JOURNAL OF PURE AND APPLIED MICROBIOLOGY, May 2015. Vol. **9**(Spl. Edn. 1), p. 131-138

Screening of Sonoran Desert Fungal Strains for Feruloyl Esterase Activity

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(Received: 18 March 2015; accepted: 20 April 2015)

During last two decades, feruloyl esterases have been intensely studied for industrial or medical applications. Also screening of microbes from thermal and extreme environments as source of this kind of enzymes with improved properties is an active research field. In this context, screening and selection of fungal strains, for feruloyl esterase activity, were developed at Sonoran Desert, one of the hottest and driest regions of America. Sampling was carried out from soil and plant residues decomposing on soil. Samples were suspended in water, diluted, spread onto agar and cultured at 40 °C. Isolated strains were spread onto selective agar, choosing those with feruloyl esterase hydrolytic halo/colony diameters ratio>2. Selected strains were identified by molecular methods and their secretomes were assessed for methyl hydroxy cinnamates specificity. Twenty thermotolerant fungal strains, showing high feruloyl esterase activity, were found and identified as *Aspergillus flavus* (2), *Aspergillus fumigatus* (2), *Aspergillus tamarii* (2), *Aspergillus terreus* (11), *Neurospora tetrasperma* (1) and *Phialophora alba* (2). At least 10 of those shown 2.5 to 3.8 higher feruloyl esterase activities, than *Aspergillus terreus* ATCC1012, a mesophile strain. Secretomes exposed scarce specificity for substrates. Selected feruloyl esterases-producing strains show high potential to be used as biotechnological tools.

Key words: Desert climate, Hydroxycinnamoyl esterases, Fungi.

Thermostable enzymes have been a topic of intense research during the last two decades, because their increased reactivity at high temperatures (70–100 °C) and long-term stability at ambient temperature make them suitable for cost effective industrial processes[1, 2, 3]. This also stimulated the screening and isolation of microbes from thermal environments as sources of new enzymes that could significantly increase the window for enzymatic bioprocess operations[4].

One of the hottest regions in North America is the Sonoran Desert, where temperatures of up to 50 °C have been recorded, although is considered as one of the wettest deserts and averages from 125 mm of rain a year[5]. Sonoran Desert has a great biological diversity and geological alternation, where many species have evolved to have specialized adaptations to the desert climate[6]. In Mexico, it covers more than half of the State of Sonora, two-thirds of the peninsula of Baja California and is present in all the islands of The Sea of Cortes. Of the seven subdivisions that comprise it, four are located in Sonora: the highlands of Arizona; the plains of Sonora; the Central Gulf Coast and the lower Colorado River Valley[6].

The feruloyl esterases (FAE) (EC 3.1.1.73) represent a diverse group of hydrolases that catalyze the cleavage and formation of ester bonds

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between polysaccharides of the cell wall of plants and phenolic acids[7]. Over the past decade, research carried out on microbial FAEs has increased intensely. The number of papers related to isolation, purification and characterization of FAEs and patents for applications in biotechnological processes, both in industrial and medical areas grew exponentially[7, 8]. Nowadays, the interest towards FAEs with new properties continues and represents an important area of research[7, 9].

According the current importance of FAEs and the particular characteristics of the Sonoran Desert, the objective of this work was to develop a screening of fungal strains for feruloyl esterase activity from samples collected from ten regions from Sonora State, Mexico.

MATERIALS AND METHODS

Sample collection

Climate charts of Sonora State, Mexico, were used in order to choose the hottest regions during the year 2009. Three regions, with records of temperatures between 45 to 50 °C, were selected. Sampling was carried out from soil and plant residues decomposing on soil with or without macroscopic evidence of fungal growth. In the case of soil samples, around 100 g from the depth of 0–10 cm and from the depth of 20–30 cm were randomly collected. All samples were transported to the laboratory in sterile zip lock bags on the same day.

