ISSN 1330-9862
(FTB-2075)

scientific note

Fungal Biodegradation of Tannins from Creosote Bush (*Larrea tridentata*) and Tar Bush (*Fluorensia cernua*) for Gallic and Ellagic Acid Production

Janeth Ventura[1], *Ruth Belmares*[1], *Antonio Aguilera-Carbo*[2], *Gerardo Gutiérrez-Sanchez*[3], *Raul Rodríguez-Herrera*[1] *and Cristóbal Noé Aguilar*[1]*

[1]Food Research Department, School of Chemistry, Universidad Autónoma de Coahuila, Saltillo, MX-25 000 Coahuila, Mexico

[2]Department of Food Science and Nutrition, Universidad Autónoma Agraria 'Antonio Narro', Buenavista, Saltillo, MX-25 000 Coahuila, Mexico

[3]Complex Carbohydrate Research Center, The University of Georgia, 315 Riverband Road, GA-30602-4712 Athens, USA

Received: July 11, 2006
Revised version: January 4, 2007
Accepted: June 13, 2007

Summary

In the present work, the production of two potent antioxidants, gallic and ellagic acids, has been studied using solid-state fermentation (SSF) of tannin-rich aqueous plant extracts impregnated in polyurethane foam. Extracts from creosote and tar bush were inoculated with *Aspergillus niger* PSH spores and impregnated in the polyurethane support. The kinetics of the fermentation was monitored every 24 h. The maximum biodegradation of hydrolysable and condensed tannins was, respectively, 16 and 42 % in creosote bush, and 40 and 83 % in tar bush. The maximal productions of gallic and ellagic acid (152 and 177 %, respectively) were reached with aqueous extracts of creosote bush. Tar bush extracts inoculated with *A. niger* PSH spores produced only gallic acid (92 %), while ellagic acid was not recovered after the fermentation process. Results demonstrated the potential use of these plants as a source for the production of antioxidants.

Key words: *Aspergillus niger* PSH, creosote bush, tar bush, gallic acid, ellagic acid

Introduction

Some plant species have developed the ability to survive long periods of dehydration, environment with high temperature and soils poor in organic matter (*1*). Plants like creosote bush (*Larrea tridentata* (DC.) Cov.) and tar bush (*Flourensia cernua* DC.) grow abundantly in Mexican desert, but have not been exploited for use or application irrespective of the fact that the leaves of these plants contain high concentrations of tannins (*2*). Some of these molecules are considered as nutraceuticals and their consumption helps in the prevention of some diseases. But at high levels of consumption, they become anti-nutritional and it has been suggested that high doses induce cellular DNA damage.

Tannins are a large class of complex phenolic compounds, comprising hydrolysable, condensed and complex tannins. Hydrolysable tannins are constituted of several organic acids, such as gallic and ellagic, which are usually linked by an ester-like bond with a glucose molecule. On acidic, basic or enzymatic hydrolysis, gallotannins produce both glucose and gallic acid. On the

*Corresponding author; Fax: ++52 844 4159 534; E-mail: cag13761@mail.uadec.mx

other hand, ellagitannins have one or more hexa-hydroxydiphenolic acid residues linked to glucose, forming a diester. Their hydrolysis results in the cleavage of glucose and hexa-hydroxydiphenolic residues that undergo spontaneous rearrangement to lactone and finally to ellagic acid (*3*,*4*).

Gallic acid (3,4,5-trihydroxybenzoic acid) is used in the manufacture of trimethoprim (TMP), an antibacterial agent, in leather industry (*5*), and as an antioxidant (*6*). Ellagic acid has been reported to have antimutagenic, anticarcinogenic, antioxidant, anti-viral and anti-inflamatory activities (*7–10*). Several fungal strains and tannin-rich substrates have been used for the production of gallic acid (*2*,*5*,*11*). For ellagic acid, few preliminary studies have been published (*12*). In the present paper, the fermentation of the phenolic extracts of creosote bush and tar bush to produce gallic and ellagic acids during a solid-state fermentation (SSF) with *Aspergillus niger* PSH was studied.

