Short Communication

α-L-Rhamnosidase and β-D-glucosidase activities in fungal strains isolated from alkaline soils and their potential in naringin hydrolysis

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α-L-Rhamnosidases (EC 3.2.1.40) and β-D-glucosidases (EC 3.2.1.21) obtained from several microbial sources are potential catalysts in food, beverage, and pharmaceutical industries. However, the enzyme preparations currently used have limitations related to the stability and activity of the enzyme as well to their reuse. A microtiter screening was carried out in 55 fungal strains isolated from alkaline soils, to obtain active α-L-rhamnosidases and β-D-glucosidases at pH 9.0. While α-L-rhamnosidase activity was detected in 45% of the strains tested, β-D-glucosidase activity was found only in 27%. Based on the fungal ability to produce α-L-rhamnosidase activity, cultures were supplemented with naringin to study the activities of the enzymes and the potential of the fungal strains on naringin hydrolysis. About 70% of the fungal strains tested increased the activities of both enzymes in the naringin-supplemented cultures as compared to non-supplemented ones. This effect was higher in *Acrostalagmus luteo-albus* LPSC 427 (15.3 fold) for α-L-rhamnosidase activity and *Metarrhizium anisopliae* LPSC 996 (51.1 fold) for β-D-glucosidase activity. All the enzyme preparations tested hydrolyzed naringin at pH 9.0, being that obtained from *Acremonium murorun* LPSC 927 cultures the one which showed highest hydrolysis. Here, different fungal species are reported for the first time for their ability to produce α-L-rhamnosidase and β-D-glucosidase activity at alkaline pH.

Keywords: Alkaline enzymes / Fungi / α-L-Rhamnosidase / β-D-glucosidase / Naringin hydrolysis

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Introduction

Naringin is a 4,5,7-trihydroxyflavonone 7-rhamnoglucoside responsible for the bitterness of citrus juices [1, 2]. α-L-Rhamnosidases (EC 3.2.1.40) and β-D-glucosidases (EC 3.2.1.21) are enzyme components of the naringinase complex, an enzyme preparation used in pharmaceutical and food industries. This enzyme system acts through the release of rhamnose and glucose from naringin [3] and other glycosides to obtain the aglycone, naringenin (4,5,7-trihydroxyflavonone), an important byproduct in biotechnology [4]. In nature, several fungi and bacteria obtain matter and energy from the degradation of specific polysaccharides, such as hairy regions of pectins and other heteropolysaccharides, and cellulose by using α-L-rhamnosidases and β-glucosidases respectively [5, 6]. However, other roles for both enzymes, such as the suppression of the C-glycosyl flavonoid phytoalexins-mediated plant defense response, and as virulence factors, have also been proposed [7, 8].

α-L-Rhamnosidases and β-D-glucosidases can be obtained from several sources such as fungi like *Penicillium*

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decumbens or bacteria, and are commercially available as different enzyme preparations that are used at industrial level [4, 9, 10]. In particular, several technical applications of fungal α-L-rhamnosidases in the food industry, such as the debittering of grapefruit juice by hydrolysis of the flavonoid naringin [11, 12], the elimination of hesperidin crystals in orange juice, and the enhancement of wine aromas by hydrolysis of terpenylglycosides [13], have been investigated. Furthermore, these enzymes, which are stable and active at acid pH values, has also been used in the determination of sugar molecules, in the production of prunin [14], which possesses anti-inflammatory and antiviral activity [15], and derhamnosylated steroids with clinical importance [16–18]. However, the use of these enzyme preparations in the technology area has some operational and economic limitations related to the enzyme stability and its activity such as one affected by alkaline pH and/or extreme temperatures as well as enzyme reuse and the solubility and fate of reaction products, achieving few significant success [4]. Therefore, new biocatalysts with a better performance, such as those active at alkaline pH, are required for industrial applications. Although a lot of information is available about the ability of fungi and bacteria to produce α-L-rhamnosidases and β-D-glucosidases as well as their catalytic properties and gene sequence [19], there are few reports on the production and characterization of these alkaline enzymes from fungi, including those associated with extreme environments [6, 20–22]. An example of this kind of environments is the native xerophilic forest dominated by the tree species *Celtis tala* Gill ex Planch (Ulmaceae) and *Scutia buxifolia* Reiss (Rhamnaceae) [23], and *Distichlis spicata* (L.) Greene grassland associated located in the eastern part of the Buenos Aires province (Argentina). This area is characterized by different soil types including alkaline-calcareous (Rendolls) and alkaline-sodic ones (Natracualfs) [24]. These soils are a potential source to isolate fungi able to produce enzymes with the capability to tolerate different pH ranges and/or highly active at extreme pHs, which might be used as biotechnological tools. Since the substrates of commercially available naranginases have low solubility in acidic solution [25], the alkaline enzymes have promising applications in the pharmaceutical industry.

