World J Microbiol Biotechnol (2010) 26:2105–2111
DOI 10.1007/s11274-010-0389-4

SHORT COMMUNICATION

Screening for alkaline keratinolytic activity in fungi isolated from soils of the biosphere reserve "Parque Costero del Sur" (Argentina)

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Received: 21 December 2009 / Accepted: 12 March 2010 / Published online: 27 March 2010

Abstract A screening was carried out on 69 fungal strains isolated from alkaline-calcareous, neutral and alkaline-sodic soils, as well as from their associated plant material, to determine their ability to grow at alkaline pH. A total of 32 fungi were selected for their ability to produce alkaline keratinase activity in submerged shaken cultures supplemented with soybean meal (SM) and tryptone and on cow hair (CH) under solid state fermentation conditions. Although several fungal strains produced keratinolytic activity on both SM and CH, they differed in the levels detected. Among them, *Aspergillus niger*, *Cladosporium cladosporioides*, *Metarrhizium anisopliae*, *Neurospora tetrasperma* and *Westerdikella dispersa* were the best producers, with levels higher than 1.2 U ml^{-1}. Different fungal species are here reported for the first time for their ability to produce keratinolytic activity at alkaline pH.

Keywords Alkaline keratinases · Argentina · Fungi · Soil · Biosphere reserve · Parque Costero del Sur

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Introduction

Keratins, which are structural proteins that represent the main constituents of the skin of vertebrates and appendages such as nails, hair, feathers, and wool, are water insoluble and extremely resistant to degradation (Gradisar et al. 2005).

Degradation of keratinous material has mostly been associated with the activity of dermatophytic fungi, yeast and bacteria. A lot of information is available on the abilities of these organisms to produce keratinase enzymes and on the catalytic properties of these enzymes (Friedrich et al. 1999; Mangiaterra et al. 2000). However, there are relatively few reports on the production and characterization of the keratinases produced by non-dermatophytic fungi, including those associated with extreme environments under stressful conditions (Gradisar et al. 2005; Elíades 2009). An example of this kind of environment is the native xerophilic forest dominated by the tree species *Celtis tala* Gill ex Planch (Ulmaceae) and *Scutia buxifolia* Reiss (Rhamnaceae) and its associated *Distichlis spicata* (L.) Greene grassland, located in the eastern region of Buenos Aires province (Argentina, Arturi et al. 1996). This assemblage of ecosystems is part of the "Parque Costero del Sur", a biosphere reserve with biogeographic, landscape, cultural and historical features (MAB-UNESCO, Goya et al. 1992; Crous et al. 2005). This area is characterized by different soil types, including alkaline-calcareous

(Rendolls), neutral (Argialbolls), and alkaline-sodic (Natracualfs) soils. These soils might be a source for the isolation of fungi able to produce enzymes tolerant to different pH values and/or highly active at extreme pH values. These enzymes can be used as biotechnological tools and have potential applications in the leather industry waste treatment (Galarza et al. 2007).

The aim of this study was to analyse the ability of 69 fungal strains, isolated on agar-medium at several pH values from various soils or from their plant material collected at the Biosphere Reserve "Parque Costero del Sur" (Argentina), to grow at alkaline pH, and on the ability of some strains to produce keratinase enzymes with activity at alkaline pH, when grownon medium with soybean meal and tryptone (SM) and on cow hair (CH) under various culture conditions, was also evaluated.

Materials and methods

Fungal isolation

The strains used for this work (Table 1) were isolated from alkaline-calcareous (Rendoll), neutral (Argialboll) and alkaline-sodic (Natracualf) soils, as well as from organic material such as decaying leaves of *Distichlis spicata* (Poaceae) collected at the Biosphere Reserve "Parque Costero del Sur" (MAB-UNESCO), located in eastern Buenos Aires province, Argentina (35°11′S, 57°17′W). The soil samples were taken from either the superficial horizon or that deeper than 20 cm. They were then processed as previously (Elíades et al. 2006a) and incubated on agar-medium at several pH values (6, 8, or 11). Similarly, *D. spicata* debris was processed as previously (Elíades et al. 2007). Plates inoculated with soil particles or debris were incubated at 25°C and observed microscopically after 5–7 days or at 1-week intervals for fungal development and isolated in axenic culture. Original taxonomic papers based on cultural and morphological features as well as the work by Domsch et al. (1993) were used to identify sporulating fungi (Cabello and Arambarri 2002). Stock cultures were kept at 4°C on 2% (w v^{-1}) agar-malt extract slants and lyophilized according to Smith and Kolkowski (1996), being then deposited in the culture collection of the Instituto Spegazzini, UNLP, La Plata, Argentina (LPSC).