Materials and Methods

Plant sampling

Samples of creosote bush and tar bush plants were collected from February to April 2005 in the southern suburban zone of Saltillo City, Coahuila, Mexico. The collected samples were placed in black polyethylene plastic bags and transported to the Food Research Department, School of Chemistry, Universidad Autónoma de Coahuila, Mexico. The leaves of both plants were separated from flowers and stems, and dried in an oven at 60 °C, for a period of 7 to 10 days. After that the dried leaves were powdered in a mill (Mini 100, Molinos Pulvex, S.A. de C.V., Mexico), and stored in plastic bottles in a dark place.

Extraction of tannins

A mass of 100 g of dried powder was placed in an Erlenmeyer flask with 400 mL of 70 % acetone. The flask was covered with aluminium foil to avoid light exposure. This mixture was refluxed at 60 °C for 12 h. After this process, the sample was filtered using Whatman filter paper no. 41 and centrifuged at 3500 rpm for 15 min. The solvent was removed using a rotary evaporator (Yamato RE540) using a temperature below 60 °C and by avoiding light exposure.

Microorganism and inoculum

Spores of *Aspergillus niger* PSH (DIA/UAdeC (Departamento de Investigacion en Alimentos/Universidad Autonoma de Coahuila), Mexico collection, Mexico) were propagated on potato dextrose agar (PDA). The spores were collected using Tween 80 (0.01 %). Czapek minimal medium was prepared using the plant extracts as the sole carbon source (pH=5.0). *Aspergillus niger* PSH spores were inoculated in this medium at a concentration of $2 \cdot 10^7$ spores/mL.

Culture conditions

Batches of ten Erlenmeyer flasks (250 mL) with 3 g of polyurethane foam (cubes of 0.5 cm^3) sterilized and impregnated (at 70 % humidity) with 7 mL of the inoculated medium were used. Reactors were covered with brown paper and incubated at 30 °C. Kinetics of the SSF process was monitored by collecting samples at 0, 24, 48, 72 and 96 h of the process. Collected samples were washed using 25 mL of distilled water; and then the fermentation liquid was recovered by compression using a sterilized 60-mL syringe. Cotton was plugged inside the syringe to avoid the passage of particles and the collected samples were stored in small plastic bottles covered with aluminium foil at freezing temperature until further analysis.

Total and hydrolysable tannins

Folin-Ciocalteu method (FAO/IAEA, 2000) was used for the analysis of tannins (*13*). In this assay, 800 μL of the sample were put into a test tube and mixed with the same volume of Folin-Ciocalteu (Sigma-Aldrich) reagent, shaken and left for 5 min. Then this solution was diluted with 5 mL of distilled water and analyzed in a UV-Visible spectrophotometer at 725 nm for the determination of total tannins and at 480 nm for hydrolysable tannins. The obtained absorbance values were analyzed against the standard curves prepared with tannic and gallic acid for total phenols and hydrolysable tannins, respectively.

Condensed tannins

The proanthocyanidin content was evaluated as catechin equivalents and analyzed by the modified HCl-butanol method (*13*). Briefly, an aliquot of 0.5 mL of the sample was placed with 3 mL of HCl/*tert*-butanol (10 %, ratio 1:9), and then an aliquot of 0.1 mL of $NH_4Fe(SO_4)_2$ in 20 % HCl was added. The mixture was sealed hermetically and heated for 1 h at 100 °C in a hot water bath. After 1 h, it was cooled and the absorbance was read at 460 nm. The catechin equivalents were calculated with reference to standard curve obtained using catechin.

Gallic acid

The technique reported by Sharma *et al.* (*14*) was used for determination of gallic acid. Citrate buffer (pH=5.0), methanolic rhodanine 0.67 % and KOH (0.5 mol/L) were needed for this assay and all reactants were pre-incubated at 30 °C for 5 min. An aliquot of 0.5 mL was mixed with 0.3 mL of methanolic rhodanine solution and incubated under the same conditions mentioned above. After that, 0.2 mL of KOH solution were added and incubated again. Finally, 4 mL of distilled water were added to the reaction mixture and incubated at 30 °C for 10 min and the absorbance was read at 520 nm.

