J. Pure & Appl. Microbiol.
April 2009
Volume 3(1): pgs. 01-386
Bhopal, India.
ISSN: 0973-7510

Journal of Pure & Applied Microbiology

AN INTERNATIONAL RESEARCH JOURNAL OF MICROBIOLOGY

www.microbiologyjournal.org

Production of *Aspergillus niger* GH1 Tannase using Solid-State Fermentation

Mario Cruz-Hernandez[1], Juan Carlos Contreras[1], Nelson Lima[2], Jose Teixeira[2] and Cristobal Aguilar[1]*

[1]Food Research Department, Universidad Autónoma de Coahuila, 25000 Saltillo, Coahuila, México.
[2]IBB - Institute for Biotechnology and Bioengineering, Centre for Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal.

(Received: 15 October 2008; accepted: 21 December 2008)

The production of tannase by *Aspergillus niger* GH1 in solid-state fermentation, under different initial concentrations of tannic acid (12.5, 25, 50 and 100 gl^{-1}). The reactors were packed with polyurethane foam impregnated with medium and inoculated with fungal spores. Tannase production was kinetically monitored by 48 h. The tannase activities extracellular and intracellular were assayed by HPLC-chromatography. Maximum extracellular and intracellular tannase activities (11.35 and 6.95 Uml^{-1} respectively) were recorded with 100 gl^{-1} of tannic acid. The substrate uptake was 100% at concentrations of 12.5, 25 and 50 gl^{-1}, while 74.4% was consumed in the presence of 100 gl^{-1} of tannic acid after 48 h of culture. These results suggest that high concentrations of tannins can be removed and tannase production can be reached in high levels. The needed to establish the production conditions under solid state fermentation, a system where the tannase is expressed extracellular in high levels.

Key words: Tannase, Tannic acid, Solid-state fermentation, *Aspergillus niger* GH1.

Tannins are molecules considered as water-soluble phenolic compounds with molecular weights ranging from 500 to 3000 Da. Tannins are present in several plants acting as pigments[1] and protecting agents against microbial attacks[2]. Several proposals to use the tannin-rich plants as a source of added value products have been made. One of them, and the most frequently one, is related with its use as substrate for the production of microbial enzymes, in particular tannase[3].

Tannin acyl hydrolase (E.C. 3.1.1.20), commonly called tannase is an important enzyme used industrially for the manufacture of gallic acid from tannin-rich materials. The tannase catalyzes the hydrolysis of ester and depside bonds in such hydrolysable tannins as tannic acid. It is generally used in food and beverage processing[4] namely, in the production of instant tea, coffee flavoured soft drinks and in the clarification of beer and fruit juices[5].

* To whom all correspondence should be addressed.
Fax: + 52 (844) 4161238
e-mail: cag13761@mail.uadec.mx

Production of tannase by various bacterial[6-7] and fungal strains[8-11] has been reported. Different works reported liquid surface, submerged or solid-state fermentation for tannase production. Among these, submerged fermentation process is mostly preferred because the sterilization and process-control methods are easier in this system[2]. Although vast amount of tannase production was achieved by submerged fermentation. This method implies some advantages mainly in the production cost and the instability of the produced enzyme[4, 12]. In this aspect, production of such enzyme from fungal strains through solid-state fermentation (SSF) is cheaper, less technology is required and its extraction easier[13]. An important advantage of SSF is that it produces only negligible amount of liquid effluents and thereby creates less pollution [14]. According our knowledge, only few reports on tannase production through SSF by *Aspergillus niger*[15, 16, 17], *Aspergillus acuealatus*[18], *Paecilomyces variotii*[19], *Aspergillus foetidus* and *Rhizopus oryzae*[10] are available. Tannase production through liquid submerged fermentation and SSF by *Aspergillus niger* GH1 was reported earlier[9] and the potential of using agro-industrial wastes as substrate for industrial tannase production through SSF has been considered. In this work, fermentation parameters for tannase production and tannic acid degradation by *Aspergillus niger* GH1 in a model SSF system were evaluated at different tannic acid concentrations.

