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IDENTIFICATION OF MEXICAN THERMOPHILIC AND THERMOTOLERANT FUNGAL ISOLATES

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ABSTRACT

Forty-four fungal strains capable of growing at temperatures above 50ºC were isolated from different samples of soil and coconut residues collected in Mexican tropical and subtropical regions. These thermophilic and thermotolerant fungal strains were identified by microscopical analysis using standard procedures. Three species were identified: *Rhizomucor pusillus* (Lindt)Schipper (19 strains), *Rhizopus microsporus* van Tieghem (6 strains), and *Aspergillus fumigatus* Fresenius (19 strains). Four strains identified as *Rhizopus microsporus* were ascribed to the variety *rhizopodiformis*; however, the other two strains showed new characteristics which require further analysis, such as a homothallic sexual reproduction. *Aspergillus fumigatus* was found in coconut residues as a common contaminant during the isolation of other thermophilic species. Strains were isolated from samples containing a high content of lipids (mainly from coconut coprah), and accordingly extracellular lipase biosynthesis was directly confirmed in Petri dishes for every strain.

Key words: Thermophilic fungi, isolation, taxonomy, lipases.

INTRODUCTION

There is a great interest to study thermophilic and thermotolerant fungi in comparison with bacteria. This interest is due mainly to the wider and more versatile fungal enzymatic battery, such as those activities involved in the degradation of organic compounds in nature. Additionally, thermophilic fungi are a potential source of enzymes with novel

properties for applications in industry [2, 7, 17].

Temperature plays an important and often decisive role in the distribution of organisms on the earth. Most fungi are mesophilic whose normal temperature for growth is about 5-35C. Some fungi are exceptionally capable of growing and living at high incubation temperatures (>50C), so they are called thermophilic or thermo-tolerant. "A thermophilic fungus is one that has a maximum temperature for growth at or above 50C and a minimum temperature for growth at or above 20C. Fungi…with maxima near 50C, but a minima well below 20C, we consider to be thermotolerant" [3]. Several studies describe new fungi discovered in extremely hot environments, such as thermal springs, industrial hot effluents, geysers and deep marine sources. These reports have shown that the maximum temperature for fungal growth is normally around 60C [10, 14]. Tropical regions are environments where thermophilic fungi can also be found. However, the isolation of this type of fungal strains from tropical regions has been poorly reported.

The total number of fungal species is estimated to be 1.5 millions; however, only about 69,000 have been described [1],. In 1886, Lindt reported a strain of *Rhizomucor pusillus* as the first thermophilic fungus ever described [9]. At present, only 50 thermophilic fungal species have been described [10]. There is accordingly an enormous potential for the isolation and characterization of new thermophilic fungi, involving applications in areas such as enzyme technology. This work deals with the isolation and identification of wild strains of filamentous fungi from tropical and subtropical regions in southern and central Mexico. The production of extracellular lipases with potential industrial applications in biotechnology was also studied.

MATERIALS AND METHODS

Culture conditions. Media having different composition were used at each experimental stage. 1) Medium for isolation (coconut coprah flour, 40 g/l; agar, 15 g/l; chloramphenicol, 250 ppm): the coconut coprah flour was previously boiled in 500 ml of water for 10 min, then filtered through a 200 µm mesh; the pH was adjusted to 5.6, and agar and chloramphenicol were added to this filtrate; then the volume was made up to 1.0 l, and a homogeneous mixture was obtained by gentle mixing. 2) Medium for identification: malt extract agar (MEA, DIFCO). 3) Mineral medium: composition as previously described [4], using sucrose as a carbon source at a final concentration of 30 g/l. 4) Medium for lipase detection: composition prepared according to Hankin and Anagnostakis [6], using peptone, 10 g/l; NaCl, 5 g/l; $CaCl_2$, 0.1 g/l; agar, 15 g/l; Tween 20, 10 ml/l.

Strains from culture collection. In order to carry out comparative studies, several strains from the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands) were included: *Rhizomucor pusillus* (CBS-183.67, CBS-253.53), and *Rhizopus microsporus* var. *rhizopodiformis* (CBS-607.73, CBS-609.81).

Origin of samples for the isolation of thermophilic fungi. Forty samples of coconut residues and soil were collected either from the Mexican tropical zone in Guerrero state or from two local industries ("Atoyac" and "La Corona", Guerrero, located in a subtropical region), which utilise coconut coprah as a raw material to manufacture soaps and detergents (**Table 1**).

Isolation and identification of thermophilic fungi. The isolation strategy of thermophilic

Table 1. Geographic origin of samples for the isolation of thermophilic fungi in this study. All strains were grouped according to their macroscopic features.