The aim of this work was to analyze the ability of several fungal strains isolated from alkaline soils to produce α-L-rhamnosidase and β-D-glucosidase activities at pH 9.0. The effect of naringin on the production of α-L-rhamnosidase and β-D-glucosidase and the potential of these enzymes on naringin hydrolysis were also evaluated.

Materials and methods

Fungal isolation

The fungal strains used in this work (Table 1) were isolated from alkaline-calcareous (Rendoll) and alkaline-sodic (Natracualf) soils from 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 according to Elíades *et al.* [24]. Washed soil particles were inoculated on malt extract agar-medium (MA: 10 g of malt extract powder (Oxoid), 2.5 g of peptone, 20 g of agar, 1 l of distilled water) of which the initial pH was adjusted to 6.0, 8.0 and 11.0 with buffers according to Nagai *et al.* [26] and incubated at 25 °C. At one-week intervals, fungal development from each soil particle was analyzed microscopically and isolated in axenic culture. The identification of the sporulating fungi was based on cultural and morphological features [27–30]. Stock cultures were kept at 4 °C on 2% (w v^{-1}) agar-malt extract slants and lyophilized according to Smith and Kolkowski [31], being then deposited in the culture collection of the Instituto Spegazzini, UNLP, La Plata, Argentina (LPSC).

Microtiter-schreening for α-L-rhamnosidase and β-D-glucosidase activities at alkaline pH

All the fungi isolated were inoculated according to Saparrat and Guillen [32] in a liquid soya-meal medium as reported by Elíades *et al.* [22], and supplemented with buffer-solutions according to Nagai *et al.* [33] to adjust the pH to 9.0. Three replicates for each fungus were incubated for 15 d at 28 ± 1.5 °C under shaking (200 rpm). The filtrate (supernatant) was collected to measure pH and α-L-rhamnosidase and β-D-glucosidase activities qualitatively in a microtiter plate (see below).

Effect of naringin on the enzyme production by selected fungi

Twenty-three fungi from the screening were grown on the soya-meal liquid medium at pH 9.0 (basal medium) [22] and in the presence of naringin (Sigma, USA, with purity higher than 99.5% purity). A stock solution of 1 mM of this glucoside in Tris-HCl 20 mM was sterilized by filtration through 0.2 μm cellulose filters and added to the basal medium before inoculating each fungus as reported by Saparrat *et al.* [6]. Three replicate cultures per isolate were grown at 200 rpm and 28 ± 1.5 °C for 15 d. The mycelium was removed from the liquid cultures by centrifugation at 20,000 × *g* for 10 min at 4 °C. The supernatant was collected to measure pH and quan-

Table 1. Fungal strains used and their ability to produce α-L-rhamnosidase and β-D-glucosidase activities at pH 9.0 using a microtiter-plate system[a].

