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Toxicity and *In vitro* Digestibility of Creosote Bush and Tar Bush Fermented under Fungal Solid State Culture Conditions

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Abstract: Creosote bush and tar bush are 2 xerophilic shrubs considered as potential animal feed after their fungal fermentation to reduce the tannins content. Kinetics of biodegradation of tannins during the fermentation of both plants by 2 strains of *Aspergillus niger* (GH1 and PSH) were evaluated under solid state culture conditions. The *in vitro* digestibility and the presence of mycotoxins were also analyzed in the fermented plants. The growth of fungal strain was directly associated to biodegradation of tannins (hydrolysable and condensed tannins) in both plants. *A. niger* GH1 degraded more than 94% of total tannins of creosote bush while, *A. niger* PSH degraded already of 77% of total tannins of tar bush. Results of digestibility of fermented materials were 9.72% for creosote bush and 13.98% for tar bush, respectively. Aflatoxins present in creosote bush were B_1 (692 ppt), B_2 (<160 ppt) and G_1 (<160 ppt) and for tar bush B_1 (182 ppt), B_2 (<160 ppt) and G_1 (<160 ppt). These results showed that animal health risk by the consumption of these kinds of fermented products is very low because are according with the official norms.

Key words: Tannin biodegradation, aflatoxins, digestibility, creosote bush, tar bush, solid state culture

INTRODUCTION

Two dominant shrubs of the northern Chihuahuan Desert are creosote bush [*Larrea tridentata* (Sess. and Moc. ex DC.) Cov.] and tar bush (*Flourensia cernua* DC.) Grasslands are being replaced by shrubs in the arid south-west region for the last 150 years (Aguilar and Gutiérrez-Sánchez, 2001). Both shrubs contain high polyphenols or tannins and hence animals do not prefer eating these shrubs. Further, when animals eat these plants, the tannins inhibit some digestive enzymes and also reduce the availability of the assimilable protein by binding with proteins, causing serious problems to animal monogastric digestive systems (Goel *et al*., 2005). The plants use their high content of tannins as a defense against animal and microbial attack (Lekha and Lonsane, 1997). It is important to note that the tannins are generally considered as inhibitors of the growth of microorganisms (Bhat *et al*., 1998). Nevertheless, it is also known that some fungi like *Aspergillus* sp. and *Penicillium* sp. have the enzymatic machinery necessary to degrade some hydrolyzable tannins, specifically the tannic acid by means of tannase enzyme (Lekha and Lonsane, 1997; Cruz-Hernandez *et al*., 2006; Aguilar *et al*., 2007).

Tannins can be classified in two groups: Hydrolysable tannins and condensed tannins. Hydrolysable tannins are formed by a molecule of sugar (generally glucose) linked to a variable number of molecules of phenolic acids (like gallic, digallic and ellagic acids). They are common to observe in dicotyledonous plants. The condensed tannins are polymers of catechin or flavanoids linked by carbon-carbon links forming polymers of the flavan-3-ol or flavan-3-4-dyol. Two differences between the hydrolysable and condensed tannins are the sugar content and the ability to be utilized as substrate by the tanasse enzyme. Condensed tannins are not linked with sugars and the tannase enzyme does not act on this kind of tannins, only on the hydrolysable tannins (Contreras-Dominguez *et al*., 2006).

The oxidative degradation of hydrolysable tannins has been studied mainly in *Aspergillus niger ssp.* and the degradation pathway of the gallic acid has been

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determined. In *Aspergillus niger*, the gallic acid is oxidised by an oxygenase to form an unstable intermediary from the citric acid cycle. This compound is descarboxylated to form the aliphatic acid and this entry into the citric acid cycle.

Lambraki and Karagouni (1998) reported that the content of tannins in carob diminished 80 % utilizing this plant as sole carbon source in liquid culture with *Aspergillus* ACTC92.

