Isolation and Evaluation of Tannin-degrading Fungal Strains from the Mexican Desert

Mario Cruz-Hernández[a], Juan Carlos Contreras-Esquivel[a], Faustino Lara[b], Raúl Rodríguez[a], and Cristóbal N. Aguilar[a,*]

[a] Food Research Department, School of Chemistry, Universidad Autónoma de Coahuila, Saltillo, Coah, México. Fax: +52 (844) 415-9534. E-mail: cag13761@mail.uadec.mx
[b] Centro Internacional de Servicios Fitosanitarios, S.A. de C.V. Saltillo, Coahuila, México

* Author for correspondence and reprint requests

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Eleven fungal strains (4 *Penicillium commune*, 2 *Aspergillus niger*, 2 *Aspergillus rugulosa*, *Aspergillus terricola*, *Aspergillus ornatus* and *Aspergillus fumigatus*) were isolated, characterized morphologically and by their capacity to degrade tannins. *Aspergillus niger* Aa-20 was used as control strain. Several concentrations of hydrolysable tannin (tannic acid) were used as sole carbon source. All strains were able to degrade hydrolysable tannins. *Aspergillus niger* GH1 and PSH showed the highest tannin-degrading capacity (67 and 70%, respectively). Also, the fungal capacity to degrade condensed tannin (catechin) was tested. *Aspergillus niger* PSH and *Penicillium commune* EH2 degraded 79.33% and 76.35% of catechin. The results demonstrated the capacity of fungi to use hydrolysable and condensed tannins as carbon source.

Key words: Fungal Strains, Screening, Tannin Degradation

Introduction

Tannins are water-soluble polyphenolic compounds, recalcitrant to biodegradation and with wide prevalence in plants. Hydrolysable and condensed tannins are the two major classes of tannins. These compounds play important roles as resistant agents to microbial decomposition, mainly due to the ability of these molecules to inhibit microbial growth by binding strongly to proteins and polysaccharides like cellulose and pectin (Lewis and Starkey, 1969; Bhat *et al.*, 1998). Condensed tannins are more resistant to microbial decomposition, while hydrolysable tannins are more easily degraded by some microorganisms (Lewis and Starkey, 1968; Lekha and Lonsane, 1997; Aguilar *et al.*, 2000, 2001; Aguilar and Gutiérrez-Sánchez, 2001). Condensed tannins are polymers of catechin or similar flavans and hydrolysable tannins are gallotannins (gallic acid and glucose) or ellagitannins (ellagic acid and glucose). Only a very limited number of microorganisms have been reported to degrade tannins, mainly bacteria. Information about fungal tannin degradation is scarce. For this reason, the mechanism of tannin degradation is not clear, especially in fungus (Bhat *et al.*, 1998).

The presence of tannins in several foods and beverages is undesirable, because their astringency and instability is a quality-detracting factor and this affects seriously the sales, which are around US$ 5 billion/year only in the case of fruit juices.

Tannase, a key enzyme in the degradation of hydrolysable tannins, is produced by a reduced group of microorganisms. This enzyme is increasingly used in a number of processes (Aguilar and Gutiérrez-Sánchez, 2001). Based on the potential use of tannase to reduce tannin levels in foods like fruit juices, the aim of this work was to isolate and select fungal strains with high capacity to degrade tannins and to produce the ability of tannase synthesis.

Materials and Methods

Microorganisms and tannin medium

Fungal strains were isolated from Mexican desert plants rich in tannins. Samples of soil and damaged tissue from *Quercus* spp., *Carya illinoensis*, *Larrea tridentata* and *Pinus sembroides* were collected and used as source of fungal strains. All strains tested are deposited in the collection DIA-UAdeC. *Aspergillus niger* Aa-20 was obtained from the collection IRD, France – UAM, México and it was used as control strain, because it has been characterized to degrade high levels of tannin (Aguilar, 2000; Ramirez-Coronel *et al.*, 2003).

Screening was performed in plates of selection medium which contained (g/l): tannin (10.0), KH_2PO_4 (4.38), $(NH_4)_2SO_4$ (8.76), $CaCl_2 \cdot 2H_2O$ (0.088), $MgSO_4 \cdot 7H_2O$ (0.88), $Na_2MnO_4 \cdot 2H_2O$ (0.0088), $MnCl_2 \cdot 4H_2O$ (0.018), $FeSO_4 \cdot 7H_2O$ (0.012) and agar (30.0). Incubation was performed at 30 °C for 5–6 d.

Isolation, conservation and identification

Strains with the ability to grow on tannin medium were selected and purified through monosporic cultures (Cruz-Hernández, 2002). These fungal strains were grown on potato dextrose agar and their growth was macro- and microscopically evaluated. Fungal spores were collected in a cryoprotector system based on skim milk and glycerol (Aguilar, 2000) and stored at −20 °C. Microscopic and biochemical analyses were used to identify genus and species following the indentification keys (Raper and Fennell, 1977; Moreno-Martinez, 1997) and the biolog™ kit, respectively.

