species that were found were *Talaromyces variabilis* (UEM 53) and *Talaromyces funiculosus* (UEM 68), which were previously classified as *Penicillium variabile* and *Penicillium funiculosum*, respectively. These two identified isolates belong to the *Talaromyces* section of the *Talaromyces* genus (Yilmaz et al., 2014).

The genera *Aspergillus*, *Penicillium*, and *Talaromyces* belong to the order Eurotiales, which includes several economically important genera (Houbraken et al., 2020). These genera are the most commonly found indoors (Visagie et al., 2014).

Filamentous fungi of the *Fusarium* genus are ubiquitous in the environment and are found in soil, water, and air (Leslie and Summerell, 2006). *Fusarium* species are primarily plant pathogens and produce toxins that can cause food poisoning (Pitt and Hocking, 2009). The genus *Fusarium* contains at least 300 species complexes or phylogenetically distinct species (O'Donnell et al., 2015).

Isolates or species of five *Fusarium* species complexes were found (Table 2, Figure 1). *Fusarium proliferatum* and *Fusarium verticillioides* belong to the *Fusarium fujikuroi* species complex (FFSC) (O'Donnell et al., 2015). The four *Fusarium equiseti* isolates belong to the *Fusarium incarnatum-equiseti* species complex (FIESC). However, the sequences obtained from the ITS-5.8SrDNA of those isolates (UEM 19, UEM 65, UEM 67, and UEM 104) were not sufficient to define to which species they belong within this complex (Leslie and Summerell, 2006; Crous et al., 2022). In addition, *Fusarium oxysporum* (UEM 102), *Fusarium lateritium* (UEM 59), and *Fusarium chlamydosporum* (UEM 60) isolates belong to species complexes with the same names (FOSC, FLSC, FCSC, respectively) (Leslie and Summerell, 2006; Crous et al., 2022); it was also not possible to define the species of these isolates within these complexes with the sequences obtained from the ITS-5.8rDNA (Table 2, Figure 1). The molecular taxonomy of *Fusarium* is more complicated and requires amplification and sequencing of other gene sequences, the most commonly used being segments of the genes for elongation factor 1α (ef1α) and the β-tubulin gene (O'Donnell et al., 2015).

The *Rhinocladiella* genus species, *Rhinocladiella similis*, is a melanized fungus from the Chaetothyriales order (Ascomycota) that is found mainly in tropical and subtropical countries and has been found to be involved in chromoblastomycosis (CBM) (Heidrich et al., 2017). CBM is a chronic granulomatous fungal infection prevalent in rural workers; it causes cutaneous and subcutaneous tissue hyperproliferation.

The only species recovered for the hyphomycete genus *Cladosporium*, *Cladosporium tenuissimum*, is a common saprobic and phytopathogenic species isolated from numerous substrates in tropical regions (Liu et al., 2019).

Two species of the *Trichoderma* genus (Hypocreaceae family) were recovered. *Trichoderma longibrachiatum* is a soil fungus found worldwide in warmer climates and produces large amounts of cellulolytic enzymes (Dong et al., 2022). *Trichoderma harzianum* is a mycoparasite used as a fungicide for growth suppression of fungal pathogens (Woo et al., 2014) and produces critical enzymes for the biofuel industry (Ferreira Filho et al., 2017).

Isolates of the genus *Aspergillus* spp. are among the leading producers of amylases (Kumar and Satyanarayana, 2009; Paul et al., 2021). Therefore, as expected, the fungal isolates with highest amylase production were species of the *Aspergillus* genus (Figure 2A). Among these isolates, the strain *Aspergillus clavatus* UEM 04 was the highest producer (35.5 U/mL of culture medium), followed by the strain *Aspergillus niger* UEM 41 (15.4 U/mL of culture medium) (Figure 2A). The *A. clavatus* UEM 04 amylase production was also higher than the α-amylase production obtained by *Aspergillus awamori* (19 U/mL), a species of the *Nigri* section of the *Aspergillus* genus, also assayed with the starch-iodine complex distaining method (Bhella and Altosaar, 1985). Production of amylases by the *A. clavatus* UEM 04 strain was later studied in our laboratory, and the results obtained were published elsewhere (Mendonça et al., 2023). This strain produces two major amylases, a glucoamylase and an α-amylase.

Amylase production by *A. niger* is well described in the literature, and a glucoamylase from this species is commercially available (Kumar and Satyanarayana, 2009). However, the *A. niger* UEM 41 isolate amylase production (15.4 U/mL) (Figure 2A) was lower than that of *A. clavatus* UEM 04, which can be explained by the low production potential of the *A. niger* UEM 41 isolate or by the characteristics of the produced enzymes. However, production of amylase by the isolate *A. niger* UEM 41 was greater than the glucoamylase production of 2 U/mL by *Aspergillus wentii*, also assayed with the starch-iodine complex distaining method (Lago et al., 2021).