Recently, we have published the composition and fungal degradation of tannins present in creosote bush and tar bush (Belmares-Cerda *et al*., 2007) and the gallic acid and tannase accumulation during fungal Solid State Culture (SSC) of creosote bush (Treviño-Cueto *et al*., 2007) demonstrating the biotechnological potential of the use of SSC in the biotransformation of these plants for the production of the antioxidant gallic acid. However, the solid residues generated by the bioprocess represent an attractive alternative to be considered as animal feed. By this reason, the aim of this research was to evaluate the toxicity and in vitro digestibility of the fermented solids obtained as by-product of the SSC using the fungal strains of *A. niger* GH1 and PSH.

MATERIALS AND METHODS

Microorganisms: For this study two strains of *Aspergillus niger* (GH1 and PSH), belonging to DIA-UAdeC collection and previously isolated, conserved and characterized by Cruz-Hernández *et al*. (2005) were used.

Plant materials: Shrubs creosote bush and tar bush were collected from the Chihuahuan Desert (Northern Coahuila, México) during the winter season (2006) and transported to the Microbiology Laboratory of the Food Research Department, Universidad Antonoma de Coahuila, Saltillo City; where their leaves were cleaned, dried, pulverized and stored at room temperature in black bags. A physicochemical characterization of the plants was previously reported by Belmares-Cerda *et al*. (2007).

Solid state culture SSC: Once the plant samples were collected and prepared, 250 g were weighted and mixed with the culture broth and adjusted an initial moisture content of 70%. Culture broth composition was (g L^{-1}): KH_2PO_4 (4.38), $NaNO_3$ (8.79), $MgSO_4.7H_2O$ (0.88), $CaCl_2.2H_2O$ (0.088), $MnCl.6H_2O$ (0.018), $NaMoO_4.2H_2O$ (0.0088) and $FeSO_4.7H_2O$ (0.012). Material to ferment was inoculated with 2×10^7 spores per gram of sample, using *A. niger* GH1 for creosote bush and *A. niger PSH* for tar bush. For the cultures, an initial pH of 5.5 and a temperature of 30°C were used. , SSC's were kinetically monitored dunring 96 h with sampling each 24 h. Once the kinetic finished, the fermented samples were weighted and dried to consider the biomass formed by difference of weights. The dry sample was stored for toxicity and *In vitro* digestibility tests.

Tannin degradation: All determinations were made in triplicates. Condensed tannins contents were determined using the HPLC method described by Makkar *et al*. (1993) and the hydrolysable tannins contents were quantified using the method reported by Waterman and Mole (1994). Standard curves were prepared with gallic acid and catechin for hydrolizable and condensed tannins, respectively.

***In vitro* digestibility:** The ruminal liquid was obtained from a fistulated bovine (fistula was made complied with all relevant Mexican guidelines and institutional policies) and stored at its original temperature. For the test, CO_2 was bubbled into ruminal liquid until reaching a pH of 7. Later, 0.5 g of fermented sample were collocated into bunsen tubes and 50 mL of McDougal reagent and buffered ruminal liquid were added and incubated at 39°C by 48 h. The blank contained only McDougal reagent-ruminal liquid. After, incubation, samples were refrigerated during 30 min and centrifuged at 3000 rpm for 10 min. Supernatants were separated and mixed with 50 mL of acid pepsin and incubated during 48 h at 39°C. Then, samples were filtered on Wathman 41 filters and washed with distilled water to avoid leaving remainders; papers filter were deposited in crucibles and were placed in the incubator to 50°C by 24 h. Samples were placed in a dryer by 10 min and they were weighed, the samples took to ashes to be able to determine the percentage of digestibility of total dry matter, the organic matter and the inorganic matter.

Toxicity: The Aflatoxins (AF) were obtained of the sample with methanol 80%, the extract was filtered and diluted with water and passed through a inmunoafinity column which contains Monoclonal Antibody (McAc) specific for AF B1, AF B2, AF G1 and AF G2. The AF were isolated, purified and concentrated in the column and later eluded with acetonitrile. The eluded was derivatizated with trifluoroacetic acid. The AF were quantified in individual form by chromatography in reverse phase and detected by fluorometry. The obtained results were expressed in parts by trillon (pt).

