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Effect of Culture Parameters on the Degradation of a Hydrolyzable Tannin Extracted from Cascalote by *Aspergillus niger*

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Tannin compounds became a contaminant in the environment because their biodegradation persistence and the increasing amount of sludge or waste water effluent to the environment in the last years (Secretaria de Desarrollo Social 1994). During leather processing using tannin, an excess of phenolic compounds are discarded as sludge or in the waste water stream. The biodegradation of tannin in contaminated sites using enzymatic system is an attractive alternative to solve the problem of pollution in the environment. *Penicilliun* and *Aspergillus* have been reported to grow at high concentrations of tannin acid as a sole C source (Scalbert 1991). Several factors affect the growth and enzyme activities of *A. niger* such as nitrogen sources, pH, temperature and oxygen diffusion. Thus, data from literature have shown that *A. niger* requires for growing and producing tannase activity: low nitrogen in the medium (Kong *et al.* 1995), alkaline pH (Porruat *et al.* 1985 and 1987) or acidic pH in the medium (Lekha and Lonsane 1994; Porruat *et al.* 1982, 1985 and 1987) and the growth temperature between 37 to 47 °C (Lekha and Lonsane 1994; Pourrat *et al.* 1982, 1985 and 1987). The oxygen level has no influence on the growth of *A. niger* (Träger *et al.* 1992a and b) but at higher levels might affect the gallic acid degradation (Scalbert 1991). Thus, those single parameters have been reported that affects the tannin degradation. However, scarce information has been reported about the combined parameters on the tannin degradation (Aguilar et al. 2001, Seth and Chand 2000).

The purpose of this study was to determine the effect of culture conditions on *Aspergillus niger* for the biodegradation of tannin extracts from cascalote (*Caesalphinia cacalaco*) under submerged fermentation.

MATERIALS AND METHODS

The strain *Aspergillus niger* CDBB H-176 was selected because it was capable to grow on tannin extract and tannin acid. *A. niger* was maintained on culture slants of potato dextrose agar (PDA, Boxon, Mx.) containing the tanning extract (2% w/v), grown for 6d at 28°C. Then the slants were stored at 4°C and subcultured every alternate month. The inoculum was grown for 8 days at 30°C until the spores concentration reached 1 x 10^8 spores mL^{-1}. The spores were removed with a 0.01% Tween 80 solution on sterile distilled water. The amount of the inoculum used was 10%.

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The hydrolysable tannin was extracted from cascalote (*Caesalphinia cacalaco*). Briefly, cascalote was mixed with the extracting solution (water: acetone: ethanol, 8:1:1) in the ratio 1:4 (w/v). The cascalote extracting solution was heated at boiling point after being acidified strongly with hydrochloric acid and a small amount of sodium bisulphite was added. These conditions allow keeping the hydrolizable extract in the soluble solution while the condensed were precipitated (Acosta-Ortiz, 1999).

The submerged fermentation culture was performed in 125 mL Erlenmeyer flask with 42 mL of Karow's medium (Prescott and Dunn 1959). The sucrose in the medium was replaced by 5% of tannin compound as a sole carbon source. The fermentation was for 10d and samples were monitored at the beginning and at the end of the experiment.

Cellular growth was measured by dry mycelia weight. The cellular growth was filtered through Whatman filter paper No 1. The moist weight mycelia biomass was dried at 60°C overnight. The supernatant was used to determine glucose by phenol-sulphuric acid method (Dubois et al., 1956) and soluble protein assayed by the method of Bradford (1976).

The separation and quantification of gallic acid and similar compounds from the tannic acid produced during the fermentation were carried out by HPLC system (Varian Inert 9012 pump, a Varian 9065 Polychrome detector and a Varian 9300 sample loop injector, coupled to a computer and managed by Star Chromatography Software version 4.01 program), set at 260 nm for gallic acid and 280 nm for phenolic compounds. The column was a C18 (Varian, 4.6 x 15 cm). The mobile phase for gallic acid was methanol: water: acetic acid (glacial) (60:39:1 v/v/v) with a flow-rate of 1.0 ml min^{-1}. The same column was used for the analysis of phenolic compounds with a mobile phase A) formic acid 1% and B) methanol. The gradient range was: 0 - 2 min, 93% A in B; 2 - 10 min, 85% A in B; 10 - 15 min, 20% A in B; 15 - 25 min, isocratic, 20% A in B.