The other isolates of *Aspergillus* also showed good growth but produced amylase at lower levels (Figure 2A) compared to the best producers. The third best-producing species was *Aspergillus flavus*, isolates UEM 05 and UEM 11, producing 3.7 and 2.6 U/mL of culture medium, respectively. In agreement with our results, a production of 2.87 U/mL of culture medium of an α-amylase by *A. flavus* NSH9, also assayed with the starch-iodine distaining method, was previously reported (Karim et al., 2018), which was also already purified and characterized. The fourth species that produced amylolytic activity at significant levels (1.65 U/mL of culture medium) was *Aspergillus parasiticus* (isolate UEM 33). Although there are no reports of amylase production by *A. parasiticus*, this fungus is the greatest producer of aflatoxin B1, a potent carcinogen (Pitt and Hocking, 2009).

Among the isolates of *Penicillium* (Figure 2B), the best producer of amylolytic activity was *Penicillium citrinum* UEM 50, producing 4.5 U/mL of culture medium (Figure 2B). Interestingly, the other two isolates of *P. citrinum*, UEM 06 and UEM 106, practically did not produce amylolytic activity in this liquid medium (0.09 and 0.38 U/mL of culture, respectively). This difference can be explained by variation between isolates and the enzymes produced, which again shows the importance of testing different isolates. The production and characterization of α-amylase and β-amylase from *P. citrinum* are reported in the literature (Metin et al., 2010; Saber et al., 2021).

The other *Penicillium* species that produced amylolytic activity were *Penicillium brevicompactum* UEM 12 (4.4 U/mL of medium), *Penicillium expansum* UEM 28 (3.5 U/mL of medium), *Penicillium olsonii* UEM 32 (2.4 U/ mL of medium), and *Penicillium crustosum* UEM 45 (1.8 U/ml of medium) (Figure 2B). Similar to what we found, there is a report in the literature on the purification and characterization of an α-amylase produced by *P. brevicompactum* in solid-state fermentation (Balkan and Ertan, 2010). There are also reports of production, purification, and characterization of α-amylases from *P. expansum*

(Doyle et al., 1989) and by *P. olsonii* (Afifi et al., 2008). However, the production of amylase by *P. crustosum* is new data.

The *T. funiculosus* isolate (UEM 68) grew less than the *T. variabilis* isolate (UEM 53) but produced greater amylolytic activity (2.9 U/mL of medium), with insignificant amylase production by *T. variabilis* (0.39 U/ml medium) (Figure 2B). This is the first report of amylase production by *T. funiculosus*, although amylase production by other species of this genus is known. For instance, there are reports of production of an α-amylase by *Talaromyces pinophilus* (Xian et al., 2015).

The isolates of the genus *Fusarium* produced amylase at lower levels than *Aspergillus* and *Penicillium* isolates (Figure 2C). The isolate *F. oxysporum* UEM 102 produced the highest amylolytic activity (2.5 U/mL of medium). There are reports of the production of amylase by *F. oxysporum* in a solid medium (Kwon et al., 2007), but no enzyme has been purified or characterized. The other *Fusarium* species studied in this work produced insignificant amounts of amylolytic activity (Figure 2C). There are few reports in the literature on amylase production by *Fusarium* species. Among them are reports of α-amylase and glucoamylase production by *Fusarium solani* isolates (Kumar and Satyanarayana, 2009), but this species was not found among our isolates.

The isolates of *R. sibilis* UEM 16, *C. tenuissimum* UEM 52, *T. longibrachiatum* UEM 59, and *T. harzianum* UEM 70 produced amylase at deficient levels (Figure 2D). Also, they did not grow well in our liquid media (Figure 2D). The best producer was the isolate of *T. longibrachiatum* UEM 52 (Figure 2D). Amylase production by *C. cladosporioides* had already been reported in a solid medium (Abe et al., 2015), but no enzyme was purified or characterized. However, there are reports of purification and characterization of amylase from *T. harzianum* (Azevedo et al., 2000).