Data analysis: Obtained results were analyzed using the software "Analisis estadisticos" licensed by the School of Agronomy of the Universidad Autónoma de Nuevo Leon, Mexico using a Tukey's test for means comparison. In addition, kinetic parameters associated to biomass formation and tannin degradation were calculated.

RESULTS AND DISCUSSION

In this study, the fungal degradation of tannins present in creosote bush and tar bush was evaluated. Also, the toxicity and *in vitro* digestibility of the fermented solids obtained from the SSC was determined.

Figure 1 shows the kinetics of growth of *A. niger* GH1 on creosote bush and *A. niger* PSH on tar bush. Fungal biomass production was 2.5 times more in the SSC of tar bush, however the specific growth rate (μ) values were not shown a significant difference (Table 1). It is important to consider that the fungal growth values were adjusted to Verhuslt-Pearl model and solved by Solver tool (Microsoft Excel) to estimate the value of μ.

It is important to note that the tannins concentration is 6 times higher in creosote bush than the level of tar bush. But the capacity to degrade tannins by *A. niger* GH1 is considerably higher, reaching a tannins degradation almost complete (Table 1). Figure 2a shows the kinetics of condensed tannins biodegradation and Fig. 2b shows the hydrolysable tannins biodegradation using the *A. niger* GH1 on creosote bush and *A. niger* PSH on tar bush.

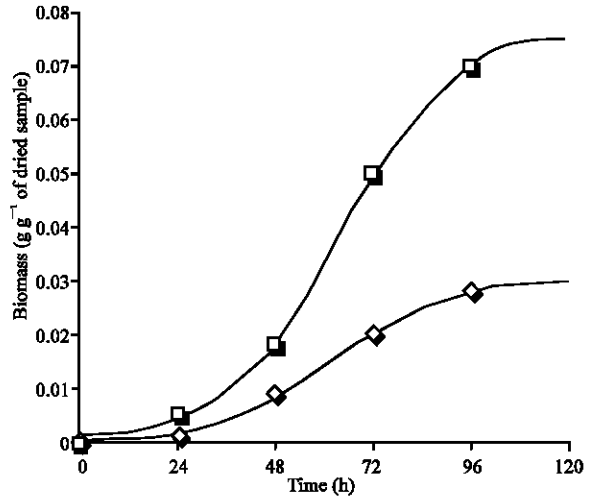

Fig. 1: Kinetics of growth of *A.niger* GH1 on creosote bush (◊) and *A. niger* PSH on tar bush (□) under solid state culture conditions. Line represents the calculated values with the Verhuslt-Pearl Model

The study of degradation of tannins is always complex due to reactivity and diversity of molecules participating in the process (Chandra *et al*., 1973; Ross and Corden, 1974). The biochemical mechanism of tannins biodegradation is supported with few published works (Bhat *et al*., 1998). Considering the fact that the degradation of tannins is based on catalytic reactions of enzymes produced during the growth of strains, the tannin acyl hidrolases play an important role in the degradation of hydrolyzable tannins, however condensed tannins are not hydrolysed by "classical tannases". Contreras-Dominguez *et al*. (2006) suggested that the initial degradation steps are carried out by mono or dioxygenases. However, further