Organism	α-L-rhamnosidase	β-D-glucosidase	Organism	α-L-rhamnosidase	β-D-glucosidase
ASCOMYCOTA			*Metarrhizium anisopliae* (996)	+	–
Acremonium cerealis (928[b])	+	–	*Microphaeropsis olivacea* (944)	+	+
Acremonium murorum (927)	+	–	*Myrothecium cinctum* (979)	–	–
Acremonium sp. (1049)	–	–	*Neosartorya stramenia* (833)	+	–
Acrostalagmus luteo- albus (427)	+	+	*Neurospora tetrasperma* (837)	–	–
Alternaria alternata (1050)	–	+	*Paecilomyces lilacinus* (952)	+	–
Aspergillus niger (845)	+	–	*Paecilomyces lilacinus* (983)	+	–
Aspergillus sidowii (931)	–	+++	*Penicillium chrysogenum* (*)	–	–
Aspergillus terreus (994)	+	++	*Penicillium retrictum* (954)	–	–
Aspergillus terreus (964)	–	–	*Penicilllium thomii* (945)	+	–
Aspergillus ustus (981)	+	+++	*Pestalotiopsis guepinii* (929)	+	+
Bipolaris ellisii (959)	–	+++	*Phialocephala* (992)	+	+
Botryotrichum piluliferum (938)	+	–	*Phialophora fastigiata* (942)	–	++
Chrysosporium sp. (951)	+	–	*Scopulariopsis brevicaulis* (947)	–	++++
Cladosporium cladosporioides (953)	+	+++	*Stachybotrys elegans* (997)	–	–
Clonostachys rosea (930)	+	–	*Stachybotrys chartarum* (922)	+	–
Curvularia lunata (934)	–	–	*Talaromyces flavus* var. *flavus* (838)	–	–
Cylindrocarpon didymun (962)	+	–	*Talaromyces stipitatus* (835)	–	+++
Cylindrocarpon olidum (923)	+	–	*Trichoderma harzianum* (*)	–	–
Cylindrocarpon lucidum (956)	–	–	*Trichoderma saturnisporum* (878)	+	–
Drechslera ravenelli (932)	–	–	*Verticillium albo- atrum* (941)	–	–
Epicoccum nigrum (940)	–	++	*Volutella cilliata* (946)	–	–
Fusarium equisetii (*[c])	+	++	*Wardomyces inflatus* (955)	+	–
Fusarium oxysporum (961)	–	–	ZYGOMYCOTA		
Fusarium semitectum (935)	–	–	*Cunninghamella elegans* (985)	–	–
Fusarium solani (936)	–	–	*Gongronella butleri* (991)	+	–
Fusarium sp. (*)	–	–	*Zigorhinchus moelleri* (*)	–	–
Humicola grisea (933)	–	–	MYCELIA STERILIA		
Melanosora zamiae (*)	–	–	*Dematiaceous sterile mycelium* (989)	–	+++

[a] Enzyme activity estimated as relative production of a yellow reaction product: +, low color intensity, ++, moderate color intensity; +++, strong color intensity; –, without differentiation of yellow coloration.
[b] LPSC number.
[c] without LPSC number

titatively α-L-rhamnosidase and β-D-glucosidase activities.

Enzyme assays

α-L-Rhamnosidase and β-D-glucosidase activities were measured at pH 9.0 through either a microtiter plate method (qualitatively) or spectrophotometric analysis (quantitatively) using the chromogen substrates *p*-nitrophenyl-α-L-rhamnopyranoside (Sigma, St. Louis, MO, USA; pnp-Rha) and p-nitrophenyl- β-D-glucopyranoside (Sigma, pnp-Glu) respectively, according to Rojas *et al.* [34]. Culture filtrates previously concentrated as reported by Elíades *et al.* [22] were used as enzyme source. Blank reactions without enzyme were also tested. The reactions were carried out in triplicate. In the microtiter-plate method, individual reactions were tested on a well from a 96-well Microplate, where sodium azide at 0.01% was included for prevening microbial growth. On each well, reaction was stopped after 24 h of incubation by adding 100 μl 100 mM NaOH, and the presence of enzyme activity was recorded when color change was observed compared to the color of blank wells. Reactions on spectrophotometer (Beckman DU 640) were carried out incubating the solution for 1.5 h at 37 °C and the absorbance was determined at 420 nm after adding 100 mM NaOH to stop the reaction. One unit (U) of enzyme activity was defined as the amount of enzyme that releases 1 μmol of p-nitrophenol in 1 min. The enzyme activity was expressed as U ml^{-1}.

Enzyme hydrolysis of naringin at pH 9.0

Naringin hydrolysis studies were performed. A solution of this substrate was prepared (3.5 mM in 20 mM Tris–HCl buffer) and adjusted to pH 9.0. Reactions were carried out by mixing 900 μl of each substrate solution and 100 μl of the enzymatic extract (0.15 U ml^{-1}). Substrate and sample blanks were performed using distilled water instead of substrate and enzyme solutions, respectively. This reaction mixture was incubated for 3 h at 37 °C. Samples of 300 μl were taken at the begin-

ning and at the end of this assay. After the incubation period, samples were added to an equal volume of 52% acetonitrile in 60 mM H_3PO_4 to stop the reaction. The remaining substrates and reaction products were quantified by HPLC using a Symmetry C18 column (3.9 × 9 × 150 mm, Waters, Milford, MA, USA) at room temperature, using a solution of acetonitrile/water (0.68 : 0.32 v v^{-1}) as solvent at a flow rate of 1 ml min^{-1}, and a UV photodiode array detector (2996, Waters). Naringin, prunin and naringenin concentrations were determined according to their respective standards (Sigma).

Statistical analysis

The data of enzyme activity were analyzed by a one-way ANOVA and means were contrasted by Tukey's test.