CONCLUSION

In conclusion, species of the genera *Aspergillus*, *Penicillium*, *Talaromyces*, *Fusarium*, *Rhinocladiella*, *Cladosporium*, and *Thichoderma* freeze-dried contaminants were identified. Amylase production by these species was studied in a liquid medium, and new amylase-producing species were found. In addition, amylase-producing isolates whose amylases had never been purified and characterized were also found. These isolates represent a biotechnological potential that can be studied in the future.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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SUPPLENTARY MATERIAL

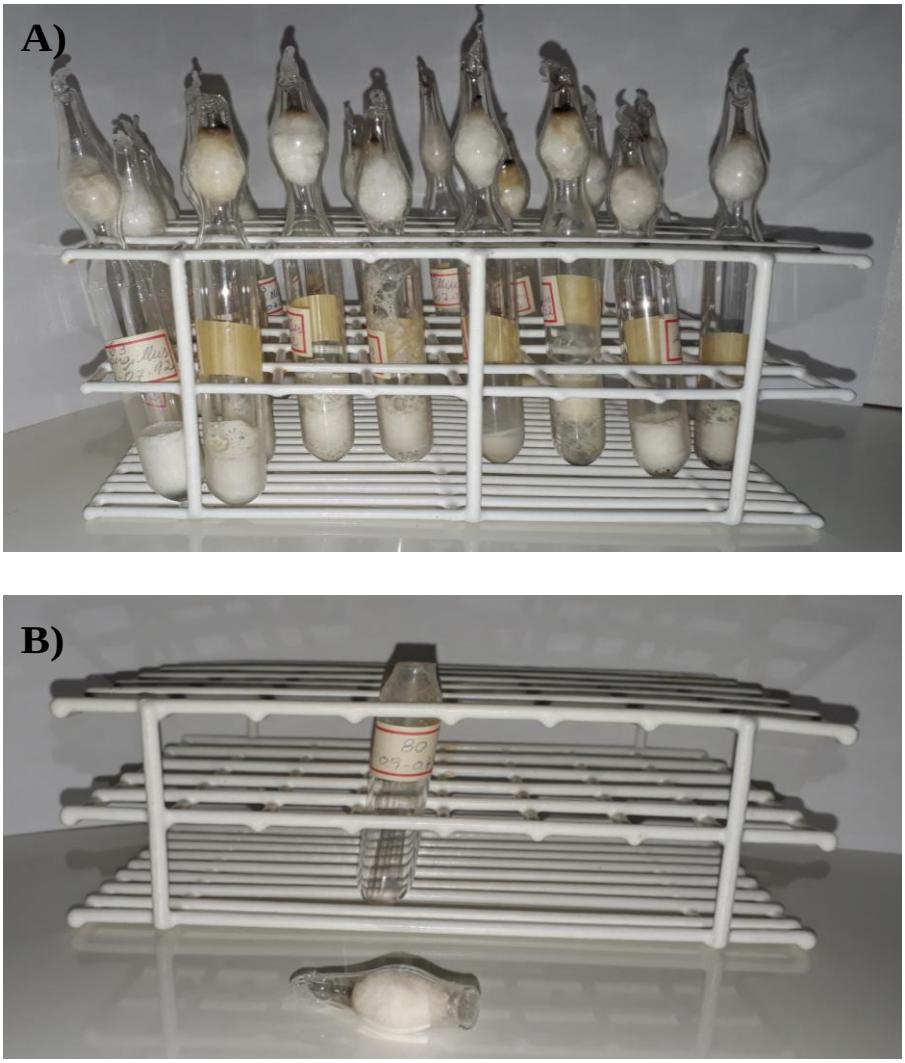

Figure S1. Fungal isolates stored by lyophilization. A) Glass ampoules with lyophilized fungal spores and/or hyphae prepared in the 1990s. B) Example of a broken ampoule for culture reactivation.