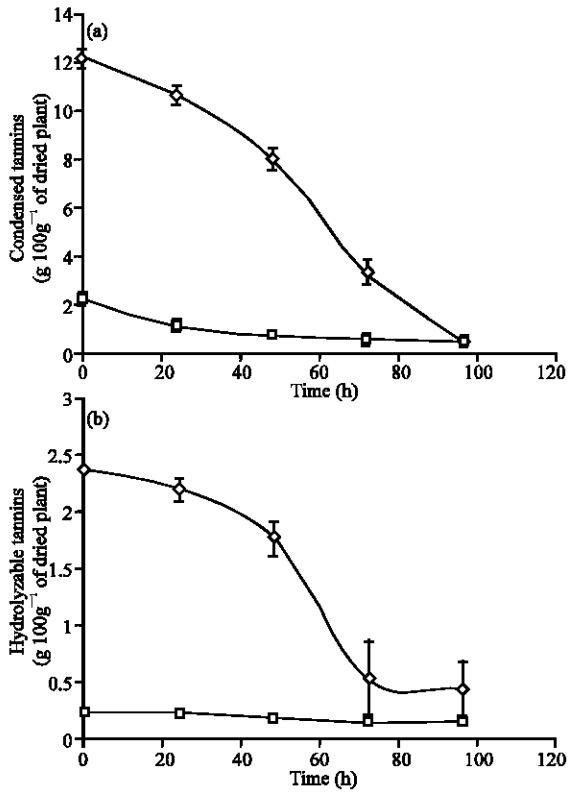

Fig. 2: Kinetics of tannins biodegradation by *A.niger* GH1 on creosote bush (◊) and *A. niger* PSH on tar bush (□) under solid state culture conditions. Condensed tannins (a) and Hydrolyzable tannins (b)

Table 1: Kinetic parameters of the fungal solid state culture of both xerophilic plants

			SSC system	
Kimetic parameter		Units	Creosote bush	Tar bush
$Y_{X/S}$	Yield	g of biomass per g of dried sample	0.002	0.037
m	Specific growth rate	1/hour	0.070	0.073
q_S	Tannins uptake quotient	g of tannins per g of biomass per hour	34.788	1.993
V	Tannin degradation rate	mg of total tannins per gram of dreid sample per hour	2.000	0.014
E	eficiency of degradation	percent	94.030	76.850

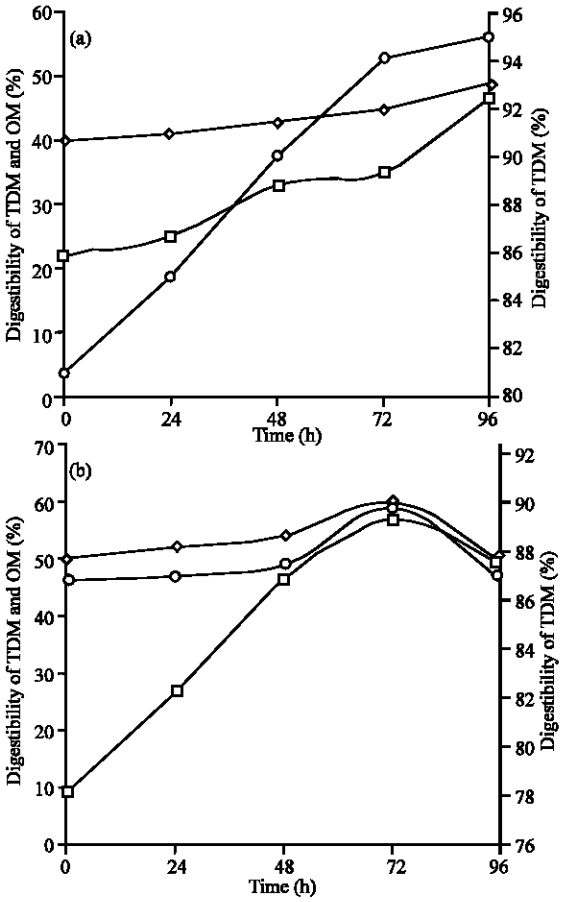

Fig. 3: Percent of digestibility of fermented solids of creosote bush (with *A. niger* GH1) and tar bush (with *A. niger* PSH). Total dry matter (♦), organic matter (□) and inorganic matter (○)

studies are needed to further characterize the degradation of condensed tannins (Aguilar *et al*., 2007).