Screening for fungal growth at alkaline pH

All fungi isolated were inoculated according to Saparrat and Guillén (2005) in a basal liquid medium as reported by Rojas et al. (2009), supplemented with ground soya meal (SM, 250–1,000 μm particles) at 1.5% (w v^{-1}) and buffer-solutions according to Nagai et al. (1995) to adjust the pH to 9.0. Three replicates were incubated for 15 days at 28 ± 1.5°C under shaking (200 rpm). Mycelial development was estimated according to Hofrichter and Fritsche (1996).

Fungal culture for producing alkaline enzymatic activity and analytical methods

Selected fungi from the screening were then grown at pH 9.0 both in submerged cultures supplemented with SM under shaking and on a solid-state fermentation SSF system using CH as growth substrate in order to analyse their ability to produce keratinase enzymes with activity at alkaline pH as well as to analyse their ability to modify the medium pH after 15 days of culture. Previous to inoculation, CH was defatted by soaking in dichloromethane, ground and fractionated into Petri plates (3 g), which were autoclaved (at 121°C for 25 min) and supplemented with buffer-solutions according to Nagai et al. (1995) for adjusting the water associated with the substrate to pH 9.0. Three replicates were carried out for each isolate in each culture system. Degradation of CH by each fungal strain was measured as percentage substrate mass reduction according to Pandey and Nagveni (2007). The pH of 15-day-old culture systems with each fungus, on the supernatants of the submerged cultures and from aqueous suspensions obtained from SSF cultures by mixing one part of substrate and five parts of water under shaking for 5 min, was also measured.

Preparation and determination of enzyme extracts

Enzyme extract from the supernatants of SM cultures was obtained by filtering the cultures with a glass OSMONICS micro-filter (size-pore 0.45 μm) and then centrifuging at 10,000×*g* for 10 min at 4°C. The clear supernatant was then passed through a PD 10 molecular exclusion column (Disposable PD-10 Desalting Columns *IMPROVED*) equilibrated with a 20 mM Tris–HCl buffer at pH 9.0.

Enzyme extract from CH-SSF cultures was obtained by mixing one part (wet mass) of substrate and three parts of 0.6 M NaCl solution and shaking the mixture at 50 rpm for 30 min at 25°C. The suspension obtained was filtered using a glass OSMONICS micro-filter (size-pore 0.45 μm). The supernatant was passed through a PD 10 molecular exclusion column (Disposable PD-10 Desalting Columns *IMPROVED*) equilibrated with a 20 mM Tris–HCl buffer at pH 9.0 and then concentrated 10× by lyophilization in an L-3 RIFICOR equipment.

Keratinase activity was determined using Keratin Azure (Sigma K-8500, 5 mg) as substrate, which was incubated with 0.4 ml borax buffer (0.1 mM pH 9.0) and 0.1 ml of