Results and discussion

We have previously reported the ability of several fungi isolated from alkaline soils to produce alkaline keratinases [25, 35]. Since other alkaline enzymes such as α-L-rhamnosidases and β-D-glucosidases have high biotechnological potential and there are few reports about these enzymatic activities in these micro-organisms [36], using a microtiter plate system we have now qualitatively analyzed the ability of 55 of those fungi to produce α-L-rhamnosidase and β-D-glucosidase activities at pH 9.0 (Table 1). Only seven of these strains showed both enzyme activities. α-L-rhamnosidase activity was detected in 45% of the strains and β-D-glucosidase activity was found only in 27% of them. Taxa such as *Acremonium murorum* LPSC 927, *Aspergillus sidowii* LPSC 931, *Chrysosporium* sp. LPSC 951, *Cladosporium cladosporioides* LPSC 953, *Cylindrocarpon didymun* LPSC 962, *C. olidum* LPSC 923, *Metharrhizium anisopliae* LPSC 996, and *Neurospora tetrasperma* LPSC 837 are reported for the first time for their ability to produce α-L-rhamnosidase activities and β-D-glucosidase at alkaline pH. However, 22 of the strains tested such as *Aspergillus terreus* LPSC 964, *Cylindrocarpon lucidum* LPSC 956, *Fusarium solani* LPSC 936, *Penicillium chrysogenum* (*) or *Volutella cilliata* LPSC 946 did showed neither glucosidase nor rhamnosidase activities under the culture conditions tested. In contrast, Saparrat *et al.* [6] have previously reported the ability of several fungal isolates from the leaf litter of *Celtis tala* and *Scutia buxifolia* forests, including *Volutella cilliata* LPSC 807 grown at pH 5.0, to produce β-D-

Table 2. Extracellular α-L-rhamnosidase activity of selected fungal cultures grown on soya-meal liquid medium at pH 9.0 (Co) and supplemented with naringin (2 mM) after 15 d of incubation[a].

Fungal strain	α-L-rhamnosidase activity (U ml^{-1})[b]		Induction effect[c]
	Co	+ naringin	
A. cerealis (928)	3.04 ± 0.14 bcdef	14.22 ± 0.73 op	+
A. murorum (927)	15.01 ± 0.3 op	15.60 ± 0.4 p	nd
A. luteoalbus (427)	0.92 ± 0.06 ab	14.04 ± 0.06 nop	+
A. niger (845)	26.50 ± 0.5 rs	24.25 ± 0.81 r	–
A. terreus (994)	6.35 ± 0.54 ghi	15.41 ± 0.16 p	+
A. ustus (981)	1.79 ± 0.03 abcd	5.18 ± 0.82 fgh	+
Chrysosporium sp. (951)	4.74 ± 0.31 efgh	9.26 ± 0.23 jkl	+
C. cladosporioides (953)	6.96 ± 2.04 hij	29.43 ± 0.6 tu	+
C. rosea (930)	3.77 ± 0.6 cdefg	4.42 ± 0.32 defgh	+
C. didymun (962)	0.67 ± 0.5 ab	4.56 ± 0.51 efgh	+
C. olidum (923)	8.37 ± 0.34 ijk	15.62 ± 1.4 p	+
F. equisetii	0.97 ± 0.93 ab	12.41 ± 0.47 mno	+
G. butleri (991)	2.12 ± 0.14 abcde	9.12 ± 0.83 jkl	+
M. anisopliae (996)	2.17 ± 1.11 abcde	0.95 ± 0.22 ab	–
M. olivacea (944)	14.05 ± 2.05 nop	29.61 ± 1.4 u	+
N. stramenia (833)	1.48 ± 0.51 abc	12.39 ± 0.4 mno	+
P. lilacinus (952)	11.37 ± 0.65 lmn	11.31 ± 0.26 lm	nd
P. lilacinus (983)	14.85 ± 0.37 op	27.31 ± 1.56 stu	+
P. thomii (945)	1.41 ± 0.4 abc	9.32 ± 0.55 jkl	+
Phialocephala sp. (992)	26.80 ± 1.05 rst	15.59 ± 0.54 p	–
S. chartarum (922)	10.52 ± 0.9 klm	19.38 ± 1.09 q	+
T. saturnisporum (878)	0.04 ± 0.05 a	0.60 ± 0.37 ab	+
W. inflatus (955)	12.47 ± 2.07 mno	15.65 ± 0.56 p	+

[a] Values are means ± S.D. of three replicates.
[b] letters indicate significant differences between fungi grown on each culture medium (Tukey's test, $P < 0.05$).
[c] Induction rating: +, increase in activity level by naringin supplementation; –, reduction in activity level by naringin supplementation; nd, No differential activity level by naringin supplementation.