Ellagic acid

The method proposed by Wilson and Hagerman (*15*) was used for the determination of ellagic acid. Inside dark test tubes 10 mg of ellagic acid or 0.1 mL of the sample were placed, and then 0.1 mL of H_2SO_4 (2 mol/L) were added. The test tubes were frozen at –15 °C for 10 min, then sealed and the air was removed with a syringe. The test tubes with ellagic acid were incubated during 24 h at 100 °C. The test tubes were washed with 3 mL of pyridine and filtered. For determination, to 1 mL of filtered sample, 1.1 mL of pyridine and 0.1 mL of

HCl (37 %) were added, then the mixture was shaken and incubated at 30 °C for 5 min. After incubation, 0.1 mL of $NaNO_2$ (0.01 %) was added and the absorbance was read at 538 nm.

Results and Discussion

To evaluate antioxidant activity, the aqueous polyphenolic extracts were used as carbon source during the solid-state fermentation process using the fungal strain of *Aspergillus niger* PSH. This fungus demonstrated its capacity to degrade hydrolysable tannins and the resulting monomers were either consumed or accumulated.

Creosote and tar bush extracts recorded the highest consumption of total phenols in the samples collected at 48 h of SSF process (Fig. 1). Initial concentration of total phenols in unfermented creosote bush extracts was 7.29 mg/g of plant, and after 48 h of fermentation, it was 6.04 mg/g of plant. In the case of raw tar bush, the total phenolic content was 2.0 mg/g of plant, and after 48 h of fermentation, it decreased to 1.0 mg/g of plant.

The obtained results demonstrated that *Aspergillus niger* PSH degraded the hydrolysable tannin polymers present in phenolic extracts of both plants. The monomers obtained by the hydrolysis of this kind of tannins were consumed by the fungus during the first 48 h of culture and then the hydrolysis products were accumulated. However, it was observed that the monomers of condensed tannins were not consumed.

The hydrolysable tannins present in the creosote bush extracts were consumed (16 %) during the first 72 h of fermentation. At 96 h, the hydrolysable tannins were degraded and approx. 15 % of the monomers of phenolic acids were accumulated. In tar bush extracts the consumption of hydrolysable tannin content at 48 h was 40 % (Table 1).

The biodegradation of condensed tannins and the respective accumulation of catechin monomers was proportional to time (Table 1). The fungal strain recorded a similar behaviour in the fermentation kinetics of both substrates tested. The highest concentration of condensed tannins was reached at 96 h of fermentation process. During the fermentation, an increase of condensed tannins of 42 and 83 % was observed for creosote and tar bush extracts, respectively.

Table 1. Kinetic evaluation of hydrolysable and condensed tannins present in aqueous phenolic extracts of creosote and tar bush

	Hydrolysable tannins	
Time/h	Creosote bush extract	Tar bush extract
	$\frac{w(\text{tannin})}{\text{mg/g}}$	
0	8.63±0.0000493	1.84±0.0000025
24	8.00±0.0000539	1.26±0.0000435
48	7.36±0.0000539	1.10±0.0000410
72	7.23±0.0000287	1.23±0.0000025
96	7.32±0.0000854	1.20±0.0000298
	Condensed tannins	
24	8.70±0.0001	0.80±0.00002
48	9.00±0.0004	0.86±0.00008
72	10.20±0.0011	1.27±0.00009
96	12.40±0.0010	1.47±0.00006

The accumulation of gallic acid indicated the depolymerization of gallotannins and after its release this substance could be used as a substrate. *A. niger* PSH consumed nearly 72 % of free gallic acid in the extract; the minimum concentration reported was 0.14 mg/g of creosote bush at 48 h of the process. After that, an accumulation of gallic acid was observed, indicating that the rate of gallotannin hydrolysis was faster than the consumption rate of gallic acid. After 96 h, there was an increase in gallic acid of 152 % and the concentration was 0.48 mg/g of creosote bush (Fig. 2). In the fermentation of tar bush extracts, the gallotannins were depolymerised after 48 h and the glucose and gallic acid were released. The highest level of gallic acid was reached at 96 h with a value of 0.08 mg/g of tar bush.

