Evaluation of Culture Conditions for Tannase Production by *Aspergillus niger* GH1

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Summary

Extra- and intracellular tannase production by *Aspergillus niger* GH1 has been evaluated using submerged (SmF) and solid-state fermentation (SSF) at different temperatures (30, 40 and 50 °C). Effects of initial substrate (tannic acid) concentration, incubation time and temperature on tannase production in SSF have been studied. *A. niger* GH1 produced the highest tannase level (2291 U/L) in SSF at 30 °C during the first 20 h of culture at tannic acid concentration of 50 g/L, and under these conditions enzyme production was entirely extracellular. The decline in tannase activity after 20 h of incubation was associated with a concomitant increase in protease activity.

Key words: tannase, culture conditions, solid-state fermentation, *Aspergillus niger*

Introduction

Tannase or tannin acyl hydrolase (E.C. 3.1.1.20) catalyzes the hydrolysis of the ester bonds present in the hydrolysable tannins and gallic acid esters. At industrial level, it is produced by microbial means using SmF, where the activity is expressed mainly in intracellular form, implying additional costs in its production (*1*). However, the commercial forms are partly pure and have low tannase activity. At the moment, the main commercial applications of tannase are in the preparation of instant tea and manufacture of acorn liquor. Tannase is also used in the production of gallic acid (*2–5*), which is an important intermediary compound in the synthesis of antibacterial drugs (*6*). Gallic acid is a substrate for the chemical or enzymatic synthesis of propyl gallate, a potent antioxidant used in the food industry (*5*). Besides, tannase is used as clarifying agent in some wines, fruit juices and coffee-flavoured drinks (*7–9*).

Recently, some studies have reported various advantages of tannase produced by SSF in comparison with that produced by SmF (*7–17*). Among these advantages are its high production level (up to 5.5 times more than in SmF), the extracellular nature of the enzyme and its stability in a wide range of pH and temperature (*1,8,12*). However, there are few microorganisms with the ability to produce tannase at high tannin concentration in spite of the fact that this compound shows antimicrobial activity.

New tannin-degrading fungal strains have been isolated from semiarid regions of North Mexico (*18*), which have been biochemically and physiologically characterized (*19,20*). The strain of *Aspergillus niger* GH1 was isolated from the leaves of »gobernadora« (*Larrea tridentate* Cov.), a native plant of the Mexican semiarid region. It is the best tannin-degrading strain and is part of the fungal collection Food Research Department UAdeC. The present study was carried out to evaluate the effect

of several culture conditions on fungal tannase production by *A. niger* GH1.

Materials and Methods

Microorganism and inoculum

Spores of *A. niger* GH1 were inoculated on potato dextrose agar using 250-mL Erlenmeyer flasks and incubated at 30 °C for 5 days. After this, spores were harvested with Tween 80 (volume ratio of 0.01 %) and these were then counted on a Neubauer chamber.

Culture conditions

Two culture systems – submerged fermentation (SmF) and solid-state fermentation (SSF) were used to produce tannase enzyme. Composition of culture medium was (in g/L): KH_2PO_4 2.19, $(NH_4)_2SO_4$ 4.38, $MgSO_4·7H_2O$ 0.44, $CaCl_2·7H_2O$ 0.044, $MnCl_2·6H_2O$ 0.009, $NaMoO_4·2H_2O$ 0.004, $FeSO_4·7H_2O$ 0.06 and tannic acid 12.5.

SmF conditions included agitation at 250 rpm, temperature 30 °C, initial pH= 5.5 and varying incubation time. Inoculum level was 10^7 spores per mL of culture medium. The solid support used in SSF was pulverized low density (20 g/L) polyurethane foam (PUF) purchased from Expomex, México, D.F. SSF was carried out at 70 % humidity. The growth kinetics of the fungal culture in SmF and SSF were periodically monitored. Crude enzymatic extracts from SmF were obtained by filtering, while the extracts from SSF were obtained by compression of the fermented material. In SmF, intracellular tannase extracts were obtained from mycelial cells retained on filter paper by washing with physiological solution, freezing with liquid nitrogen and milling in a mortar, while in SSF the fermented material (cells and PUF) was similarly treated; in both cases the macerate was recovered with buffer solution (acetate 100 mM, pH=5.5). All experiments were conducted in triplicate.

Tannase assay

The tannase activity was evaluated by the spectrophotometric method reported by Sharma *et al.* (*21*), using methyl gallate prepared in 0.05 M citrate buffer (pH=5) as substrate, citrate buffer 0.05 M (pH=5) as control and the crude enzymatic extract. For the colour reaction the methanolic rhodanine (0.67 % by mass per volume) and potassium hydroxide (0.5 M) were used. This reaction was monitored by measuring absorbance at 520 nm. One unit of tannase was defined as the amount of enzyme able to release one μmol of gallic acid formed per min under assay conditions (temperature and time).