Isolation and cultivation of fungi

Petri dishes containing potato dextrose agar (PDA, procured from Difco) were employed to isolate strains. 10g of soil sample were diluted in 125 ml of sterile tween 80 solution (0.05% v/v) by vortexing. 1.5 g of milled and sieved sample, containing plant residues, was diluted in 13.5 ml of sterile tween 80 solution (0.05% (v/v)) by vortexing. Then, 0.5 ml of serial diluted supernatant was spread onto the surface of the medium and incubated at 40°C for 3 d. Isolated strains were preserved at 4 °C in sloped tubes containing PDA, covered with sterile mineral oil. They were used to propagate fungi on PDA Petri dishes used as inocula.

Screening of fungal strains for FAE activity

Screening was performed using a minimal medium described by Rodríguez *et al.*, (2006) with little modifications in carbon source. Petri dishes containing selective agar (1, 2 or 3 g/l of ethyl ferulate, 4 g/l of urea, 5 g/l of K_2HPO_4, 1 g/l of $MgSO_4$, 15 g/l of bacteriological agar and 1% (v/v) bromocresol purple aqueous solution (0.1% (w/v), pH 6.5) were prepared. The released ferulic acid from ethyl ferulate by FAE activity decrease pH, discoloring or changing to yellow the purple color of the medium. Magnesium sulfate and potassium phosphate dibasic were procured from J.T. Baker; urea, bromocresol purple and ethyl ferulate from Sigma-Aldrich-Fluka (Mexico) and bacteriological agar from Difco.

Inoculum was picked with sterile wooden stick and transfer by touching each dish. For preliminary screening, plates were incubated at 40°C for 5 d and then, hydrolytic-halos and colony diameters were measured in order to establish halo/colony ratios. For secondary screening *Aspergillus terreus* ATCC 1012, a known FAEs-producing strain was included as positive control. Also, *Aspergillus niger* ATCC 16888 was used as a second control because it is a known FAEs-producing strain which has been extensively studied[11, 12]. Pre-selected and mesophilic control strains were incubated both at 25 and 40°C in order to compare efficiency of FAEs production.

For classifying the selected FAEs producing strains as mesophilic, thermotolerant or thermophilic according Mouchacca (1997)criterion, they were inoculated in a PDA medium using 24 wells microplates, incubated at 20°C and 50°C for 5 d.

Molecular identification of isolates

All strains were grown on PDA at 30°C for 72 h. Subsequently, fungal mycelium was collected by scraping the surface of the solid culture and DNA was extracted with the commercial kit Dneasy® Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. PCR was carried out with Illustra ™ PureTaq Ready-To-Go PCR Beads (GE Healthcare, Uppsala, Sweden) on a Veriti™ 96-well thermal cycler (Applied Biosystems, Foster City, USA). Primers ITS1 and ITS4 were employed to amplify an approx. 600 bp fragment from the ribosomal gen cluster under the same reaction conditions described elsewhere[14]. PCR products were sent to Macrogen USA Corp. for sequencing of both strands. Consensus sequences were obtained with

the CLC Main Workbench 5.5 software package (CLCBio, Aarhus, Denmark) and compared to recorded sequences from GenBank database using the BLAST algorithm[15].

Solid fermentation and secretome extraction

Selected strains for FAE activity were culture on solid-state fermentation. 125 ml Erlenmeyer flasks containing 7 g of a milled and sieved (40 mesh) mixture of corn bran and sugar cane bagasse (2:1 w/w) were sterilized at 121°C, 15 min. Then, 4.5 ml of inoculum (3×10^7 spores/ml) and 8.5 ml of minimal medium (4 g/l of urea, 5 g/l of K_2HPO_4, 1 g/l of $MgSO_4$) were added and incubated at 30°C for 48 h. Then, cultures were dried at 30°C for 24 h and milled and sieved (60 mesh). 1 g of sieved material was extracted with 5 ml MOPS buffer (2.5 mM, pH 7.2) by vortexing during 1 min and steeping for 10 minutes in ice bath. This mixture was centrifuged at 10000 ×g, 4°C, for 10 min and recovered supernatants containing secretomes were kept at 4°C for further analyses.