MATERIALS AND METHODS

Microorganism and culture medium. Spores of the strain *Aspergillus niger* GH1 (DIA/UAdeC collection) were preserved at –20 °C in protect-crioblocks (bead storage system, Technical Service Consultants Limited). Inoculum was prepared transferring the spores to potato dextrose agar (PDA BD Bioxon®), incubated at 30 °C for 5 days. After the fungal growth the spores were scraped into 0.01 % Tween 80 sterilized solution and counted in a Neubauer chamber. Medium for tannase production was the same as reported contained (gl^{-1}): KH_2PO_4, 5; NH_4NO_3, 10; $MgSO_4.7H_2O$, 1; $CaCl_2.6H_2O$, 0.1; $MnCl_2.6H_2O$, 0.02; $NaMoO_4.2H_2O$, 0.01; $FeSO_4.7H_2O$, 0.125. Salt-containing medium was autoclaved at 121 °C for 15 min. Tannic acid (Sigma, U.S.A.) solution was filter-sterilised (nylon membrane 47 mm diameter, 0.2 µm pore size, Millipore) and added to a final concentration of 12.5, 25.0, 50.0 and 100.0 gl^{-1}

Solid state fermentation (SSF)

The SSF involved the use of polyurethane foam (PUF) (Expomex, México) as a support to absorb the liquid medium. PUF was washed as reported (20) and then pulverised in a plastic-mill. Column reactors (25 × 180 mm) were packed with 10 g of inoculated (2×10^7 spores g^{-1} of dry inert PUF) support. Culture conditions were: temperature 30 °C, aeration rate 20 ml of air per gram of support per min, initial pH 5.5, and initial moisture content 65 % and an incubation time of 48 h. Samples were taken every 6 h. At each sampling point, the fermented mass of reactor was removed and homogenized. For enzyme leaching, the content of each reactor was mixed with distilled water (10:1 w/v) and vortexed for 1 min. Solid particles were filtered (Whatman International Ltd. Springfield Mill, Maidstone, Kent, England, Filter No. 41) and the clear filtrate was assayed for extracellular tannase activity. The remaining solids were washed three times with 50 ml of distilled water. Intracellular enzyme was recovered by deep-freezing the cells in liquid nitrogen and by macerating in a chilled mortar. The recovery process was previously reported [21]. The process was carried out using acetate buffer, pH 5.5 to recovery the enzyme from the debris.

Analytical methods. Tannase assay was carried out using the HPLC-methodology proposed[22]. One unit of enzyme (U) was defined as the amount of enzyme able to release 1 µmol gallic acid per ml per min. Biomass formation in SSF was determined by technique reported[23], where 0.5 g of fermented solid is impregnated with phosphoric acid (0.15 $moll^{-1}$) and heated in a boiling water bath to hydrolyse the mycelium during 7 min; the sample is then cooled and centrifuged to obtain a mycelial protein solution; finally, 200 ml of sample were mixed with 800 ml of biuret reagent and the blue color was measured at 595 nm. Tannic acid concentration was evaluated spectrophotometrically using the phenol-sulphuric acid method reported (Aguilar et al. 2001). Briefly, the method implies thermal reaction of 1 ml of sample with 2 ml of phenol-sulfuric reagent ($1 mgml^{-1}$) during 5 min into a boiling water bath,

then the sample was cooled and the absorbance was recorded at 480 nm.

Kinetics parameters

Growth curves were fitted by a Maquardt "Solver" computer program (Excel, Microsoft) using logistic equation[1] as follows:

$$X = \frac{X_{max}}{1 + \left[\frac{X_{max} - X_0}{X_0}\right]} e^{-\mu t} \quad ...(1)$$

Where X (gl^{-1}) represents the biomass calculated, X_0 and X_{max} (gl^{-1}) are the initial and maximum biomass value, respectively, μ (h^{-1}) is the specific growth rate, and t (h) is the culture time. The algorithm minimizes te sum of least square errors comparing experimental data with the theorical values obtained.

The biomass/substrate yield, $Y_{x/s}$, is calculated by the equation [2]:

$$Yx/s = \frac{(X_{max} - X_0)}{(S_0 - S_{final})} \quad ...(2)$$

Where X_{max} and X_0 (gl^{-1}) are the maximum and initial biomass values obtained, respectively, and S_0 and S_{final} (gl^{-1}) are the initial and final substrate concentration values, respectively.

The specific substrate uptake rate, q_s, is defined by the equation [3]:

$$q_s = \frac{\mu}{Y_{x/s}} \quad ...(3)$$

Where q_s is giving as grams of substrate consumed per gram of biomass per hour.