The molecular weight of tannin compounds was monitored throughout the fermentation and they were determined by gel chromatography. The column was packed sephadex G-15 with an average particle size of 80 μm. The collected fractions were monitored in a spectrophotometer (UV/VIS Perkin-Elmer Lamda 3A) at 266 and 271 nm. Blue dextran and different markers were used to calculate the empty volume and the molecular weight, results were expressed in Daltons.

An experimental design was conducted to elucidate the effect of selected factors on the tannin degradation compounds. Eight experiments with four factors and two levels were based on half fraction factorial experiment design 2^{n-1}. Table 1 shows the factors and the levels tested. The four factors involved were: urea (U), pH (P), temperature (T) and stirring (S). Experiments were performed by confounded the interaction UPT with the effect of factor S (defining contrast I, UPT) (Montgomery 1991).

All the measurements are the mean of three replicates. Analysis of variance (ANOVA, one way) and standard error were processed with a statistical package

(SAS, version 6.01). Response variable were gallic acid concentration, absorbance, dry biomass and glucose concentration. The kinetic was performed with the best conditions obtained from the ANOVA test.

Table 1. Factorial Experiment with four factors and two levels for tannin biodegradation by *Aspergillus Niger* CDBB H-176.

Factor	Level	
	Low (-)	**High (+)**
Urea (U) (g L^{-1})	0.5	1.0
pH (P)	3.5	6.5
Temperature (T) (°C)	28.0	39.0
Stirring (S) (rpm)	0.0	150.0

RESULTS AND DISCUSSION

The tannin content in the extract (Table 2) was lower than those reported on main industrial tannin extract (80%) and from gall nuts (77%), and higher than tara pods and sumac leaves (56 and 34% respectively) (Barthomeuf et al. 1994).

Table 2. Characteristics of vegetable tanning extract.

Characteristic	
Color	Yellow
Glucose	860.40 μg mL^{-1}
Light sensitive	Yes
Molecular weight	707.28 Dalton
pH	4.5 - 5.0
Protein	No detected
Tannins	60 %
Total nitrogen	0.00135 %

The conditions used in the kinetic were the results of fractional factorial design: Temperature 28°C, pH 3.5, stirring 150 rpm, urea 1.0 g L^{-1} using a 5% of tannin extract concentration (Fig. 1). The absorbance increased sharply at 4d and glucose produced at 6d incubation. The increase of glucose might be due to the release of glucose from the tannin structure (Beverini and Metche 1990; Deschamps et al. 1983; Kumar1992; Kumar et al. 1995). After 4d, glucose decreased up to less than 4 mg mL^{-1} at 11d incubation. Simultaneously, fungal biomass at 12d increased significantly up to 3 fold compared to 5d incubation. These results suggest that glucose was used as a C source for fungal growth.

The growth rate obtained by Monod's equation was 0.18 d^{-1} and the doubling time 4d.