Sample	Origin	Physical type	Strains	Group
1	"Atoyac"	Soil	-	
2	"Atoyac"	Grated coconut	2a	I
3	"La Corona"	Coconut fragments	3a	III
4	"La Corona"	Coconut fragments	4a, 4b	I, III
5	"La Corona"	Coconut fragments	5a, 5b	I, III
6	"La Corona"	Coconut fragments	6a	I
7	"La Corona"	Coconut fragments	7a	I
8	"La Corona"	Coconut fragments	8a	I
9	"La Corona"	Soil and coconut fragments	9a	III
10	"La Corona"	Soil and coconut fragments	10a, 10b	I, II
11	"La Corona"	Soil and coconut fragments	11a	II
12	"La Corona"	Soil and coconut fragments	12a	I
13	Guerrero	White sponge	13a	II
14	Guerrero	Black sponge	-	
15	Guerrero	White grated coconut	-	
16	Guerrero	White grated coconut	16a	I
17	Guerrero	Brown grated coconut	17a	I
18	Guerrero	Coconut fragments	18a	III
19	Guerrero	Coconut fragments	19a, 19b, 19c	I, III, III
20	Guerrero	Coconut fragments	-	
21	Guerrero	Coconut fragments	-	
22	Guerrero	Coconut fragments	22a	I
23	Guerrero	Coconut fragments	-	
24	Guerrero	Soil	24a, 24b	I, III
25	Guerrero	Soil	-	
26	Guerrero	Soil	26a	III
27	Guerrero	Soil	27a, 27b	II, III
28	Guerrero	Soil	28a, 28b	II, III
29	Guerrero	Soil	29a, 29b	I, III
30	Guerrero	Soil	30a, 30b	III, III
31	Guerrero	Soil	31a	III
32	Guerrero	Soil	32a, 32b	I, III
33	Guerrero	Soil	33a, 33b	I, I
34	Guerrero	Soil	34a, 34b, 34c, 34d	I, III, III, III
35	Guerrero	Soil	35a	I
36	"Atoyac"	Coconut coprah cake	36a	II
37	"Atoyac"	Coconut coprah cake	-	
38	"Atoyac"	Coconut coprah cake	-	
39	"Atoyac"	Coconut coprah cake	-	
40	"La Corona"	Coconut coprah cake	-	

I= Strains having features similar to those reported previously for *Rhizomucor pusillus* (CBS-183.67, CBS-253.53).
II= Strains having features similar to those reported previously for *Rhizopus microsporus* (CBS-607.73, CBS-609.81).
III= Strains having features similar to those reported previously for *Aspergillus fumigatus***.**

Table 2. Culture phenotypes of thermophilic and thermotolerant strains grown on malt extract agar at 30ºC.

Culture characters	**Groups**			***Rhizomucor pusillus***		***Rhizopus microsporus***	
	I	**II**	**III**	**CBS 183.67, CBS 253.53 ***	**A**	**CBS 607.73 CBS 609.81 ***	**B**
Sporulation color	Light brown	Dark brown	Green	Light brown	Grey or sepia	Dark brown	Dark brown
Pigmentation of the medium	Brown	Brown	Beige-green	Brown	nr	Brown	nr
Growth in sucrose	+	-	+	+	+	-	nr
Height of aerial mycelium (mm)	1.90 ± 1	26 ± 5.6	1.0 ± 0.2	2 ± 0,1	2-3	22 ± 4	nr
Radial growth rate (mm/h)	0.26 ± 0.04	0.6 ± 0.04	0.2 ± 0.2	0.25 ± 0.02	nr	0.6 ± 0.02	nr
Lag phase (h)	25 ± 5.6	12 ± 2	18 ± 3.0	17 ± 0.35	nr	12 ± 2.4	nr

* Mean and standard deviation of two strains from culture collection.
A= Culture phenotypes according to Schipper [22].
B= Cultural characters according to Schipper and Stalpers [23] for *Rh. microsporus* var. *rhizopodiformis*.
nr= Not reported.

and thermotolerant fungi consisted of combining a selective medium and a high incubation temperature. The samples were aseptically inoculated in the isolation medium, and incubated for five days at 50C. Later, healthy fungal colonies were purified through ten successive subcultures. Each strain isolated was incubated at 30C for one week. Fungal species were identified by microscopic analysis using appropriate literature and standard taxonomic procedures.