FAE substrate specificity

Ninety six well plates containing 100 µl of methyl ferulate, methyl caffeate, methyl *p*-coumarate and methyl sinapinate solutions (0.1% (w/v)) and 1% (v/v) bromocresol purple aqueous solution (0.1%(w/v)) were added with 50 µl of secretomes. Discoloration, or change to yellow, in the purple color of the medium after 5 min of secretome addition was considered positive for FAE activity. Depol™ 740L (Biocatalysts Ltd., Wales, UK), a commercial enzymatic extract with feruloyl esterase activity was used as positive control.

RESULTS

Preliminary screening of fungal strains for FAE activity

Sampling localities were grouped into three regions according their proximity among them and their location in the State of Sonora, Mexico (fig 1). A total of 205 fungal strains were isolated from the collected samples. Although, more than 90% of the strains were able to growth in the culture medium containing ethyl ferulate as sole carbon source, just those with a halo/colony diameter ratio > 2, at least at one of the three concentrations of ethyl ferulate employed, were considered as good candidates for FAE activity. The halo/colony diameter ratio is a broad accepted criterion for selecting strains which produce %enzymes of

Table 1. Georeferenced sampling places and molecular characterization of the selected isolates.

Region	North latitude	West longitude	Isolated	Reference strain	Identity (%)
I	30°09'54.10"	111°14'44.30"	B80	*Aspergillus fumigatus(ATCC1022*	99
	28°00'55.90"	111°03'4.10"	B94	*Aspergillus terreus(ATCC1012) (ATCC1012)*	100
	28°04'05.50"	110°45'12.90"	B102	*Aspergillus terreus(ATCC1012)*	100
	28°18'59.60"	111°14'40.60"	B17	*Aspergillus terreus(ATCC1012)*	100
			B13	*Phialophora alba(ICMP17034)*	100
	28°48'09.70"	111°12'13.20"	A70	*Aspergillus terreus(ATCC20526)*	99
			A144	*Aspergillus terreus(ATCC20526)*	100
			A62	*Neurospora tetrasperma(ATCC MYA-4615)*	99
			B9	*Aspergillus terreus(ATCC1012)*	100
	28°33'50.80"	111°00'28.40"	B65	*Aspergillus flavus(ATCC20043)*	99
	29°16'20.30"	111°05'43.60"	B23	*Aspergillus terreus(ATCC1012)*	99
			B38	*Aspergillus fumigatus(ATCC1022)*	99
II	29°31´48´´	109°31´48´´	CG1-2	*Aspergillus terreus(ATCC20526)*	100
			CG5-2	*Aspergillus terreus(ATCC1012)*	100
			CG16	*Aspergillus tamarii(NRRL427)*	100
			CG4	*Phialophora alba(ICMP17034)*	100
			CG4-1	*Aspergillus tamarii(NRRL427)*	100
III	31°31'55.01"	113°25'40.05"	SB1	*Aspergillus terreus(ATCC1012)*	99
			B10	*Aspergillus terreus(ATCC1012)*	100
			SB9	*Aspergillus flavus(ATCC20043)*	99

interest[12, 16, 17]. According to this condition, 20 fungal strains were selected and classified as thermotolerant since they were able to growth at 20 and 50°C, following the criterion suggested by Mouchacca (1997). Growth at 20°C was higher than growth at 50°C after 5 d of incubation. Twelve strains were selected from region I, five from region II and three from region III. From all selected strains, just three were isolated from soil samples (0–10 cm depth) in the region I and the rest of strains from plant material.

It is known that FAEs are involved in the degradation of lignocellulosic materials[18], thus most selected fungal strains were isolated from this kind of material; even the strains isolated from soil which include samples collected from the surface, rich in products resulting from decomposition of dead vegetal matter. Moreover, 85% of the selected strains come from region I and region II where a great variety of vegetation is found, including trees as "mesquite" (*Prosopis* sp.), sarcocaule shrub and microphyll desert shrub.

Molecular characterization

Of the 20 selected FAEs-producing strains, two were identified as *Aspergillus flavus*, two as *Aspergillus fumigatus*, two as *Aspergillus tamarii*, eleven as *Aspergillus terreus*, one as *Neurospora tetrasperma* and two as *Phialophora alba* (table 1).