Table 1 Fungal strains used and their growth on SM cultures at pH 9

Organism	Isolation substrate[a]	Gowth on SM[b]	Organism	Isolation substrate	Growth on SM
ASCOMYCOTA					
Acremonium cerealis (928[c], a[d])	A (20), 11	+	*Neosartorya stramenia* (833, t)	N (S), 8	+
Acremonium kiliense (958, a)	A (20), 11	+	*Neurospora tetrasperma* (837, t)	N (S), 6	+++
Acremonium murorum (927, a)	R (20), 11	++	*Nigrospora sphaerica* (971, a)	N (L), 6	+
Acremonium sp. (1049, a)	R (S), 11	+	*Paecilomyces lilacinus* (952, a)	R (20), 11	+++
Acrostalagmus luteo-albus (427, a)	R (20), 11	++	*Paecilomyces lilacinus* (983, a)	N (S), 6	+++
Alternaria alternata (1050, a)	N (20), 11	++	*Penicillium chrysogenum* (*, a)	A (20), 11	++
Aspergillus niger (845, a)	R (20), 11	+++	*Penicillium thomii* (945, a)	A (S), 6	+
Aspergillus sidowii (931, a)	N (20), 11	++	*Penicillum restrictum* (954, a)	N (20), 8	+
Aspergillus terreus (994, a)	N (S), 11	++	*Periconia minutissima* (943, a)	N (L), 6	+
Aspergillus terreus (964, a)	R (S), 11	++	*Pestalotiopsis guepinii* (929, a)	R (20), 11	+
Aspergillus ustus (981, a)	R (20), 11	++	*Phialocephala* sp (992, a)	N (20), 6	+
Bipolaris cynodontis (995, a)	N (L), 6	+++	*Phialophora fastigiata* (942, a)	N (S), 6	+
Bipolaris ellisii (959, a)	N (L), 6	+++	*Scopulariopsis brevicaulis* (947, a)	R (S), 11	+
Bipolaris sorokiniana (1000, a)	N (L), 6	+++	*Sporormia fimetaria* (339, t)	N (L), 6	++
Botryotrichum piluliferum (938, a)	R (S) 11	+	*Sporormia* sp. (*, t)	N (L), 6	++
Chaetomium globosum (950, t[e])	A (20), 8	+	*Stachybotrys chartarum* (922, a)	N (S), 8	++
Chrysosporium sp. (951, a)	N (S), 6	+	*Stachybotrys elegans* (997, a)	N (S), 11	++
Cladosporium cladosporioides (953, a)	R (20), 6	++	*Talaromyces flavus* var. *flavus* (838, t)	R (S), 8	+
Cladosporium herbarum (949, a)	N (S), 6	++	*Talaromyces stipitatus* (835, t)	N (S), 8	+
Clonostachys rosea (930, a)	R (20), 11	++	*Talaromyces trachyspermus* (831, t)	A (S), 6	+
Curvularia lunata (934, a)	N (L), 6	+	*Trichoderma harzianum* (*, a)	R (S), 8	++
Curvularia protuberata (975, a)	N (L), 6	+	*Trichoderma saturnisporum* (878, a)	R (S), 8	++
Cylindrocarpon didymum (962, a)	A (S), 6	+	*Verticillium albo-atrum* (941, a)	R (S), 8	+
Cylindrocarpon lucidum (956, a)	R (20), 6	+	*Verticillium nigrescens* (939, a)	A (S), 11	+
Cylindrocarpon olidum (923, a)	R (20), 6	++	*Volutella ciliata* (946, a)	N (S), 8	+
Doratomyces stemonitis (984, a)	A (S), 8	+	*Wardomyces inflatus* (955, a)	R (20), 8	+
Drechslera halodes (999, a)	N (L), 6	+	*Westerdikella dispersa* (830, t)	A (S), 8	++
Drechslera ravenelli (932, a)	N (S), 11	+			
			Zygomycota		
Emericellopsis minima (839, t)	A (S), 11	+	*Absidia spinosa* (986)	A (S), 8	+++
Epicoccum nigrum (940, a)	R (20), 8	+	*Cunninghamella elegans* (985)	N (20), 8	+
Exserohilum rostratum (*[f], a)	N (L), 6	+	*Gongronella butleri* (991)	A (20), 8	+
Fusarium oxysporum (961, a)	R (20), 11	+++	Mycelia Sterilia		
Fusarium semitectum (935, a)	R (20), 11	−	Dematiaceous sterile mycelium (989)	R (S), 11	++

Table 1 continued

Organism	Isolation substrate[a]	Gowth on SM[b]	Organism	Isolation substrate	Growth on SM
Fusarium solani (936, a)	A (20), 11	+++			
Humicola grisea (933, a)	N (20), 11	+			
Metarrhizium anisopliae (996, a)	N (20), 8	+			
Microsphaeropsis olivacea (944, a)	R (S), 6	+			
Myrothecium cinctum (979, t)	N (S), 11	+			

[a] Type of soil from where each fungal strain was isolated (Argialboll, A; Natracualf, N; Rendoll, R) on the superficial horizon (S), the horizon deeper than 20 cm (20) or from decaying leaves of *D. spicata* (L) on agar-medium at several pH values (6, 8, or 11)

[b] Growth rating: + minor growth (formation of poorly visible hyphae), ++ formation of conspicuous hyphae, +++ formation of mycelial mats, – no growth

[c] LPSC number

[d] Anamorphic state

[e] Telemorphic state

[f] Without LPSC number

each enzyme extract at 28°C according to Galarza et al. (2004). After 7 h, the absorbance at 595 nm of the supernatant of the mixture reaction, which had been previously stopped by adding 0.5 ml 10% trichloroacetic acid (TCA), was measured spectrophotometrically. A blank reaction without enzyme extract (replaced by buffer) was also carried out. The reactions were carried out in triplicate. Based on preliminary studies, optimal assay times were known to fall in the linear range of enzyme kinetics. One enzymatic activity unit (U) was defined as the amount of enzyme that produces 0.1 absorbance$_{595\ nm}$ unit per hour. Data were expressed as U ml^{-1} and analysed by a one-way ANOVA, and means were contrasted by Tukey's test ($P < 0.05$, Statistix 8).