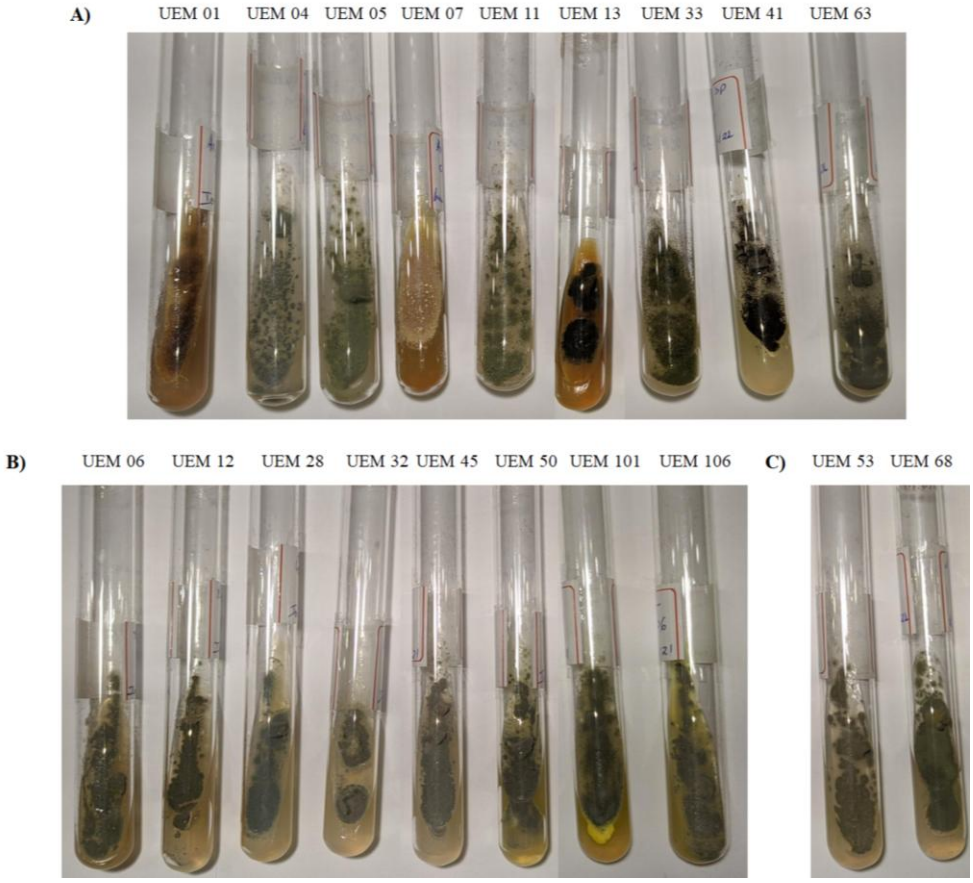

Figure S2. Isolates of A) *Aspergillus* spp. B) *Penicillium* spp. C) *Talaromyces* spp.

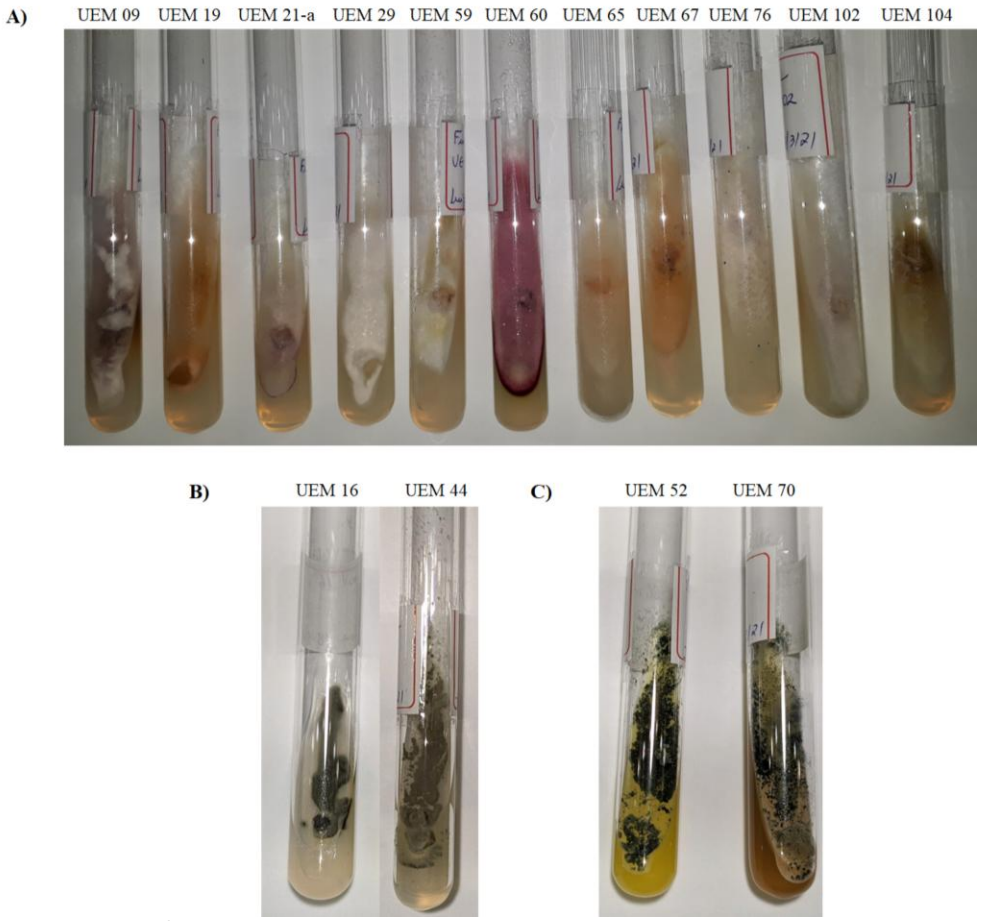

Figure S3. Isolates of A) *Fusarium* spp. B) *Rhinocladiella* sp. and *Cladosporium* spp. (UEM 16 and UEM 44). C) *Trichoderma* spp. (UEM 52 and UEM 70).