Tannase/biomass yield, $Y_{E/X}$, is estimated from de linear correlation between tannase activities, E (Ul^{-1}) and biomass concentrations, X (gl^{-1}). The yield coefficient is defined as $Y_{E/X}$ (units of tannase per gram of X). The specific rate of formation of enzymes, q_p, is definided in [4]:

$$q_p = \frac{\mu}{Y_{E/X}} \quad ...(4)$$

Where q_p is the units of tannase produced per gram of biomass per hour, $Y_{E/X}$ is the tannase/ biomass yield.

RESULTS AND DISCUSSION

Both extracellular as intracellular tannase activity produced by *A. niger* GH1 in the SSF process were seriously affected by the initial tannic acid concentration. Figure 1a shows the kinetics of extracellular tannase production, while Figure 1b shows the results obtained for intracellular tannase production. A consistent behaviour was found, in which the increment in the initial tannic acid concentration is related with an increase in tannase activity. In this study, a maximum of tannase activity was reached with 100 gl^{-1} of tannic acid at 48 h. In other SSF systems evaluated, a marked decrement in the tannase production after to reach the maximum level is obtained However, in this study that decrement was not found probably due to the low level of concomitant protease activity[21]. High levels of tannase produced were excreted to the culture medium. Similar results were obtained[24] using sugar cane baggase as support of SSF demonstrating that the fungal tannase activity was only detected in the extracellular crude extract and not in the intracellular extract.

This study revealed that the use of higher tannic acid concentrations promotes the excretion of tannase intracellular activity; these results may be explained by the existence of a substrate monitoring inside the cells that at high substrate concentrations inhibits the tannase excretion process. In this case, the extracellular/intracellular ratio was 1.3:1 for 50 gl^{-1} and 1.6:1 for 100 gl^{-1}; while for 12.5 and 25 gl^{-1} of tannic acid present, the intracellular activities were low and the extracellular/intracellular ratios were superior to 6:1 in both cases.

The results of tannase production obtained in SSF system can be partially explained by those reported for pectinesterase and polygalacturonase[25]. These authors suggested that the high levels of enzymatic production obtained in SSF are due to changes in the composition of membrane fatty acids provoked by stress conditions favoured on this enzyme production process (SSF) when the substrate concentration is increased. Also, these results are explained by the previous reports of our group[21,17] where a different pattern of induction/repression of tannase was observed depending of the culture system and the

culture conditions. The most recent idea to explain the high levels of enzyme produced by SSF was proposed[26] assuming the dependence of enzyme expression with the substrate and oxygen diffusivities on the solid support. However, this phenomenon has not been satisfactorily explained. Table 1 shows a summary of the kinetic parameters evaluated in SSF systems, being clearly demonstrated the high tannic acid-degrading capacity of *A. niger* GH1. It is important point out that only at the highest substrate concentration there is no total consumption of tannic acid. This aspect is very important if the biodegradation of phenolic compounds or tannins of waste water and tannin-rich materials (i.e. coffee pulp, creosote bush, etc.) is to be considered.

Table 1. Summary of kinetic parameters considered in the tannase production and tannic acid degradation by SSF

Tannic acid(gl^{-1})	$\mu(h^{-1})$	$Y'_{x/s}(gXgS^{-1})$	$Y_{P/X}$ ($UmgX^{-1}$)	$q_s(gXgS^{-1}h^{-1})$	$q_p(UgX^{-1}h^{-1})$	Substrate uptake (%)
12.5	0.288	0.306	1.385	0.942	0.399	100
25.0	0.295	0.163	1.443	1.812	0.426	100
50.0	0.223	0.091	1.469	2.440	0.328	100
100.0	0.217	0.074	2.183	2.929	0.474	73.03

On SSF, a particular behaviour of the kinetic parameters is observed, as the product yield (Y_p), specific substrate uptake rate (q_s) and specific product formation rate (q_p) were higher for the initial tannic acid concentration of 100 gl^{-1}. Also, the higher whole tannase activity and lower uptake substrate percentage conversion were obtained. The analysis of the kinetic parameters demonstrated that SSF this is a good system to be applied in the degradation of hydrolysable tannins and tannase production.