***In vitro* digestibility:** Digestibility of fermented solids from kinetics of SSC was evaluated *in vitro*. Figure 3a contains the results obtained in the SSC of creosote bush with *A. niger* GH1. For the case of the Inorganic Matter (IM) the digestibility increased 18% as can be observed at 96 h of culture, which can be due to the intestinal flora that is in rumen of animals, which use the mineral presents in the plant for the activation of different mechanisms, because the minerals serve like activators or catalysts as reaction. In the case of the Total Dry Matter (TDM) a high percentage of digestibility can be observed at the end of SSC, which means an increment of the 22% of the initial percentage. In the case of the Organic Matters (OM) a greater percentage of digestibility can be observed at 96 h, with a 46,82% of digestibility, which can be due to that the proteins produced by the microorganisms are available and therefore, they can be assimilated more easily, because the pepsins and the microbial flora present in rumen act of one better way on these molecules.

Table 2: Content of Aflatoxins present in creosotebush *A. niger* GH1

Time of the fermented sample	Aflatoxin type	Concentration (ppt)
GOB/GH1-48 h	AF B_1	457.5
	AF B_2	< 160
	AF G_1	< 160
	AF G_2	< 160
GOB/GH1-72 h	AF B_1	< 160
	AF B_2	< 160
	AF G_1	< 160
	AF G_2	< 160
GOB/GH1-96 h	AF B_1	692.8
	AF B_2	< 160
	AF G_1	< 160
	AF G_2	< 160

Table 3: Content of Aflatoxins present in tarbush *A. niger* PSH

Time of the fermented sample	Aflatoxin type	Concentration (ppt)
HOJ/PSH-48 h	AF B_1	409.6
	AF B_2	< 160
	AF G_1	< 160
	AF G_2	< 160
HOJ/PSH-72 h	AF B_1	182.3
	AF B_2	< 160
	AF G_1	< 160
	AF G_2	< 160
HOJ/PSH-96 h	AF B_1	612.5
	AF B_2	< 160
	AF G_1	< 160
	AF G_2	< 160

Figure 3b shows the results of digestibility of fermented solids from kinetics of SSC of tar bush with *A. niger* PSH. For the case of the IM a greater increase in the digestion is observed the 48 h of a 13.32% of the initial content of inorganic material present in the material. For the case of the TDM, a greater percentage of digestibility can be observed 72 h, with a 63.46% of digestibility, which means an increase of the 13.98% of the initial percentage. In the case of the OM, a greater percentage of digestibility can be observed the 96 h, with a 61.41% of digestibility, which means an increase of the 14.73% of the initial percentage, which can be due to that the proteins an biomass produced by the fungus are available and therefore, they are assimilated easily.

Toxicity: Table 2 and 3, respectively show the contents of Aflatoxins (AF B1, high AF B2, AF G1 and AF G2) present in the samples of creosote bush fermented with *A. niger GH1* (GOB/GH1) and of tar bush with *A. niger PSH* (HOJ/PSH). They show the type of aflatoxins and the concentration in parts by trillon (ppt).

The content of aflatoxins present in the fermented vegetal materials was considerably minimum and taking into account the maximal levels allowed in feeds established in the Mexican Official Norm (NOM188, 2002)

(300 μg Kg^{-1}) and in Mexican Official Norm (NOM061, 1999) (20 ppb for the high frequency B1), it is possible to demonstrate that the fermented vegetal materials contain fungal toxins, but in amounts that do not cause poisonous effects in the animals.

The obtained results show that in the fermented materials exist fungi toxins. For the case of the creosote bush, particularly for the AF B1, at 48 h of culture a concentration of 457,5 ppt was detected and in agreement to the fermentation process carried out the concentration increases until reaching a maximum concentration of 612,5 ppt. At 96 h, which could be dur to the stress condition of the fungus. For the case of tar bush, it happens something similar and to the 96 h the greater concentration was obtained.

CONCLUSION

Solid state culture process is an attractive alternative to bioprocess the plants creosote bush and tar bush because during the fungal process the anti-physiological characteristic is eliminated due to the tannins degradation increasing the digestibility, although it is necessary to optimize the processes of fermentation to obtain better yields. Also, the fermented vegetal material presents fungi toxins, but in negligible concentrations and below the maximum levels established in the Mexican Official Norms; therefore, it could be used like feed for the cattle and other animals.

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