Results and discussion

We have previously reported morphological and ecophysiological features of the most representative fungi growing on several soil types and on litter from forests of *Celtis tala* and *Scutia buxifolia* and *Distichlis spicata* grassland in Argentina, including the size of their reproductive structures and their response to pH (Elíades et al. 2004, 2006a, b, 2007; Saparrat et al. 2007, 2008). In the present work, we isolated 69 fungal strains at pH 11, 8 and 6 (38, 27, 35%, respectively) from the three soil types tested and identified them taxonomically (Table 1). While 31 strains were isolated from Natracualf soils, 24 and 14 were isolated from Rendoll and Argialboll soil, respectively. Most of the fungi found were anamorphic states of Ascomycota, although teleomorphic ones as well as some representatives from Zygomycota such as *Absidia spinosa*, *Cunninghamella elegans* and *Gongronella butleri*, were also identified.

Fungi were screened for growth at alkaline pH in a basal liquid medium supplemented with SM at pH 9.0. Except for *F. semitectum* LPSC 935, all the strains revealed ability to grow in SM, showing formation of conspicuous hyphae and mycelial pellets in 30 and 15% of the strains tested, respectively. However, more than half of the strains presented poor growth (Table 1).

On selected fungi from the previous screening, they were grown at an initial pH of 9.0 both in submerged cultures supplemented with SM under shaking and on a SSF system using CH as growth substrate. Most of the fungi caused a decrease in the pH level of the system (Table 2), being *A. niger* LPSC 845 the isolate that caused the greatest decrease in the SM medium. This may be related to the typical fungal activity that generates acidification as a consequence of the use of C and N compounds (Moore-Landecker 1996). In contrast, *Periconia minutissima* LPSC 943 grown in SM medium increased the pH levels slightly to 9.5, being this increase probably due to

Table 2 Extracellular pH and keratinolytic activity from 15 day-old fungal cultures grown on SM and CH at pH 9

Fungal strain	SM		CH	
	Final culture pH	Keratinolytic activity[a] (U ml^{-1})	Final culture pH	Keratinolytic activity (U ml^{-1})
A. kiliense 958	8.16	0.58 efgh	8.89	0.52 fghijk
A. murorum 927	9.00	1.00 cdefgh	8.35	1.13 bc
Acremonium sp. 1049	9.03	0.99 cdefgh	8.93	0.87 bcdefg
A. luteo-albus 427	9.26	0 fgh	8.28	0.65 cdefgh
A. niger 845	5.64	1.70 cdef	8.48	2.00 a
A. sidowii 931	8.82	1.23 bcde	9.05	1.14 bc
A. terreus 994	7.91	0.90 defgh	9.05	0.77 cdefg
A. terreus 964	7.54	0.87 efgh	8.90	0.53 efghij
Chrysosporium sp. 951	7.31	1.26 bcde	8.90	1.06 bcd
C. cladosporioides 953	8.78	1.91 abcd	8.89	1.28 b
C. rosea 930	8.74	0.71 efgh	8.98	0.56 efghi
C. didymum 962	9.29	0.97 cdefgh	9.09	1.03 bcde
C. olidum 923	9.27	1.10 cdefg	9.05	0.98 bcdef
F. solani 936	7.82	0.85 efgh	9.15	0.58 defghi
M. anisopliae 996	8.72	2.34 a	8.37	2.09 a
N. tetrasperma 837	8.21	2.34 a	8.36	1.82 a
P. lilacinus 952	8.45	0.93 dfgh	8.38	0.93 bcdef
P. lilacinus 983	9.07	1.52 cdefgh	8.33	0.96 bcdef
P. minutissima 943	9.50	0.96 cdefgh	8.32	0.51 fghijkl
S. fimetaria 339	9.33	1.95 abc	8.36	0.l m
V. albo-atrum 941	8.44	0.75 efgh	9.03	0.59 defghi

[a] Letters indicate significant differences between fungi grown on each culture medium (Tukey's test, $P < 0.05$). The following list includes the species (and LPSC number of strains) that showed levels lower than 0.5 U ml^{-1}: *A. cerealis* 928, *A. ustus* 981, *F. oxysporum* 961, *S. chartarum* 922, *T. stipitatus* 835 and *T. trachyspermus* 831 on both SM and CH cultures, and only on SM *D. stemonitis* 984 and *Phialocephala sp.* 992 only on SM

the release of NH_4^+ as a result of fungal degradation of organic N (Saparrat et al. 2007).