Table 3. Extracellular β-D-glucosidase activity of selected fungal cultures grown on soya-meal liquid medium at pH 9.0 (Co) and supplemented with naringin (2 mM) after 15 d of incubation[a].

Fungal strain	β-D-glucosidase activity (U ml^{-1})[b]		Induction effect[c]
	Co	+ naringin	
A. cerealis (928)	0.02 ± 0.008 a	1.64 ± 0.35 abc	+
A. murorum (927)	7.36 ± 0.6 f	0.26 ± 0.08 ab	–
A. luteoalbus (427)	13.20 ± 1.6 h	19.19 ± 1.26 m	+
A. niger (845)	0.39 ± 0.16 ab	4.49 ± 0.22 e	+
A. terreus (994)	16.11 ± 0.37 l	21.47 ± 0.5 n	+
A. ustus (981)	15.20 ± 0.31 ijkl	15.09 ± 0.92 ijkl	nd
Chrysosporium sp. (951)	15.56 ± 0.54 jkl	27.59 ± 0.51 o	+
C. cladosporioides (953)	21.90 ± 0.94 n	33.70 ± 1.03q	+
C. rosea (930)	7.46 ± 1.01 f	0.77 ± 0.05 abc	–
C. didymun (962)	1.16 ± 0.17 abc	12.55 ± 0.5 h	+
C. olidum (923)	13.50 ± 0.66 hi	16.25 ± 0.22 l	+
F. equisetii	13.98 ± 0.21 hij	18.62 ± 0.14 m	+
G. butleri (991)	14.18 ± 0.33 hijk	15.08 ± 0.87 ijkl	+
M. anisopliae (996)	0.09 ± 0.01 a	4.60 ± 0.3 e	+
M. olivacea (944)	27.60 ± 0.95 o	30.01 ± 0.09 p	+
N. stramenia (833)	16.25 ± 0.3 l	27.34 ± 0.6 o	+
P. lilacinus (952)	0.01 ± 0.001a	0.01 ± 0.002 a	nd
P. lilacinus (983)	0.02 ± 0.006 a	2.47 ± 0.12 cd	+
P. thomii (945)	10.19 ± 0.32 g	15.83 ± 0.26 kl	+
Phialocephala sp. (992)	2.04 ± 0.03 bc	1.78 ± 0.11 abc	–
S. chartarum (922)	0.28 ± 0.05 ab	4.21 ± 0.11 de	+
T. saturnisporum (878)	0.50 ± 0.06 ab	6.94 ± 0.96 f	+
W. inflatus (955)	0.01 ± 0.006 a	12.65 ± 0.74 h	+

[a] Values are means ± S.D. of three replicates.
[b] letters indicate significant differences between fungi grown on each culture medium (Tukey's test, $P < 0.05$).
[c] Induction rating: +, increase in activity level by naringin supplementation; –, reduction in activity level by naringin supplementation; nd, No differential activity level by naringin supplementation.

glucosidase activity. On the other hand, after 15 d of incubation, most of the strains tested changed the initial pH value (9.0) to values between 7.0 and 8.0. However, *Aspergillus niger* LPSC 845 and *Myrothecium cinctum* LPSC 979 caused the greatest decrease in the pH of the medium to levels lower than 6.0, whereas *Fusarium equisetii* (*) and *Volutella cilliata* LPSC 946 increased the pH levels slightly to 9.5. Although the acidification is a general feature of the fungal culture liquids as incubation develops, an increase in pH by some fungi might be related to the release of NH_4^+ as a result of fungal degradation of organic N [20].

Based on their promising ability to produce α-L-rhamnosidase activity, 23 selected fungi were grown on soya-meal liquid medium at pH 9.0 to analyze the effect of naringin (2 mM) on α-L-rhamnosidase and/or β-D-glucosidase activities (Tables 2 and 3). Although cultures pH was not modified by the addition of naringin (data not shown), the activities of both enzymes were increased in its presence in 70% of the fungi tested. This effect was higher in *A. luteo-albus* LPSC 427 (15.3 fold) and *Fusarium equisetti* (*) (12.7 fold) cultures for α-L-rhamnosidase activity, and in *M. anisopliae* LPSC 996 (51.1 fold), *S. chartarum* LPSC 922 (15 fold) and *T. saturnisporum* LPSC 878 (13.9 fold) cultures for β-D-glucosidase activity. Monti *et al.* [37] also reported the inducibility of α-L-rhamnosidases by naringin in *Aspergillus aculeatus*, *A. terreus* and *Emericella nidulans*, although they did not reveal this enzyme activity constitutively. In both fungi and bacteria, α-L-rhamnosidase activity is downregulated by fructose and glucose and upregulated by L-rhamnose and their flavonoid glycosides, including naringin [38, 39]. Since *Phialocephala* sp. LPSC 992 and *C. rosea* LPSC 930 when cultured in the presence of naringin for 15 d showed a reduction in α-L-rhamnosidase activity (58.2%) and in β-D-glucosidase activities (10.3%) respectively, some mechanism of catabolite repression by the products from naringin hydrolysis such as glucose, might be involved. In this sense, this latter enzyme control has also been found for other fungal species producing β-D-glucosidase activity [40].