Biodegradation of ellagitannins to ellagic acid and its accumulation was proportional to the fermentation time for both substrates. Initial ellagic acid concentration was 2.72 and 2.49 mg/g for tar bush and creosote bush, respectively. After 96 h, the ellagic acid accumulated to 92 and 177 % in creosote and tar bush extracts respectively (Fig. 3), reaching values of 4.74 mg/g of creosote bush and 7.56 mg/g of tar bush.

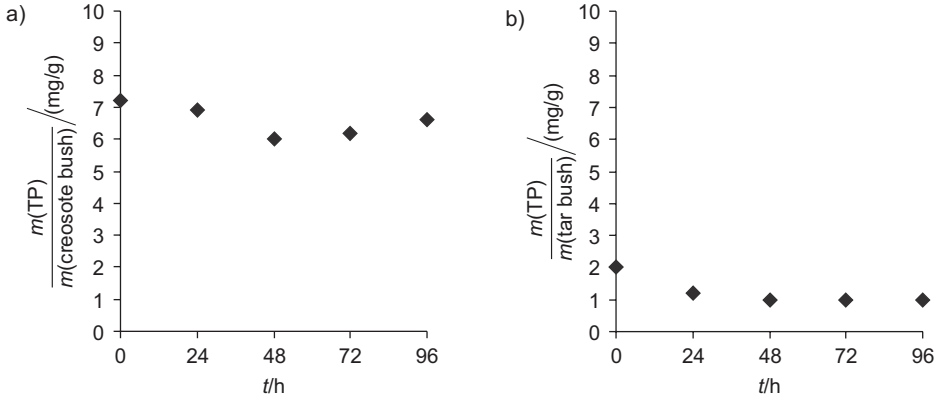

Fig. 1. Degradation of total phenolics (TP) by *Aspergillus niger* PSH in: a) aqueous phenolic extract of creosote bush, b) aqueous phenolic extract of tar bush

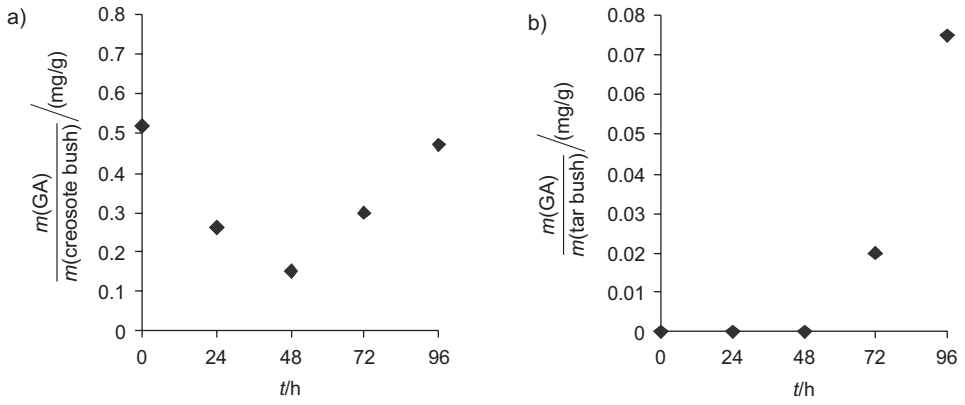

Fig. 2. Production of gallic acid (GA) by *Aspergillus niger* PSH in: a) aqueous phenolic extract of creosote bush, b) aqueous phenolic extract of tar bush

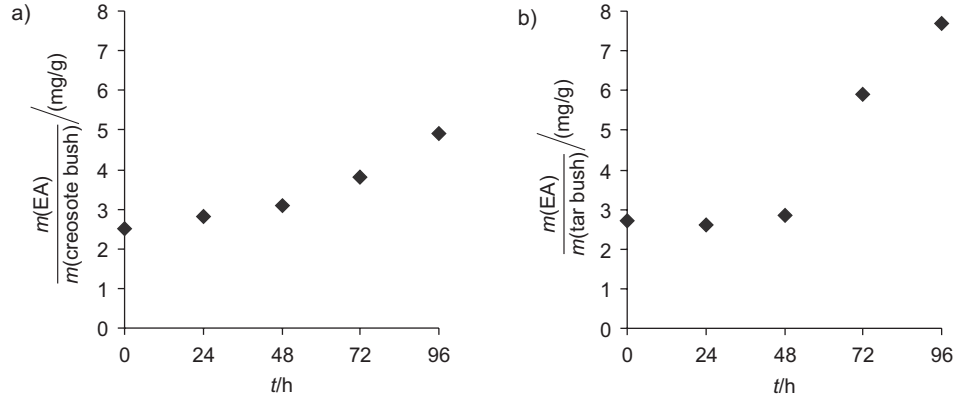

Fig. 3. Production of ellagic acid (EA) by *Aspergillus niger* PSH in: a) aqueous phenolic extract of creosote bush, b) aqueous phenolic extract of tar bush