RESULTS AND DISCUSSION

Strain isolation. The strategy and techniques used for the selective fungal isolation from 40 soil and coconut samples, allowed the isolation of 44 new thermophilic and thermotolerant fungi capable of growing at high temperatures (up to 50C). Moreover, all isolates were classified into three different groups, according to main macroscopic morphological features (**Table 2**). Analysis of these results revealed that coconut residues from the tropical region of Guerrero State only presented one thermophilic strain in every sample. In contrast, soil samples from the same region contained several strains. On the other hand, samples of coconut coprah cake from the local industries ("Atoyac" and "La Corona") did not have any thermophilic strains. Thus, among the samples analysed, tropical soils presented a higher variation in the fungal populations and were the best source for the isolation of thermophilic fungi.

Identification of isolated thermophilic and thermotolerant fungi

Culture characters. Groups I, II and III comprised strains having features similar to those reported previously for *Rhizomucor pusillus* (CBS-183.67, CBS-253.53), *Rhizopus microsporus* (CBS-607.73, CBS-609.81), and *Aspergillus fumigatus*, respectively (**Table 2**) [15, 16].

Table 3. Morphological characteristics used to identify levels of class (A), genus (B), and species (C) of thermophilic and thermotolerant fungi isolated in this work.

A

Groups I-II	**Group III**
Hyphae not septated Asexual spores contained in a (sporangiospores)	Hyphae septated Asexual spores not formed in a sporangium sporangium or ascus Sexual reproduction unknown
➔ ***Zygomycetes***	➔ ***Deuteromycetes***

B

Group I	**Group II**	**Group III**
Hyaline hyphae Isolated and ramified sporangiophore Stolons and rhizoids sometimes present Sporangia without apophysis Asexual smooth spores Thermophilic	Abundant mycelium Radial growth rate extremely fast Pigmented and non-ramified sporangiophores with rhizoids and stolons Sporangia brown-black, globular with apophysis Columella subglobose Asexual smooth spores	Raising conidiophores and blown up in vesicles Vesicle containing phialide which produces conidia associated in chains
➔ ***Rhizomucor***	➔ ***Rhizopus***	➔ ***Aspergillus***

C

Group I	**Group II**	**Group III**
Sporangiophores ramified against the height, monopodial or sympodial Heterothallic sexual reproduction	Good growth at 50°C	Dark green conidiophores, arranged in compact columns Spatula-shaped vesicle Phialides directly inserted in the top of the vesicle Conidia subglobose with granulose surface Fast growth at 37°C
➔ ***Rhizomucor pusillus***	➔***Rhizopus microsporus***	➔***Aspergillus fumigatus***
	Strains 10b, 27a, 28a, 36a Heterothallic reproduction	Strains 11a, 13a Homothallic reproduction
	Rh. microsporus var. ***rhizopodiformis***	Variety unknown

Morphological characteristics. Main microscopical characteristics for every group (I-III) are shown in **Tables 3-4.**

Group I: Morphological characteristics and dimensions of aerial hyphae from 19 wild strains belonging to this group were close to those observed in strains of *Rh. pusillus* (CBS-183.67, CBS-253.53). They were also similar to those characteristics reported by Schipper [15] for *Rh. pusillus* (**Tables 3-4**). Strains of *Rh. pusillus*, as well as wild strains, were thermophilic as good growth was observed at 50C (each strain colonized the Petri dish within 3 days). By

Table 4. Comparison of microscopical structures from strains belonging to Groups I (*Rh. pusillus*) and II (*Rh. microsporus*), and those from culture collection, which were grown on malt extract agar at 30°C for a week.

	Rhizomucor pusillus			***Rhizopus microsporus***				***Rh. homothallicus***
Strains	19 wild	CBS-183.67	**D1**	10b, 27a,	11a, 13a	CBS-607.73	**D2**	**D2**
Structures (μm)		CBS-253.53*		28a, 36a		CBS-609.81 *		
Sporangia diameter	45 ± 6	47 ± 7	50	74 ± 4	75 ± 11	69 ± 6	80	100-125
Columella								
Width	25 ± 3	32 ± 11	35	44 ± 11	46 ± 15	45 ± 19	nr	>50
Length	29 ± 3	37 ± 9	45	52 ± 11	61 ± 22	54 ± 21	nr	nr
Spore diameter)	3-4	3-4	3-5	5-6	5-6	5-6	5-6	7.5-8
Sporangiophore length	nr	nr	nr	266 ± 42	346 ± 41	254 ± 51	150-500	>850
Zygosporediameter	65	70	70	nd	34	nd	nr	>125

* Mean and standard deviation of two strains from culture collection.

D1= Dimensions reported by Schipper [22].

D2= Dimensions reported by Schipper and Stalpers [23] for *Rh. microsporus* var. *rhizopodiformis*.

nr= Not reported.

nd= Not determined.

contrast, these strains showed poor growth at 20C, developing mycelial colonies of only 5mm after 13 days.