Finally, it must be referred that several studies indicate that submerged fermentation (SmF) is not suitable for tannase production due to the long fermentation times (low productivity)[27-28] and to the intracellular nature of the enzyme (2). The results presented in this work, using polyurethane foam as inert support, complement

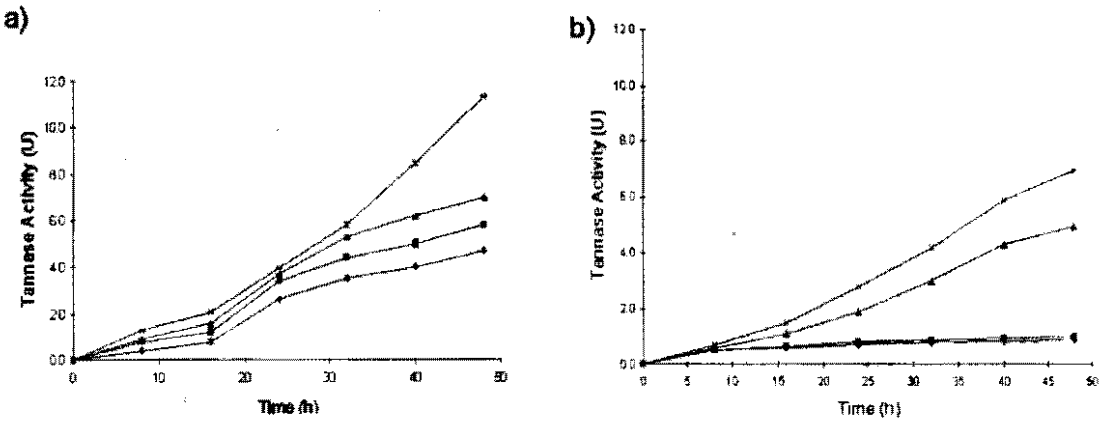

Fig. 1. Extracellular (a) and intracellular (b) tannase production by *A. niger* GH1 in SSF with tannic acid concentrations of 12.5 (◆), 25.0 (■), 50.0 (Δ) and 100.0 (×) gl^{-1}.

those reported[15,29,18,12], and clearly demonstrate that SSF presents significant advantages as compared to submerged fermentation for tannase production, the fermentation time is improved, significant amounts of extracellular enzyme are produced and the system is not inhibited by high concentrations of tannic acid

Obtained results clearly indicate the capability of *Aspergillus niger* GH1 to produce high levels of tannase in SSF suggesting that this production system should be considered for large-scale production of tannase and gallic acid and that cheap and simple agricultural waste like coffee pulp or creosote bush may be used in the future as substrates.

ACKNOWLEDGEMENTS

The present work was performed as part of a cooperative agreement between the Universidad Autonoma de Coahuila (UAdeC, México) and the Universidade do Minho (UM, Portugal) within a specific international exchange program (VALNATURA project, alfa network from European Union) undertaken at the Biological Engineering Department (UM, Portugal). M.A. Cruz-Hernandez thanks to Concejo nacional de Ciencia y Tecnología (CONACYT) the fellowship to study the doctoral program at the Department of Biotechnology (UAdeC).

REFERENCES

1. White T., Tannins, their occurrence and significance. *J Sci Food Agric* 1957; **8**: 377-385.
2. Lekha PK, Lonsane BK., Production and application of tannin acyl hydrolase: state of the art. Adv Appl Microbiol 1997; **44**: 215-260.
3. Banerjee D, Mondal KC, Pati BR., Production and characterization of extracellular and intracellular tannase from newly isolated *Aspergillus aculeatus* DBF 9. *J Basic Microbiol* 2001; **41**(6): 313-318.
4. Aguilar CN, Rodríguez R, Gutiérrez-Sánchez G, Augur C, Favela-Torres E, Prado-Barragan L A, Ramírez-Coronel A, Contreras-Esquivel JC., Microbial tannases: advances and perspectives. *Appl Microbiol Biotechnol* 2007; **76**: 47-59.
5. Belmares R, Contreras-Esquival JC, Rodriguez-Herrera R, Coronel AR, Aguilar CN., Microbial production of tannase: an enzyme with potential use in food industry. *Lebensm Wiss Technol* 2004; **37**: 857-864.
6. Osawa R, Kuroiso K, Goto S, Shimizu A., Isolation of tannin degrading Lactobacillus from humans and fermented foods. *Appl Environ Microbiol* 2000; **66**: 3093.
7. Nishitani Y, Sasaki E, Fujisawa T, Osawa R., Genotypic analysis of lactobacilli with a range of tannase activities isolated from human feces and fermented foods. *Syst Appl Microbiol* 2004; **27**: 109.
8. Rana NK, Bath TK., Effect of fermentation system on the production and properties of tannase of *Aspergillus niger* van Tieghem MTCC 2425. *J Gen Appl Microbiol* 2005; **51**: 203-212.
9. Cruz-Hernandez M, Augur C, Rodriguez R, Contreras-Esquivel JC Aguilar CN., Evaluation of culture conditions for tannase production by *Aspergillus niger* GH1. *Food Technol Biotechnol* 2006; **44**(4): 541-544.
10. Mukherjee G, Banerjee R., Effect of temperature, pH and additives on the activity of tannase produced by a co-culture of *Rhizopus oryzae* and *Aspergillus foetidus*. *W J Microbiol Biotechnol* 2006; **22**: 207.
11. Battestin V, Macedo GA, Effects of temperature, pH and additives on the activity of tannase produced by *Paecilomyces variotii*. *Electronic Journal of Biotechnology* 2007; **10**: 2-9.
12. Banerjee D, Mondal KC, Pati BR., Tannase Production by *Aspergillus aculeatus* DBF9 through solid-state fermentation. *Acta Microbiologica et Immunologica Hungarica* 2007; **54**(2): 159-166.
13. Pandey A, Selvakumar P, Soccol C R, Nigam P., Solid-state fermentation for the production of industrial enzymes. *Curr Sci* 1999; **77**: 149.
14. Pandey A, Radhakrishnan S, The production of glucoamylase by *Aspergillus niger* NCIM 1245. *Proc Biochem* 1993; **28**: 305.
15. Lekha PK, Lonsane BK., Comparative titres, location and properties of tannin acyl hydrolase produced by *Aspergillus niger* PKL 104 in solid-state, liquid surface and submerged fermentations. *Proc Biochem* 1994; **29**:407-503.
16. Sabu A, Pandey A, Dvad MJ Szakacs G., Tamarind seed powder and palm karnel cake: two novel agro residues for the production of tannase under solid state fermentation by *Aspergillus niger* ATCC 16620. *Biores Technol*