The highest consumption of total phenols and hydrolysable tannins in the phenolic extracts of both plants was reached at 48 h of fermentation. This could be due to the fact that the phenolic extracts of both plant materials have complex polysaccharides, and moreover, the studied strain preferred to consume free monophenols and glycosides like gallic acid and glucose present in the extracts before the production of hydrolytic enzymes to degrade tannins.

Some tannin-rich sources and several microorganisms (*2,5,11,16*) have been used for gallic acid production and the hydrolytic enzyme responsible for its production is the tannase or tannin acylhydrolase (Table 2). It had been reported earlier that tannase can also hydrolyse ellagitannins. But the results of the present study did not show this pattern and hence we consider that this enzyme is unable to degrade ellagitannins.

Results obtained in this study are similar to those reported by Shi *et al.* (*12*) for valonea tannins (79.2 % at 168 h). Comparing these results, the lower rate of hydrolysis in valonea tannins could be due to low protein levels in its phenolic extracts. However, Belmares-Cerda *et*

Table 2. Biotechnological production of gallic and ellagic acids

Gallic acid				
Production/%	Culture status	Microorganism	Time/h	Reference
85.67	solid	*Rhizopus oryzae*	60.0	(*11*)
90.48	solid	*Aspergillus foetidus*	72.0	
0.14	solid creosote bush	*A. niger* PSH	48.0	(*16*)
>100.00	solid tar bush		24.0	
90.90	solid	*Rhizopus oryzae*	72.0	(*5*)
15.00	solid	*A. niger* Aa 20	43.5	(*2*)
Ellagic acid				
79.2 21.0	solid liquid	Co-culture (*A. niger*-*Candida utilis*)	168.0	(*12*)

al. (*17*) reported better results using the same substrates as tested in this study. This could be explained by the fact that they used the leaves of creosote and tar bush as a substrate, and this matrix has a high content of protein and tannin-protein complexes (*18*).

Several fungal species such as *Penicillium*, *Chaetomium*, *Fusarium*, *Rhizoctonia*, *Cylindrocarpon* and *Trichoderma* (*19*) were reported to use the monomers of gallic acid as a substrate for the oxidative breakdown to a simple oxidative acid, which then enters the citric acid cycle (*20*) and is converted to pyrogallol. Finally, our results demonstrated the possibility of considering that both tested plants in this study, creosote bush (*Larrea tridentata* (DC.) Cov.) and tar bush (*Flourensia cernua* DC.), could be employed in the microbial production of antioxidants due to their high tannin content.

Conclusions

Phenolic extracts of creosote and tar bush can be used as a carbon source by *A. niger* PSH. The gallic acid can be consumed by the fungus during the solid-state fermentation while ellagic acid is not used as a substrate. It is possible to decrease or increase the content of phenolic compounds by controlling the process of solid--state fermentation. This study demonstrated the feasibility of the production of potent nutraceuticals. This is the first work about the use of aqueous phenolic extracts of Mexican semi-desert plants as a substrate for solid--state fermentation and for the production of important nutraceuticals like gallic and ellagic acid. However, it is necessary to optimise the fermentation process.

Acknowledgements

This work was supported by the Mexican Council of Science and Technology (CONACYT), Project: CONAFOR-CONACYT-2004-C01-13.