The presence of a large number of strains belonging to the genus *Aspergillus* coincides with the report of Bonnin *et al.,* (2008), which points out species of *Aspergillus* genus as efficient producers of enzymes degrading plant cell wall. Other species of *Aspergillus* reported as producers of FAEs are *A. niger*, *A. awamori*, *A. nidulans*, *A. oryzae*, *A. tubingensis*, *A. flavipes* and *A. foetidus*[7, 20]. The existence of *Aspergillus terreus* and *Aspergillus fumigatus* in the Sonoran Desert areas were already reported by Cruz *et al.,* (2005) and Kithsiri *et al*., (2003).

Higher ratios of native strains at 40°C suggest adaptive modification to environmental conditions that promote higher production of

Table 2. Selection of feruloylesterases-producing strains using ethyl ferulate as substrate.

Isolated	[a]Ratio					
	25 °C			40 °C		
	[b]EF(1 g/L)	EF(2 g/L)	EF(3 g/L)	EF(1 g/L)	EF(2 g/L)	EF(3 g/L)
A. niger(ATCC 16888)	0	0	0	0	0	0
A. terreus(ATCC 1012)	2.8	4	3.25	2	3	3
B102	6.25	3.75	4	1.25	9.37	11.25
SB1	4	2	1.25	4	3.5	3
B23	5	2	2	1	7.25	6
B17	0	1	1.25	6	5	2
A70	0	2.5	1	5.5	3	4
CG1	0	1	1	5.5	7.5	3.5
A144	0	2.5	1	4	4	3
B10	2.5	3.25	1.25	1.75	11.25	11.25
CG5-2	3	1	1.75	1.75	9.37	8.75
B94	2.5	1	1	1	10.25	5.75
B9	2	3	4.25	5.25	5.5	7.25
B80	5	3.5	4.25	1.25	11.25	8.75
B38	5.5	4.5	5	1.25	11	8.75
SB9	3.5	3.25	3.75	1.25	10	6
B65	2	3	4.5	1.25	5	5.75
CG16	7.5	8.25	8.25	1.75	10	8.5
CG4-1	7.5	7.25	9.25	1.25	7.5	10
CG4	3.75	2.5	2.5	1	3.5	2.25
B13	3.75	2.5	2.5	1	2.5	2
A62	0	1	1	1	2.5	1.75

[a]Ratiobetween hydrolytic halo and colony diameters; [b]Ethylferulate

enzymes with FAE activity and/or enzymes with higher catalytic efficiency. It is also expected a higher enzymatic thermostability according the sources, such was observed with enzymes isolated from thermophiles[23].

Phylogenetic tree

The phylogenetic tree shows the selected strains which were identified as feruloyl esterase producers (Fig. 2). It should be noted that the fungal strains identified as *A. terreus* are divided into two groups. The first one is composed by strains SB1-2, CG5-2, B94, B9, B17, B102, B10 and B23, being the last the most different among them. This group turned out to be similar to the strain *A. terreus* ATCC1012. In the case of the second group, which is composed by strains CG1-2, A144 and A70, it was proven to be similar to the strain of *A. terreus* ATCC20526, being A70 strain the most different among them.

Secondary screening of fungal strains for FAE activity

Since 90% of the selected strains during the preliminary screening correspond to *Aspergillus* genus, for secondary screening *Aspergillus terreus* ATCC 1012 and *Aspergillus niger* ATCC 16888 were included as controls. Table 2 shows FAE activity tests developed at 25 and 40°C, at three ethyl ferulate concentrations, in order to establish differences on growth, enzymatic activities and possible advantages of native strains compared with controls. At any ethyl ferulate concentration, *A. terreus* ATCC 1012 established higher ratios at 25° C than 40°C since it is considered a mesophile strain. However, *A. niger* ATCC 16888 was unable to growth, even at the lowest ethyl ferulate concentrations because this strain may not produce the amount or type of enzymes required to hydrolyze and metabolize ethyl ferulate which also is toxic for many species of fungi. The antimicrobial activity of cinnamic acid derivatives has been largely reported[24, 25, 26]. At 25°C and at any ethyl ferulate concentration, native strains of *Aspergillus* genus and *Phialophora alba* established similar or lower ratios than *A. terreus ATCC* 1012. Just *A. tamarii* strains showed higher ratios (80–180%) than control. However, at 40°C, native strains increased their ratios between 70 to 800%, while *A. terreus* ATCC 1012 decreased around 8 to 30%. Stand out some *A. terreus* native strains, like B102, B23, CG1, B10, CG5-2, B94, and *A. flavus, A. fumigatus*, and *A. tamarii* strains which halo/colony diameter ratios results around 2.5 to