Table 2 shows the results of the screening of the 32 fungi selected, in both liquid (SM) and solid (CH) conditions. Although several of the fungal strains tested produced keratinolytic activity on both SM and CH, they differed in the levels detected. *Aspergillus niger* LPSC 845, *Cladosporium cladosporioides* LPSC 953, *Metarrhizium anisopliae* LPSC 996, *Neurospora tetrasperma* LPSC 837 and *Westerdikella dispersa* LPSC 830 were the highest producers in SM and CH systems. While *Humicola grisea* LPSC 933 and *Pestalotiopsis guepinii* LPSC 929 did not reveal extracellular keratinase activity in both culture conditions tested, *A. luteo-albus* LPSC 427 did not produce it only in SM. *Doratomyces stemonitis* LPSC 984, *Phialocephala* sp. LPSC 992 and *Sporormia fimetaria* LPSC 339 failed to show activity on CH.

Different fungal species such as *Acremonium murorum*, *Aspergillus sidowii, Chrysosporium* sp., *Cladosporium cladosporioides, Cylindrocarpon didymum, C. olidum, Metharrhizium anisopliae*, *Neurospora tetrasperma* and *Westerdikella dispersa* are reported for the first time for their ability to produce keratinolytic activity at alkaline pH.

All the strains that grew on CH revealed different levels of development on the solid substrate. About 50% of them showed a velvet-like mycelial surface, 12.5% of them mycelial mats, and 37% scarce growth. This fungal ability to develop more on CH than in SM could be related to the fact that the soil where fungi were isolated had animal waste produced by the livestock activity of the region (Sánchez et al. 1976; Cabello and Arambarri 2002).

Reduction in CH mass by fungal activity was also measured, being observed for several fungal cultures (Table 3). *Metarrhizium anisophliae* LPSC 996 and *Chrysosporium* LPSC 951 sp. showed the highest reductions 26.86 and 24.98%, respectively. The scarce growth of *Metarrhizium anisophliae* on this substrate type and the high keratinase activity found are in agreement with previous findings about efficient performance of this fungal species as an entomopathogenic organism attaching organic N-rich substrates (Mohan and Pillai 1982).

In summary, in the present work we reported the ability of fungi isolated from three types of soils from the Biosphere Reserve "Parque Costero del Sur" (Buenos Aires, Argentina) to produce keratinolytic activity at pH 9.0. In

Table 3 Fungal growth on CH at pH 9 and their mass loss after 15 days of incubation

Fungal strain	Growth on CH[a]	CH mass loss (%)[b]
A. cerealis 928	+	7.40
A. kilense 958	+	9.00
A. murorum 927	+	7.78
Acremonium sp. 1049	+	6.15
A. niger 845	++	18.25
A. sidowii 931	++	5.08
A. terreus 994	++	9.64
Chrysosporium sp. 951	++	24.98
C. cladosporioides 953	++	16.35
C. olidum 923	+	6.18
F. oxysporum 961	++	6.35
M. anisopliae 996	+	26.83
N. tetrasperma 837	+++	13.53
P. lilacinus 983	+++	8.87
P. guepinii 929	++	5.41
W. dispersa 830	+++	15.88

[a] Data are the mean of three replicates

[b] Variation coefficient was 34%. The following list includes the species (and LPSC strain number) that showed levels lower than 5% of CH mass loss: *A. luteo-albus* 427 (+++), *A. terreus* 964 (++), *A. ustus* 981 (++), *C. rosea* 930 (++), *C. didymum* 962 (+), *D. stemonitis* 984 (++), *F. solani* 936 (++), *H. grisea* 933 (++), *P. lilacinus* 952 (++), *P. minutissima* 943 (+), *Phialocephala sp.* 992 (+), *S. fimetaria* 339 (+), *S. chartarum* 922 (++), *T. stipitatus* 835 (+), *T. trachyspermus* 831 (+), *V. albo-atrum* 941 (++). The parentheses indicate growth rating

this sense, these isolates growing on media with protein-rich substrates, such as CH and SM, differed in their enzyme production levels as well as in their contribution to CH mass loss. Further experiments are in progress to characterize the enzymes from strains revealing high enzyme potential.

Acknowledgments LA Elíades, CE Voget and MCN Saparrat are Members of Carrera del Investigador CONICET. Cabello MN is a Member of Carrera del Investigador CICPBA. This research was supported by grants from UNLP (11/N527 Proyect), CIC, CONICET (PIP 1422), Agencia Nacional de Promoción Científica y Tecnológica (PICT 2007 01233).

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