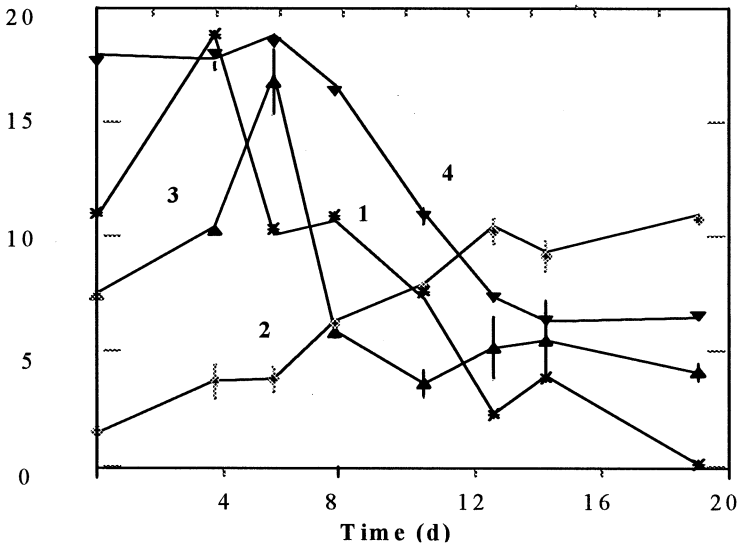

Figure 1. Kinetic of tannins biodegradation by *Aspergillus niger CDBB H-176* 1 - Absorbance x 100 (271 nm) (*). 2 - Dry mycelia weight (mg mL^{-1}) (•). 3 - Glucose concentration (mg mL^{-1}) (▲) and 4 - Protein concentration x 10 (μg mL^{-1}) (▼). Bars are ±1 standard deviation

These results suggest that *A. niger* grew slower using tannin extracts and tannic acid as sole carbon source compared to glucose (Tinger et al. 1969). Analysis of variance shows that stirring had a significant effect on fungal growth and glucose produced, but not for galllic acid production and absorbance (Table 3).

The results are in agreement with Seth and Chad (2000) who reported that increasing

Table 3. ANOVA analysis for gallic acid, Abs, dry biomass and glucose concentration at 10 days fermentation (% significance).

Interaction	Gallic acid	ABS	Dry Biomass	Glucose
	(% significance)			
U	42.86	27.19	45.60	96.02
P	48.85	9.66	33.35	22.49
T	89.02	51.33	67.86	6.08
S	12.10	98.63	0.44*	0.26*
UP	99.22	8.20	90.56	69.14
UT	77.35	43.20	56.26	25.11
US	25.89	90.42	87.33	97.94

U (urea), P (pH), T (temperature), S (stirring)
* Significant at $P<0.05$

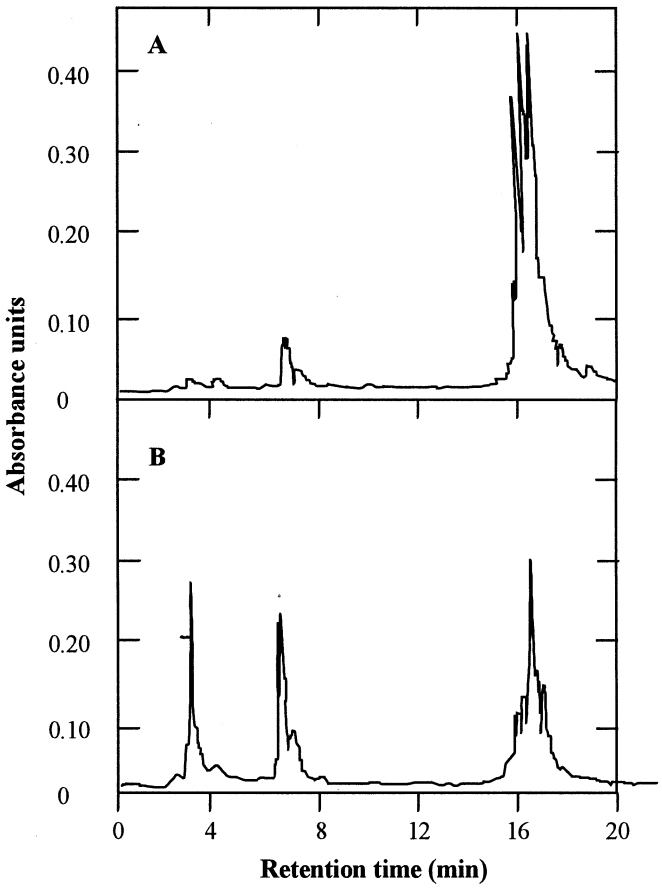

Figure 2. HPLC chromatograms of tannin compounds at 0d (A) and tannin biodegradation products at 11d (B) incubation.

the agitation rate affect the oxidation of tannin and cell growth. They carried out the fermentation with *Aspergillus awamori* with agitation speed of 300, 350 and 400 rpm.