Group II: Wild strains belonging to this group showed a strong similarity to the general morphological characteristics observed and reported [23] from strains CBS-607.73 and CBS-609.81 of *Rh. microsporus* (**Tables 3-4**). This was so particularly for strains 10b, 27a, 28a and 36a. These strains were accordingly identified as *Rh. microsporus* var. *rhizopodiformis*. Moreover, the homothallic sexual reproduction showed by strains 11a and 13a (**Fig. 1**) has not previously been reported from *Rh. microsporus* var. *rhizopodiformis*. This type of reproduction is known from *Rh. homothallicus*; however, this species is not capable of growing at temperatures higher than 50C. Furthermore, shape and size of aerial hyphal structures in strains 11a and 13a were different from those of *Rh. homothallicus* (**Table 4**). The zygospore size of *Rh. homothallicus* is usually bigger (>125 μm) [16] than those of strains 11a and 13a (34μm). Based on these data, it is possible that strains 11a and 13a belong to a new variety or even to a new species, which requires further studies. Strains from Group II can be considered as thermotolerant as they were capable of growing at 50C and 20C, covering the entire culture medium in the Petri dish within 3 and 6 days, respectively.

Group III: The culture and morphological characteristics of 19 strains studied were identical to those reported for *Aspergillus fumigatus* (**Tables 2-3**), so there was no need of comparative studies with standard strains. Strains belonging to this group were also thermotolerant because they were capable of growing at 50C and 20C, covering the entire culture medium in the Petri dish within 7 and 23 days, respectively.

Despite of having carried out studies of materials from tropical and subtropical

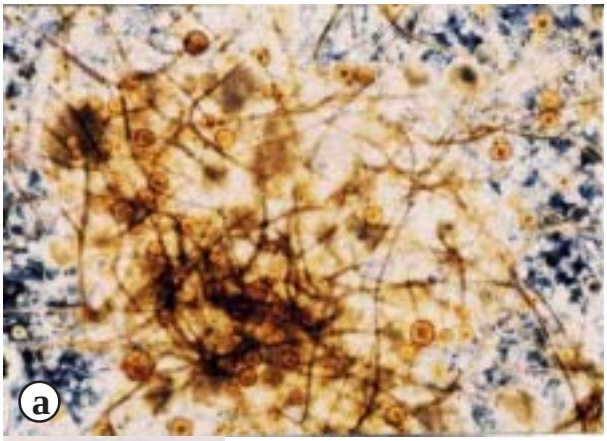

Fig. 1. Homothallic sexual reproduction of strain 11a (*Rhizopus* sp.). Zygospores obtained after one week on malt extract agar at 30°C. Microscopical magnification: 10x (a) and 100x (b).

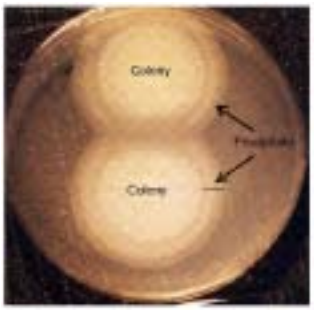

Fig. 2. Biosynthesis of extracellular lipases of thermophilic fungi. Strain 8a (*Rhizomucor pusillus*) is shown, confirming the biosynthesis by the precipitate (calcium laureate) formed around the fungal colony on agar plates.

regions, only three fungal thermophilic and thermotolerant species were found: *Rhizomucor pusillus* (Lindt)Schipper, *Rhizopus microsporus* van Tieghem, and *Aspergillus fumigatus* Fresenius, representing 43%, 14% and 43%, respectively, of mycobiota isolated. In fact, these species have been isolated from a wide variety of environments, such as guayule plants (*Parthenium argentatum*) [3], organic composting [2], cereal grains [5], soil [17], air [18], seaweed enriched environments [11], poultry feed products [12], and palm oil [13]. Ecosystem diversity and isolations strategies culture media, temperatures) are important factors affecting the isolation of thermophilic microbiota.

During the isolation of thermophilic fungi, *A. fumigatus* is commonly found as a contaminant. This fungus is a pathogen affecting mammals through aspergillosis and mycotoxins [8]. For this reason, we discarded isolates found in this study. However, the high incidence of *A. fumigatus* in coconut coprah could be a potential health problem in regions where this residue is used as a raw material for the industry or animal feed. Safety precautions are recommended.

Lipase (esterases) synthesis was confirmed in 44 isolates, and the exogenous production of this enzyme was made evident by the precipitate present around and below the fungal colony grown on agar medium (**Fig. 2**). Samples studied (mainly coconut residues) contained high amounts of lipids, so this is an appropriate strategy for finding new strains producing lipases. Further studies are now being carried out in order to characterize the stability and production lipases in thermophilic strains isolated.

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