Protease activity assay

Protease activity was assayed by the method of Dosoretz *et al.* (*22*). Substrate solution was prepared with 5 mg of azocasein dissolved in 1 mL of citrate buffer (50 mM, pH=5). To this solution, 0.05 mL of the crude enzymatic extract were added and the mixture was incubated for 1 h at 30 °C. Then 0.15 mL of trichloroacetic acid (5 %, volume ratio) were added and the mixture centrifuged at 12 000 rpm for 2 min. The obtained supernatant was spectrophotometrically evaluated at 520 nm.

One unit of protease activity was defined as the amount of enzyme necessary to release 1 μmol of chromophoric group of the substrate, which produced a change of absorbance of 0.001 under assay conditions.

Substrate degradation

Substrate uptake was determined by the spectrophotometric method reported by Dubois *et al.* (*23*) and modified by Aguilar *et al.* (*24*), using tannic acid as standard.

Protein content

For protein content estimation of the crude enzymatic extract, the microassay of Bradford (Bio-Rad® Protein Assay, No. 500.0002) was used. Samples were measured at 595 nm.

Statistical analysis

All results were analyzed using the general lineal procedure and comparison of means by Tukey's test (p= 0.05). A statistical computer program developed by the Universidad Autónoma de Nuevo León was employed.

Results and Discussion

Results obtained from this comparative study of tannase production using SmF and SSF systems are reported in Table 1. The strain of *A. niger* GH1 had a higher tannase activity on SSF (more than four times) as compared to SmF, and this observation is similar to the results previously reported (*1,7–17*). It is important to note that for tannase production, the used model (substrate-support) resulted in enhanced enzyme induction. This result also indicates that *A. niger* GH1 strain can be adapted for SSF system and utilizes the nutrients in a better form than when it is grown in other culture systems, like SmF.

Table 1. Comparative tannase production by *Aspergillus niger* GH1 using submerged and solid-state fermentation (at 30 °C for 30 h)

Culture system	Extracellular tannase activity/(U/L)	Intracellular tannase activity/(U/L)	Total tannase/(U/L)
SmF	537	n.d.	537
SSF	2291	n.d.	2291

n.d. Enzyme activity was not detected under culture conditions after 30 h

After the selection of the culture system, the effect of initial level of substrate-inducer on tannase production was evaluated. Fig. 1 shows the tannase activity produced under these conditions. It was observed that by increasing the substrate concentration from 12.5 to 50 g/L, the tannase activity increased (Fig. 1a). At 20 h of incubation, when an initial concentration of 100 g/L was used, no tannase activity could be detected as the fungal growth was in a prolonged lag phase. Fig. 1b shows that by using an initial tannic acid concentration of 50 g/L, the specific activity was higher than with other levels of the tested substrate.
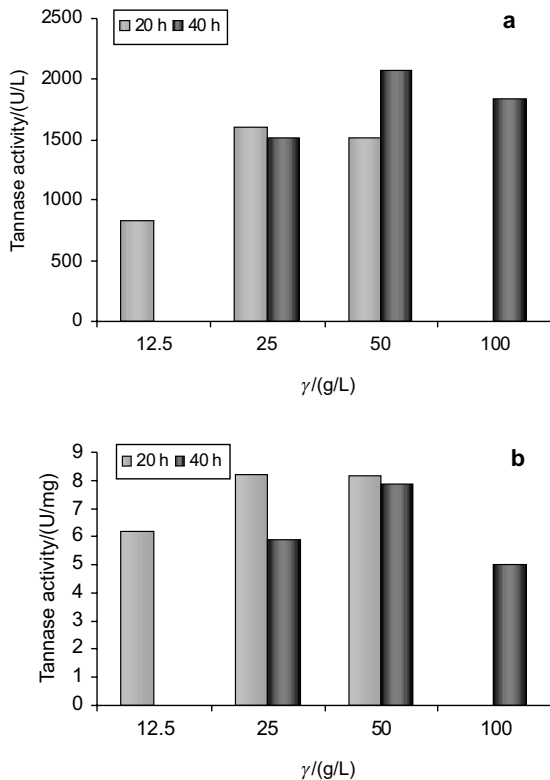
Fig. 1. Effect of initial substrate-inducer concentration on tannase activity (a) and specific activity (b) in SSF of *A. niger* GH1 at 30 °C

Fig. 2. Substrate utilization during the course of tannase production by *A. niger* GH1 at 30 °C in SSF with 12.5 g/L of tannic acid

Fig 3. Tannase (a) and protease (b) activities produced by *A. niger* GH1 strain at 30 °C in SSF with tannic acid concentration of 50 g/L

These results demonstrated the capacity of the fungus to grow on high concentrations of a compound characterized by its antimicrobial activity. Only *Aspergillus* species are able to tolerate tannic acid in concentrations higher than 20 g/L. Aguilar *et al.* (*24*) reported the effect of the concentrations of tannic acid on tannase production by *A. niger* Aa-20.