The ability of the enzyme preparations from the selected cultures to hydrolyze naringin *in vitro* at pH 9.0 was also analyzed. This ability was estimated through its residual level and that of prunin and naringenin by HPLC after 3 h of incubation compared to mixtures without enzyme. Although all the enzyme preparations

Table 4. Concentration of main flavonoids as detected by HPLC analysis in reaction mixtures with naringin at pH 9.0 after 3 h of incubation (control) and in the presence of enzyme preparations from several fungal cultures[a].

Reaction mixture	Naringin (g l^{-1})	Prunin (g l^{-1})	Naringenin (g l^{-1})	NH (%)[b]
Control (without enzyme)	10.39 ± 0.55	0.20 ± 0.01	0.09 ± 0,0047	0
A. murorum LPSC 927	8.68 ± 0.46	1.76 ± 0.09	0.12 ± 0,0063	16.4
A. luteo-albus LPSC 427	9.75 ± 0.51	0.42 ± 0.02	0.17 ± 0,0090	6.2
M. olivacea LPSC 944	9.66 ± 0.51	0.27 ± 0.01	0.04 ± 0,0021	7.02
P. lilacinus LPSC 983	9.49 ± 0.50	0.52 ± 0.03	0.04 ± 0,0021	8.7
S. chartarum LPSC 922	9.54 ± 0.50	0.17 ± 0.01	0.06 ± 0,0032	8.2

[a] Values are referred to 1 ml of reaction. All HPLC analyses were replicated, the mean values being reported. [b]NH, hydrolysis percentage of naringin.

tested hydrolyzed naringin at pH 9.0, that obtained from *A. murorum* LPSC 927 cultures showed the highest ability (16.4% of naringin reduction compared to that from mixtures without enzyme), which produced also the highest level of prunin (Table 4). However, the incubation of naringin at pH 9.0 in the presence of an enzyme preparation from *A. luteo-albus* LPSC 427 cultures revealed a high level of naringenin. Scaroni *et al.* [41] found higher hydrolysis percentages in other fungi but at lower pH values (up 6.5), though they also found activity in naringin at alkaline pH.

In summary, in the present work we reported the ability of fungi isolated from alkaline soils to produce α-L-rhamnosidase and β-D-glucosidase activities at pH 9.0. In this sense, isolates growing on a liquid medium supplemented with soybean at pH 9.0 differed in their enzyme production levels and in their response to the addition of naringin as an inducer of enzyme production as well as in their ability to hydrolyze naringin at pH 9.0. Further experiments are in progress to characterize the enzymes from strains revealing high enzyme potential. To our knowledge, this is also the first report on several fungal taxa which are able to produce α-L-rhamnosidases and β-D-glucosidases activities at alkaline pH.

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References

[1] Hau-Yang, T., Gee-Kaite, Y., 1991. Limonin and naringin removal from grapefruit juice with naringinase entrapped in cellulose triacetate fibers. J. Food Sci., **56**, 31–34 DOI: 10.1111/j.1365-2621.1991.tb07968.x.

[2] Hongfei, L., Hesheng, D.M., Mao, Y., Zhang, D., 2004. Effect of naringinase on debittering of citrus juice. Wang (Department of Food Science and Engineering, Ningbo University, Ningbo 315211, China).

[3] Mazzaferro, L.S., Orrillo, G.A., Ledesma, P., Breccia, J.D., 2008. Dose-dependent significance of monosaccharides on intracellular α-L-rhamnosidase activity from *Pseudoalteromonas* sp. Biotechnol. Lett., **30**, 2147–2150.

[4] Puri, M., Kalra, S., 2005. Purification and characterization of naringinase from a newly isolated strain of *Aspergillus niger* 1344 for the transformation of flavonoids. World J. Microb. Biotechnol., **21**, 753–758.