Inoculum and culture conditions

For each fungus, 50 ml of PDA was placed in 1000 ml flasks and inoculated with spores from the cryo-protector system. Flasks were incubated at 30 °C for 5 d. A spore suspension was obtained using sterile Tween 80 (0.01%) and the content of spores per ml was calculated by counting them in a Neubauer chamber. Cultures were carried out in 250 ml flasks each containing 50 ml of medium which contained different types and quantities of tannins. Tannic acid and catechin were obtained from Sigma-Aldrich© products (Cat. No: T02001000 and C1788–5, respectively). Control cultures were inoculated with spores of *Aspergillus niger* Aa-20. Flasks were inoculated with 5×10^6 spores. This procedure was repeated for each of the fungi. Flasks were incubated at 30 °C, 200 rpm and monitored at 50 h of incubation period.

Analytical methods

Biomass formed in each of the flasks was filtered through Whatman paper No. 41, and dried at 60 °C over night. The biomass content was gravimetrically calculated. Tannic acid was determined by the phenol-sulfuric acid method (Dubois *et al.*, 1956) following the modifications reported by Aguilar *et al.* (2000). The catechin content was evaluated by the reverse phase HPLC method developed by Ramirez-Coronel and Augur (2003). Tannase activity was spectrophotometrically assayed (Sharma *et al.*, 2000). One tannase unit was defined as enzyme amount needed to release 1 μmol of gallic acid per min.

Results and Discussion

Isolation and characterization of fungal strains

Eleven fungal strains grew on plates with tannin as sole carbon source. These filamentous fungi were purified by monosporic cultures and stored at − 20 °C. All fungal strains isolated were characterized microbiological and biochemically. Results are according to literature data because only species of *Aspergillus* and *Penicillium* were able to grow on tannin (Lekha and Lonsane, 1997; Aguilar and Gutiérrez-Sánchez, 2001).

Tannin degradation

Initial tannic acid concentrations tested were 10, 20, 50 and 100 g/l. All strains tested against the first three initial tannic acid concentrations grew easily. Fig. 1 presents the values of maximal biomass of the fungus at 50 h grown from cultures with tannic acid at 50 g/l. All strains were able to grow well under these conditions. However, when an initial tannic acid concentration of 100 g/l was used, only two *Aspergillus* strains (PSH and GH1) grew, degrading higher levels (70 and 67%, respec-

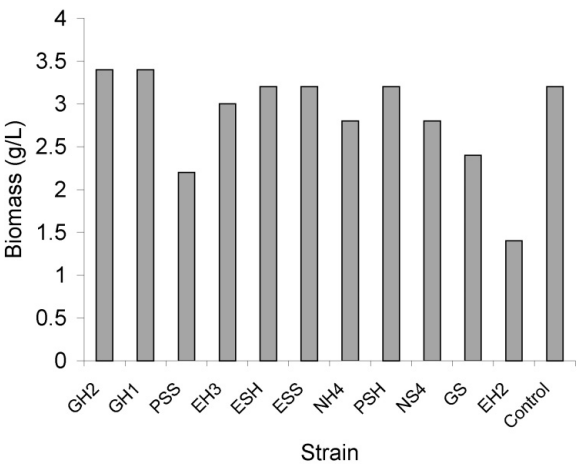

Fig. 1. Maximal fungal biomass values in cultures with an initial tannic acid concentration of 50 g/l. Strains: *P. commune* GH2, EH2, EH3 and ESS, *A. terricola* PSS, *A. ornatus* ESH, *A. rugulosa* NS4 and NH4, *A. niger* PSH, GH1, and Aa-20 (control).

tively) of tannic acid than the control strain (65%) at 50 h of incubation period.

At difference with the use of high tannic acid concentrations, low initial catechin concentrations of 2, 3, 5 and 10 g/l were tested. At initial catechin concentration higher than 5 g/l all strains were unable to grow. Fig. 2 shows the percentage of catechin degradation at the lowest catechin concentration tested. It is clear that all strains have a higher capacity to degrade catechin in comparison with the control strain, *A. niger* Aa-20, which has been characterized as a good tannin-degrading fungus (Aguilar *et al.*, 2000, 2001). It is important to consider that, while hydrolysable tannins can be utilized by several microorganisms, only very few members of the genera *Aspergillus* and *Penicillium* have been reported to grow on condensed tannins derivated from catechin (Lewis and Starkey, 1969; Grand, 1976; Ramirez-Coronel, 2002).