Fig. 2 shows the substrate concentration during the kinetics of tannase production using 50 g/L of tannic acid. It is easy to determine the fermentation time, due to a high concentration of substrate consumed by the strain in the first few hours of growth.

The tannase activity, which was initially detected at 18 h of culture, reached a peak at 24 h. Tannase production decreased and protease activity increased at 30 h (Fig. 3a). The effect of protease on tannase activity observed in the present study confirms the observations reported by Aguilar *et al.* (*15*) that protease influences the production of tannase and causes a decrease in its activity. These results also indicate the need for further purification of the enzyme.

The incubation time required for the production of the tannase enzyme by *A. niger* GH1 in SSF was lower than the culture time of 120 h reported by Sharma *et al.* (*21*), using a strain of *A. niger* van Tieghem.

The above results have significant implications for tannase production because the strain *A. niger* GH1 has the capacity to degrade tannic acid rapidly and at high concentrations. Other authors have reported tannase production in media with glucose and tannic acid and fermentation time of 100 h (*25–27*). Most of the published
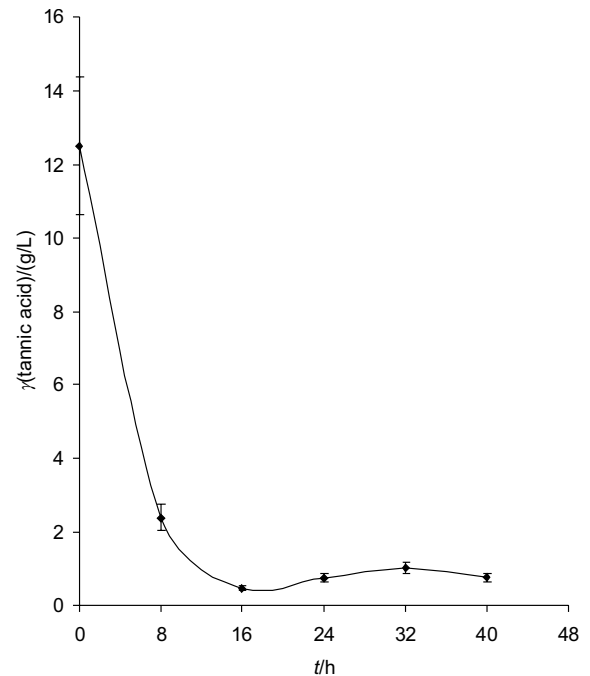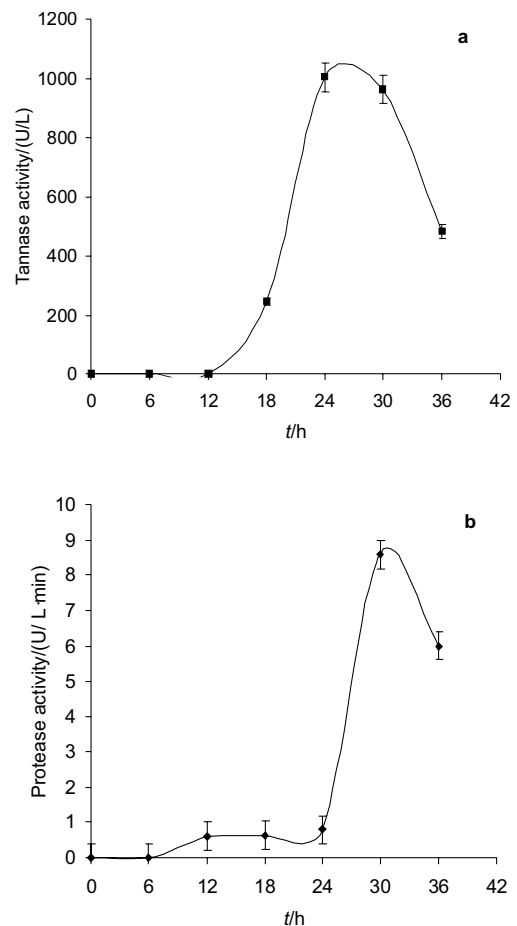
work on tannase purification reports the use of mycelial mass to obtain the protein due to its intracellular nature (*25,28,29*). The other advantage in the use of this strain is the form of expression of the enzyme, because in SSF it is wholly extracellular. These conditions represent a system for an easy recovery of enzyme protein using fewer steps with high yield.

Conclusions

The culture conditions for the production of tannase enzyme from *A. niger* GH1 strain were evaluated and standardized. These conditions were: solid-state fermentation with polyurethane as support, incubation temperature of 30 °C, fermentation time of 24 h, substrate concentration of 50 g/L of tannic acid. These culture conditions can be used for further studies on the purification, immobilization and applications of tannase from *A. niger* GH1.

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