[5] Hashimoto, W., Nankai, H., Sato, N., Kawai, S., Murata, K., 1999. Characterization of α-L-rhamnosidase of *Bacillus* sp. GL1 responsible for the complete depolymerization of gellan. Arch. Biochem. Biophys., **368**, 56–60.

[6] Saparrat, M.C.N., Rocca, M., Aulicino, M.B., Arambarri, A., Balatti, P., 2008. *Celtis tala* and *Scutia buxifolia* leaf litter decomposition by selected fungi in relation to their physical and chemical properties and the lignocellulolytic enzyme activity. Eur. J. Soil Biol., **44**, 400–407.

[7] Rizk, A.F., Wood, G.E., 1980. Phytoalexins of leguminous plants. Crit. Rev. Food Sci. Nutr., **13(3)**, 245–295.

[8] Kerppola, T.K., Serwold-Davis, T., Gross, D.C., Kahn, M.L., 1987. Effect of increased B-glucosidase activity on virulence of *Erwinia amylovora*. Appl. Environ. Microbiol., **53**, 677–682.

[9] Young, N.M., Rosemary, A.Z., Johnston, J.C.R., 1989. Purification of the α-L-rhamnosidase of *Penicillium decumbens* and characterisation of two glycopeptide components. Carbohydr. Res., **191(1)**, 53–62 DOI:10.1016/0008-6215(89)85045-1.

[10] Puri, M., Kaur, A., Sarupsingh, R., Singh, A., 2009. Response surface optimization of medium components for naringinase production from *Staphylococcus xylosus MAK2*. Appl. Biochem. Biotechnol., **162(1)**, 181–191.

[11] Soares, N.F.F., Hotchkiss, J.H., 1998. Naringinase immobilization in packaging films for reducing naringin concentration in grapefruit juice. J. Food Sci., **63**, 61–65.

[12] Soria, F.F., Cuevas, C., Ellenrieder, G., 1999. Purification and some properties of a-L rhamnosidase of *Aspergillus terreus*. Appl. Biol. Sci., **5**, 109–120.

[13] Caldini, C., 1994. Kinetic and immobilization studies on fungal glycosidases for aroma enhancement in wine. Enzyme Microb. Technol., **16**, 286–291.

[14] Roitner, M., Schalkhammer, T., Pittner, F., 1984. Preparation of prunin with the help of inmobilized naringinase pretreated with alkaline buffer. Appl. Biochem. Biotech., **9**, 483–488.

[15] Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head bacteriophage t4. Nature, **227**, 680–685.

[16] Elujoba, A., Hardman, R., 1987. Diosgenin production by acid and enzymatic hydrolysis of fenugreek. Fitoterapia, **58**, 299–303.

[17] Monti, D., Pisvejcova', A., Kren, V., Lama, M., Riva, S., 2004. Generation of an a-rhamnosidase library and its application for the selective derhamnosylation of natural products. Biotechnol. Bioeng., **87**, 763–771.

[18] Yu, H., Gong, J., Zhang, C., Jin, F., 2002. Purification and characterization of ginsenoside-α-L-rhamnosidase. Chem. Pharm. Bull., **50**, 175–178.

[19] Manzanares, P., Fajardo, S., Martin, C., 2001. Production of lignolytic activities when treating paper pulp effluents by *Trametes versicolor*. J. Biotechnol., **43**, 125–132.

[20] Saparrat, M.C.N., Arambarri, A.M., Balatti, P.A., 2007. Growth response and extracellular enzyme activity of *Ulocladium botrytis* LPSC 813 cultured on carboxy-methylcellulose under a pH-range. Biol. Fertil Soils, **44**, 383–386.

[21] Elíades, L.A., 2009. Estudio de la micobiota alcalofílica y alcalino-tolerante del suelo de los bosques de *Celtis tala* Gill (ex Planch) y *Scutia buxifolia* Reiss en el Partido de Magdalena. Provincia de Buenos Aires. Tesis Doctoral. Facultad de Cs. Naturales y Museo. UNLP pp. 196.

[22] Elíades, L.A., Voget, C.E., Saparrat, M.C.N., Cabello, M.N., 2010. Screening for alkaline keratinolytic activity in fungi isolated from soils of the biosphere reserve "Parque Costero del Sur" (Argentina) World J. Microb. Biotechnol. **26 (11)**, 2105-2111. DOI: 10.1007/s11274-010-0389-4. 2010.