The results are in agreement with Seth and Chad (2000) who reported that increasing the agitation rate affect the oxidation of tannin and cell growth. They carried out the fermentation with *Aspergillus awamori* with agitation speed of 300, 350 and 400 rpm.

Figure 2 shows the chromatogram of tannin acid concentration at the beginning and at 11d of incubation. The main molecular weight of tannin biodegradation products and the extract (Table 4) confirm the modification of the initial tannic acid structure.

Extracellular enzymatic activity was not detected after 10d of incubation. Tannase is exclusively intracellular in submerged fermentation processes during the first 48 hr of fermentation; subsequently a large portion of the enzyme is excreted in submerged fermentation with an intracellular to extracellular tannase ratio of 1:6 (Lekaha and Lonsane 1994). Reasons for not detecting of enzymatic activity could be due to the

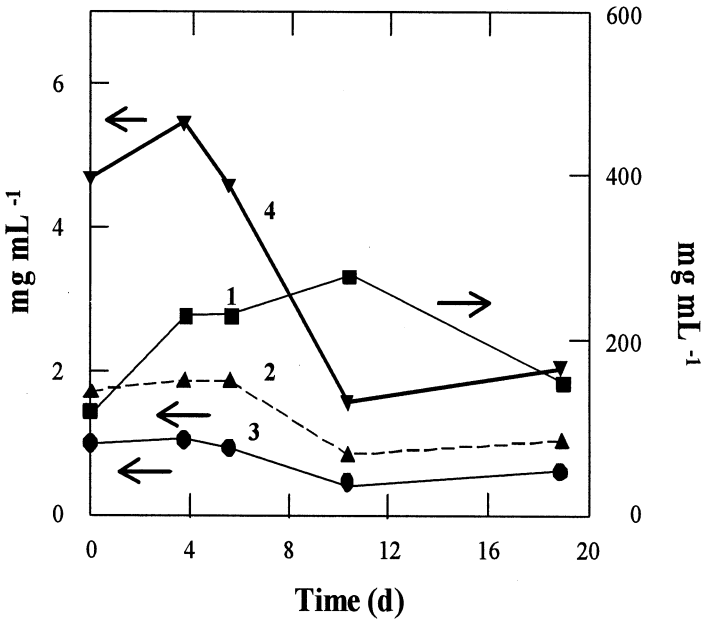

Figure 3. Formation of gallic acid and compounds similarly to tannic acid during the biodegradation process by *A. niger* CDBB H-176. 1- Gallic acid concentration (■), Compound similarly to tannic acid: 2- A = 16.28 min (▲) 3- B = 16.75 min (●) 4- C = 17.07 min (▼). Bars of standard deviation are hidden by the size of the symbols.

competitive inhibition by the gallic acid concentration. (Mahadevan and Sivaswamy 1985). Thus, Bradoo et al. (1997) reported that either gallic acid or glucose at 300 μg mL^{-1} concentration inhibited 90% of tannase activity.

In this work the concentration of gallic acid were between 120 to 280 mg mL^{-1} and glucose between 3.8 to 16.0 mg mL^{-1}. These results indicate that gallic acid or glucose concentration might inhibit tannase activity. Removal of phenols from industrial waste

Table 4. Molecular weight of tannin biodegradation products.

Time	**Molecular weights estimated (Daltons)**				
(days)	**707.28**	**664.62**	**586.86**	**335.25**	**278.18**
0	+	-	-	-	-
13	-	+	-	-	-
15	-	+	+	+	-
20	-	-	+	-	+

+ fraction present
- fraction not present

effluents is an important practical problem since many of the compounds are toxic and their presence on irrigated wastes or drinking water is a health hazard.

Figure 3 shows the kinetic of gallic acid and the three phenolic compounds produced (at 11d incubation) degraded by *A. niger*. The maximum gallic acid content occurs at 11d incubation (133.4% fold than at 10d incubation) and coincided with the maximum decrease of phenolic compounds (68.5, 56 and 70.8% less than the initial concentration of A, B and C respectively).

The approach described in this work could lead to the development of a simple and rapid procedure to detect tannin compounds produced in the biodegradation of tannin extracts.

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