Table 3. Feruloyl esterase activity of the selected strainson different substrates

Isolated	Strains	[a] MF	[b] MC	[c] MpC	[d] MS
B102	*Aspergillusterreus*	+	-	+	+
SB1	*Aspergillusterreus*	+	+	+	+
B23	*Aspergillusterreus*	+	-	+	-
B17	*Aspergillus terreus*	+	+	+	+
A70	*Aspergillus terreus*	+	+	+	+
CG1	*Aspergillus terreus*	+	+	+	+
A144	*Aspergillus terreus)*	+	+	+	+
B10	*Aspergillus terreus*	+	-	+	+
CG5-2	*Aspergillus terreus*	+	-	+	+
B94	*Aspergillus terreus*	+	+	+	+
B9	*Aspergillus terreus*	+	+	+	+
B80	*Aspergillus fumigatus*	+	+	+	+
B38	*Aspergillus fumigatus*	+	-	+	+
SB9	*Aspergillus flavus*	+	+	+	+
B65	*Aspergillus flavus*	+	+	+	+
CG16	*Aspergillus tamarii*	+	+	+	-
CG4-1	*Aspergillus tamarii*	+	-	+	-
CG4	*Phialophora alba*	+	+	+	+
B13	*Phialophora alba*	+	+	+	+
A62	*Neurosporatetrasperma*	+	+	+	+

[a]Methylferulate; [b]Methylcaffeate; [c]Methyl *p*-coumarate; [d]Methylsinapinate

3.8 times higher than *A. terreus* control.

FAE substrate specificity

Feruloyl esterase specificity was tested using four synthetic substrates as shown in table 3. Such substrates are used in the classification of Crepin *et al.,* (2004) which states that there are four FAEs types (A, B, C, D). This classification is based on the substrate specificity and primary sequence. In the case of *A. terreus*, secretomes obtained from B102, B23, B10 and CG5-2 strains were not able to hydrolyze methyl caffeate suggesting type A FAEs production which have preference for substrates whose substituents are methoxy groups. However these strains showed the largest halo/diameter colony ratios (table 3). On the contrary, most of the strains with the lowest halo/colony ratios, produced secretomes capable of hydrolyzing the four substrates showing that contain unspecific FAEs (type C or D) or FAEs mixtures. The larger halo/colony ratios may be due to a higher enzyme specificity and thus to a higher catalytic efficiency instead to an increased enzyme production.

Fig. 1. A: Map of México. B: Map of Sonora State, including the regions where FAEs-producing strains were isolated.

In the case of *A. tamarii* CG16, strain secretome was not able to hydrolyze methyl sinapinate suggesting type B FAEs production, since they have a strong preference for hydroxylated substrates. Just in two cases (B23 and CG4-1), methyl sinapinate and methyl caffeate were not hydrolyzed at our analysis conditions,