[23] Arturi, M.F., Barrera, M.D., Brown, A.D., 1996. Caída y masa de hojarasca en bosques de *Celtis tala* Gill ex Planch y *Scutia buxifolia* Reiss del este de la provincia de Buenos Aires, Argentina. Rev. Fac. Agr. La Plata, **101**, 151–158.

[24] Elíades, L.A., Cabello, M.N., Voget, C.E., 2006. Soil microfungi diversity in *Celtis tala* and *Scutia buxifolia* forests in Eastern Buenos Aires Province (Argentina). J. Agric. Tech., **2(2)**, 229–249.

[25] Ellenrieder, G., Blanco, S., Daz, M., 1998. Hydrolysis of supersaturated naringin solutions by free and immobilized naringinase. Biotechnology Techniques, **12**, 63–65.

[26] Nagai, K., Suzuki, K., Okada, G., 1998. Studies on the distribution of alkalophilic and alkali- tolerant soil fungi II: Fungal flora in two limestone caves in Japan. Mycoscience, **39**, 293–298.

[27] Domsch, K.H., Gams, W., Anderson, T., 1993. Compendium of soil fungi IHW-Verlag. Eching. Pp. 859.

[28] Cabello, M.N., Arambarri, A.M., 2002. Diversity in soil fungi from undisturbed and disturbed *Celtis tala* and *Scutia buxifolia* forests in the eastern Buenos Aires province (Argentina). Microbiol. Res., **157**, 115–125.

[29] Barron, L.G., 1972. The genera of Hyphomycetes from soil. R.E. Krieger Publishing Company. New York, p. 364.

[30] Ellis, M.B., 1971. Dematiaceous Hyphomycetes. C.A.B. England. Pp. 608.

[31] Smith, D., Kolkowski, J., 1996. Fungi. In: Maintaining Cultures for Biotechnology and Industry (Hunter-Cevera, J. C. and Belt, A., eds). Academic Press, Inc. California.

[32] Saparrat, M.C.N., Guillén, F., 2005. Ligninolytic ability and potential biotechnology applications of the South american fungus *Pleurotus laciniatocrenatus*. Folia Microbiol., **50**, 155–160.

[33] Nagai, K., Sakai, T., Ratiatmodjo, R.M., Suzuki, K. *et al*., 1995. Studies on the distribution of alkalophilic and alkali- tolerant soil fungi I. Mycoscience, **36**, 247–256.

[34] Rojas, N.L., Cavallito, S.F., Cabello, M.N., Hours, R.A., Voget, C.E., 2009. Alkaline polysaccharidases produced in solid state cultures by alkalophilic fungi isolated from Argentina. J. Pure & Appl. Micro., **2(1)**, 1–10.

[35] Elíades, L.A., Cabello, M.N., Saparrat, M.C.N., Voget, C., 2010. Alkalophilic and alkali-tolerant soil fungi from *Celtis tala* and *Scutia buxifolia* forests in eastern Buenos Aires province (Argentina). Current Topics in Biotechnology. In press.

[36] Rodríguez, M.E., Lopes, C.A., Valles, S., Caballero, A.C., 2010. Characterization of α-rhamnosidase activity from a Patagonian *Pichia guilliermondii* wine strain. J. Appl. Microbiol., **109(6)**, 2206–2213.

[37] Monti, D., Pišvejcová, A., Křen, V., Lama, M., Riva, S., 2004. Generation of an α-L-rhamnosidase library and its application for the selective derhamnosylation of natural products. Biotechnol. Bioeng., **87(6)**, 763–771.

[38] Ávila, P.M., Jaquet, M., Moine, D., Requena, T. *et al*., 2009. Physiological and biochemical characterization of the two a-L-rhamnosidases of *Lactobacillus plantarum* NCC245. Microbiology, **155**, 2739–2749.

[39] Mendoza-Cal, A., Cuevas-Glory, L., Lizama-Uc, G., Ortiz-Vázquez, E., 2010. Naringinase production from filamentous fungi using grapefruit rind in solid state fermentation. Afr. J. Microbiol. Res., **4(19)**, 1964–1969.

[40] Magnelli, P., Forchiassin, F., 1999. Regulation of the cellulase complex production by *Saccobolus saccoboloides*: induction and repression by carbohydrates. Mycologia, **91**, 359–364.

[41] Scaroni, E., Cuevas, C., Carrillo, L., Allenreider, G., 2002. Hydrolytic properties of crude α-L-rhamnosidases produced by several wild strains of mesophilic fungi. Lett. Appl. Microbiol., **34**, 461–465.