References

1. D.F. Gaff, Desiccation-tolerant flowering plants in southern Africa, *Science, 174* (1971) 1033–1034.
2. B. Treviño-Cueto, M. Luis, J.C. Contreras-Esquivel, R. Rodríguez, A. Aguilera, C.N. Aguilar, Gallic acid and tannase accumulation during fungal solid state culture of a tannin-rich desert plant (*Larrea tridentata* Cov.), *Bioresour. Technol. 98* (2007) 721–724.
3. C.N. Aguilar, G. Gutiérrez-Sánchez, Review: Sources, properties, applications and potential uses of tannin acyl hydrolase, *Food Sci. Technol. Int. 7* (2001) 373–382.
4. Y.Y. Ow, I. Stupans, Gallic acid and gallic acid derivatives: Effects on drug metabolizing enzymes, *Curr. Drug Metab. 4* (2003) 241–248.
5. B. Kar, R. Banerjee, B.C. Bhattacharyya, Microbial production of gallic acid by modified solid state fermentation, *Ind. J. Microbiol. Biotechnol. 23* (1999) 173–177.
6. K.W. Lee, Y.J. Kim, H.J. Lee, C.Y. Lee, Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine, *J. Agric. Food Chem. 51* (2003) 7292–7295.
7. A.S. Meyer, M. Heinonen, E.N. Frankel, Antioxidant interactions of catechin, cyanidin, caffeic acid, quercetin and ellagic acid on human LDL oxidation, *Food Chem. 61* (1998) 71–75.
8. A.A. Bohn, C.S. Forsyth, G.D. Stoner, D.J. Reed, A.A. Frank, Effect of cocaine, 95 % oxygen and ellagic acid on the development and antioxidant status of cultured rat embryos, *Toxicol. Lett. 95* (1998) 15–21.
9. Y. Ogawa, K. Kanatsu, T. Iino, S. Kato, Y.I. Jeong, N. Shibata, K. Takada, K. Takeuchi, Protection against dextran sulphate sodium-induced colitis by microspheres of ellagic acid in rats, *Life Sci. 91* (2002) 827–839.
10. K.I. Priyadarsini, S.M. Khopde, S.S. Kumar, H. Mohan, Free radical studies of ellagic acid, a natural phenolic antioxidant, *J. Agric. Food Chem. 50* (2002) 2200–2206.
11. G. Mukherjee, R. Banerjee, Biosynthesis of tannase and gallic acid from tannin rich substrates by *Rhizopus oryzae* and *Aspergillus foetidus*, *J. Basic Microbiol. 44* (2004) 42–48.
12. B. Shi, Q. He, K. Yao, W. Huang, Q. Li, Production of ellagic acid from degradation of valonea tannins by *Aspergillus niger* and *Candida utilis*, *J. Chem. Technol. Biotechnol. 80* (2005) 1154–1159.
13. *Quantification of Tannins in Tree Foliage*, A laboratory manual for the FAO/IAEA, FAO /IAEA Working Document, IAEA, Vienna, Austria (2000) Sec. 3.05–4.09.
14. S. Sharma, T.K. Bhat, R.K. Dawra, A spectrophotometric method for assay of tannase using rhodanine, *Anal. Biochem. 279* (2000) 85–89.
15. T.C. Wilson, A.E. Hagerman, Quantitative determination of ellagic acid, *J. Agric. Food Chem. 38* (1990) 1678–1683.
16. R.E. Belmares-Cerda, Composition and fungal biodegradation of some polyphenolic compounds present in the Mexican semiarid region, *MSc Thesis*, Universidad Autónoma de Coahuila, Mexico (2004) pp. 7–30 (in Spanish).
17. R.E. Belmares-Cerda, J.C. Contreras-Esquivel, R. Rodríguez, A.F. Aguilera, C.N. Aguilar, Biotechnological approach of natural resources of the Mexican desert, *Claridades Agropeccuarias, 3* (2006) 25–27 (in Spanish).
18. M. Naczk, R. Amarowicz, D. Pink, F. Shahidi, Insoluble condensed tannins of canola/rape seed, *J. Agric. Food Chem. 48* (2000) 1758–1762.
19. T.K. Bhat, B. Singh, O.P. Sharma, Microbial degradation of tannins – A current perspective, *Biodegradation, 9* (1998) 343–357.
20. J.A. Field, G. Lettinga: Biodegradation of Tannins. In: *Metal Ions in Biological Systems, Vol. 28*, H. Sigel, A. Sigel (Eds.), Marcel Dekker Inc., New York, USA (1992) pp. 61–97.