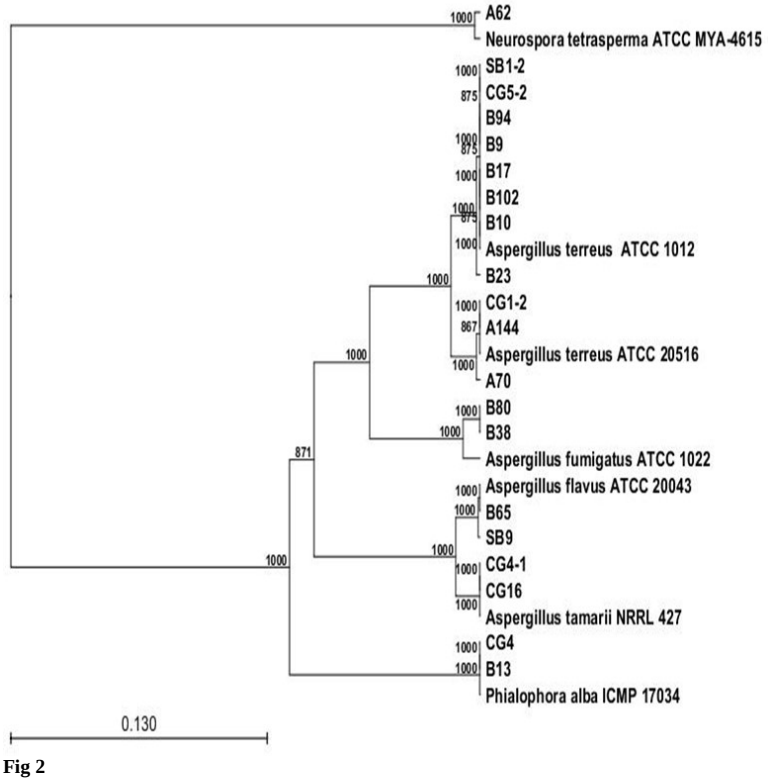

Fig 2

Fig. 2. Phylogenetic tree for FAEs producing strains from the Sonoran Desert collection.Numbers at the nodes are bootstrap values (of 1,000 replicate)

probably due to a low enzyme concentration produced by these strains.

Several strains of *Aspergillus terreus* have been reported as producers of different types of FAEs that can be induced by the employed substrate. *Aspergillus terreus* strain MUCL35566 was reported as a producer of type B FAE when grown on sugar beet pulp as it showed activity on methyl caffeate but not on methyl sinapinate. Also, *A. terreus* strain MUCL35503 was reported as type B FAEs producer when it was grown on oat xylan. However, when this strain was grown on sugar beet pulp, hydrolytic activity on methyl caffeate and methyl sinapinate was observed, suggesting type A and B FAEs production[28]. It was reported that *A. terreus* strain GA2 exhibits FAE activity, although classification was not included[29]. On the other hand, it is also reported that *A. terreus* MTCC11096 produces three types of FAEs, one type A and two type C[30]. Bouzid *et al*., (2006) found that *Aspergillus flavus* strain BRFM821, grown on sugar beet pulp, presented activity on methyl sinapinate and methyl caffeate as substrates, suggesting the production of type A and B FAEs. When *A. flavus* strain BRFM821 was grown on oat xylan, it produced just a type B FAE which showed activity on methyl caffeate but not on methyl sinapinate. In the case of *Aspergillus tamarii*, three strains have been reported producing type B FAEs (*A. tamarii* MUCL14048, *A. tamarii* MUCL 18828, *A. tamarii* MUCL 43440) when they were grown on sugar beet pulp and oat xylan[28]. No reports about FAEs production by *Aspergillus fumigatus*, *Neurospora tetrasperma* and *Phialophora alba* were found and this work is the first that notice these fungal species as FAEs-producing strains. Currently, secretomes are being employed to obtain zymograms in order to establish the number of FAEs and FAE's specificity.

CONCLUSION

Results show that the ecosystem of the Sonoran Desert is a good source of FAEs-producing thermotolerant fungi, which show advantages over other FAEs-producing mesophile strains. The characterization of FAEs produced and their applications are interesting and promising topics for future studies due to characteristics of the environment in which microorganisms develop and the diversity of types of FAEs that could be induced by specific substrates and culture conditions.

ACKNOWLEDGMENTS

The authors acknowledge technical assistance of the Q.B. Socorro Vallejo Cohen, M.C. César Oropeza and M.C. Aldo Gutiérrez. FJ Bacame-Valenzuela thanks to the Mexican Council for Science and Technology (CONACYT) for his PhD scholarship (No. 326666). The study was financially supported by FOMIX-CONACYT-Jalisco (No. 2012-07-190698).

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