2005; **96**: 1223.

17. Aguilar CN, Augur C, Favela E, Viniegra G, Production of tannase by *Aspergillus niger* Aa-20 in sumerged and solid state fermentation: influence of glucose and tannic acid. *J Ind Microbiol Biotechnol* 2001b; **26**: 296-302.
18. Kumar R, Sharma J, Singh R., Production of tannase from *Aspergillus rubber* under solid-state fermentation using jamun (*Syzygium cumini*) leaves. *Microbiological Research* 2007; **162**(2): 384-390.
19. Battestin V, Saavedra GA, Macedo G., Biochemical characterization of tannases from *Paecilomyces variotii* and *Aspergillus niger*. *Food Sci Biotechnol* 2007; **16**(2): 243-248.
20. Zhu Y, Smith J, Knol W, Bol J., A novel solid state fermentation system using polyurethane foam as inert carrier. *Biotechnol Lett* 1994; **16**: 643-648.
21. Aguilar CN, Augur C, Favela E, Viniegra G, Induction and repression paterns of fungal tannase in solid-state and submerged cultures. *Proc Biochem* 2001; **36**(6): 565-570.
22. Beverini M, Metche M, Identification, purification and physicochemical properties of tannase of *Aspergillus oryzae*. *Sci Aliments* 1990; **10**: 807-816.
23. Córdova-Lopez J, Gutiérrez-Rojas M, Huerta S, Saucedo-Castañeda G, Favela-Torres E., Biomass estimation of *Aspergillus niger* growing on real and model supports in solid state fermentation. *Biotechnol Tech* 1996; **10**:1-6.
24. García-Peña I., Producción, purificación y caracterización de tanasa producida por *Aspergillus niger* en ferrmentación en medio sólido. Tesis de maestría Universidad Autónoma Metropolitana-Unidad Iztapalapa México 1996.
25. Maldonado MC, Strasser de Saad AM., Production of pectinesterase and polygalacturonase by Aspergillus niger in submerged and solid state systems. *J Ind Microbiol Biotechnol* 1998; **20**: 34-38.
26. Viniegra-González G, Favela-Torres E., Why solid-state fermentation seems to be resistant to catabolite repression? *Food Technol Biotechnol* 2006; **44**(3): 397-406.
27. Ganga PS, Nandy SC, Santappa M., Effect of environmetal factors on the production of fungal tannase. Leather Sci 1978; **23**: 203-209.
28. Pourrat H, Regerat F, Pourrat A, Jean D., Production of tannase by a strain of *Aspergillus niger*. *Biotechnol Lett* 1982; **4**: 583-588.
29. Chaterjee R, Dutta A, Banerjee R, Bhattacharyya BC, Production of tannase by solid-state fermentation. *Bioprocess eng* 1997; **14**: 159-162.