Tannase activity was only detected in those cultures with tannic acid as substrate and the fungi were unable to produce tannase during the catechin degradation. Fig. 2 shows the results of tannase activity produced at 50 h of culture by the fungi when an initial tannic concentration of 50 g/l was employed. It has been reported that tannase acts only on hydrolysable tannins releasing gallic acid and glucose. While in experiments with catechin, the fungus used its phenoloxidase system

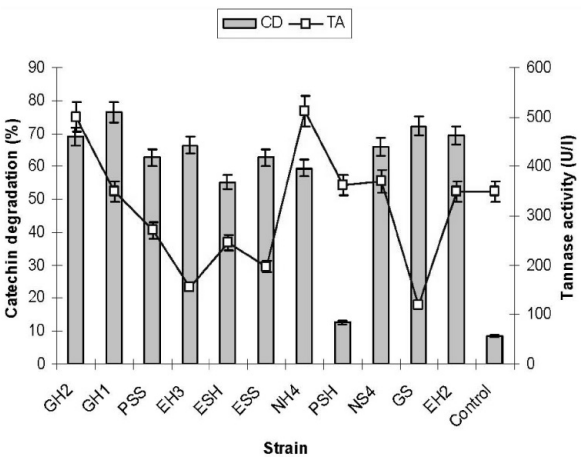

Fig. 2. Percentage of catechin degradation (CD) by fungal strains in cultures with an initial catechin concentration of 2 g/l, and maximal tannase activity (TA) values produced in cultures with an initial tannic acid concentration of 50 g/l.

(laccase, peroxidase and tyrosinase) *i.e.*, culture broths were oxidized.

Knudson (1913) first reported that tannic acid could be degraded by a strain of *Aspergillus niger.* Filamentous fungi, especially species of *Penicillium* and *Aspergillus* have been implicated in tannin degradation. Lewis and Starkey (1969) reported that pure cultures of some soil fungi grew on media containing tannins as sole carbon source. Different sources of tannins were compared and both condensed and hydrolysable tannins were used as substrates. *Aspergillus*, *Penicillium*, *Fusarium*, *Polyporus* and *Trametes* were shown to grow better on tannic acid (gallotannin) than on chestnut tannin (ellagitannin) or wattle tannin (condensed tannin). Most of the fungal species that have been used for biodegradation of tannery effluent belong to the genera *Aspergillus* and *Penicillium.* Other fungi, including *Chaetomium*, *Fusarium*, *Rhizoctonia*, *Cylindrocarpon*, and *Trichoderma*, are capable of degrading tannery waste constituents (Mahadevan and Muthukumar 1980). *Psalliata campestris* was found to oxidize catechin and *A. niger* could degrade gallic acid (Mahadevan and Sivaswamy 1985). *Cis*-aconitic, -ketoglutaric and citric acids were the intermediates of this degradation. Gallotannins, besides catechin, were degraded by *A. fumigatus* to gallic acid in 6–8 d. Subsequently, other workers also reported that species of *Aspergillus* and *Penicillium* could utilize catechin, gallotannin and gallic acid as carbon sources (Saxena *et al.*, 1995). In a number of fungal systems, tannins have been found to be degraded rapidly in the presence of other metabolisable substances. Ganga *et al.* (1977) found that *A. niger* and *Penicillium* spp. grew profusely in a medium containing glucose and wood-apple tannin. With wattle tannin at 0.3% and glucose at 166.7 mM concentration, growth of *A. niger* improved. Additional carbon and nitrogen sources favoured rapid production of tannase which, in turn, cleaved tannins and provided a continuous supply of carbon source for growth. The effects of certain factors, such as temperature, pH value and carbon sources on the decomposition of tannic acid and gallic acid by *Penicillium chrysogenum*, was studied by Suseela and Nandy (1985). However, their findings varied from the observations of earlier works. The decomposition of tannic acid and gallic acid was maximal in shake cultures at 28 °C, and both these acids were found to be completely decomposed in 3 d, whereas sugars present as additional carbon

source at 3% level retarded their degradation. From tannery liquors and xylophagous insects, which showed growth and hydrolytic action on tannins in culture media containing various concentrations of gallotannins, the tannin degrading enzymatic system of *Candida* was found to utilise gallotannins as substrate (Auki *et al.*, 1976a, b). This yeast tannase hydrolysed the ester and depside linkages of tannic acid. Later, a number of yeasts were reported which could degrade condensed tannins (wattle tannins) (Otuk and Deschamps, 1983; Vennat *et al.*, 1986). The strains isolated and studied were of *Candida guilliermondii*, *C. tropicalis* and *Torulopsis candida.* The degradation was determined by the estimation of leucoanthocyanidin and flavan-3-ol groups after treatment with the yeasts. A strain of *C. guilliermondii* degraded the flavan-3-ol structures but did not affect the leucoanthocyanidin components. Most yeasts were efficient degraders of quebracho tannins and reduced the tannin content of pine and gaboon wood bark extracts by 70 to 80% in five days (Otuk and Deschamps, 1983).

In conclusion, in this work all fungal strains were able to use tannins as sole carbon source. *Aspergillus niger* PSH and *Penicillium commune* EH2 exhibited their biotechnological potential because they can utilize condensed tannins while *Aspergillus niger* PSH and GH1 degraded high tannic acid levels used as carbon source. They could be used to produce tannase and degraded tannins directly in residues of coffee pulp, being a good alternative to solve the big problem that represents the accumulations of this agroindustrial byproduct in some countries like Mexico, Colombia or Brazil. Use of higher initial catechin concentrations and the enzymatic activities related are under investigation.

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