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Induction and repression patterns of fungal tannase in solid-state and submerged cultures

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Abstract

Induction and repression patterns of tannase production by *Aspergillus niger* Aa-20 in solid-state (SSC) and submerged culture (SmC) were established. Tannic acid and glucose were used as carbon sources. Induction and repression ratios were obtained with different concentrations of tannic acid and glucose, respectively. Tolerance to high concentrations of tannic acid by *A. niger* Aa-20 was lower in SmC than in SSC. In SSC an increase in tannic acid enhanced the expression of tannase activity. The addition of glucose (>20 g l^{-1}) resulted in strong catabolite repression in SmC system. The tannase/biomass yield in SSC was at least 2 times higher than in SmC. The results presented demonstrate the capacity of SSC to minimize catabolite repression. The role of gallic acid in tannase regulation was also studied. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Tannase; Solid-state culture; Submerged culture; Induction; Catabolite repression

1. Introduction

Tannase or tannin acyl hydrolase (EC, 3.1.1.20) catalyses the breakdown of hydrolysable tannins or gallic acid esters. This enzyme is used in the industrial processing of fruit juices and coffee-flavoured soft drinks as a clarifying agent, but the major commercial applications of the tannase are in the manufacture of instant tea and in the production of gallic acid [12]. Production of tannase has been reported both by submerged culture (SmC) [1,6,8,9,13] and solid-state culture (SSC) [6,13] techniques.

Differences in enzymic activity titres have been obtained when an enzyme is produced in SSC and SmC. Higher enzyme activities have been reached using the SSC system. Enzymes such as α-amylase,2 pectinases, [3–5] tannin acyl hydrolase [6] and proteases [7] have been considered in these comparative studies. In many of the cases, high sugar concentrations have been related to high enzyme production in SSC, whereas in SmC, enzyme production was repressed [1,5]. Several hypothesis have been proposed to explain this behaviour. Ramesh and Lonsane [1] suggested that the SSC system minimizes the catabolite repression phenomenon; Maldonado and Strasser de Saad [4] showed that these differences were related to changes in some fatty acids in membranes of the fungal cells. Except for this last report, none of the studies mentioned show experimentally that glucose represses enzyme synthesis by catabolite repression mechanism in SmC system and that SSC minimizes this regulatory mechanism. A precise description of the phenomenon is necessary before proposing explanations concerning possible causes.

Some studies concerning optimum production and regulatory aspects of tannase by moulds have been carried out in SmC systems by Bradoo et al. [8] and Bajpai and Patil [9]. Tannases are induced by tannic acid or by some of its derivatives [8] but the regulatory mechanism of its production remains uncertain. In the present study, tannase was used as a model system to view experimentally the differences in enzyme regulation mechanism in both culture systems.

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An attempt was, therefore, made to explain the differences in enzyme titres in SSC and SmC. In order to establish a comparison, the induction and repression patterns of tannase were studied using tannic acid as inducer and glucose as repressor. In addition, the tannase/biomass yields were compared. The role of gallic acid in the tannase regulatory mechanism was also investigated.

2. Materials and methods

2.1. Microorganism and culture media

Aspergillus niger Aa-20 spores (IRD-UAMI collection) used in this work were stored at −20°C in protect-crioblocks (bead storage system, Technical Service Consultants Limited). Inocula were prepared by transferring the spores to PDA medium and incubating at 30°C for 5 days. The spores were then scraped into a 0.01% Tween 80 solution and counted in a Neubauer chamber.

The medium for tannase production was the same as reported previously by Lekha and Lonsane [6] using $(NH_4)_2SO_4$ as nitrogen source. Salt-containing medium was autoclaved at 121°C for 15 min. In the induction study, a tannic acid (Sigma, USA) solution was filter-sterilized and added to a final concentration of 12.5, 25, 50 or 100 g l^{-1}. In SSC, an additional concentration was studied (160 g l^{-1}). The glucose repression effect was measured by adding different glucose concentrations (6.25, 12.5, 25 and 50 g l^{-1}) to the base medium with 25 g l^{-1} of tannic acid as inducer. In SSC the glucose concentration ranged from 6.25 to 200 g l^{-1}. For SmC and SSC systems, the liquid broths were the same when cultures were compared.

2.2. Submerged culture (SmC)

Erlenmeyer flasks (250 ml) with 50 ml of liquid sterile broth, at an initial pH 5.5, were inoculated with 2.5 ml of the spore suspension (2×10^7 spores per ml of suspension) and incubated at 30°C on a rotary shaker (220 rpm) for 48 h. Samples were removed at 8 h intervals. Biomass was separated by filtration through Whatman No. 41 filter paper. The cell-free culture broth was assayed for extracellular tannase activity and evaluated for tannic acid content. The mycelium was washed three times with saline solution, frozen with liquid nitrogen, macerated in a chilled mortar and mixed with acetate buffer (200 mM, pH 5.5). The fungal debris was removed by centrifugation and the resulting supernatant was assayed for tannase activity.

2.3. Solid state culture (SSC)

The SSC involved the use of low density (around 20 g l^{-1}) polyurethane foam (PUF) (Expomex, México) as a support to absorb the totality of the liquid broth, since PUF was found to absorb 20 times its dry weight in liquid broth. The foam was washed as reported by Zhu et al. [10] and then pulverized in a plastic-mill. Column reactors (25 × 180 mm) were packed with 10 g of inoculated medium having 3.5 g of dry PUF and 6.5 g of liquid broth inoculated with 7×10^7 spores. Culture conditions were: 30°C; aeration rate, 20 ml of air per g of support per min; initial pH, 5.5; initial moisture content of 65% and, incubation time of 48 h. Samples were taken every 6 h. For enzyme leaching, the content of each reactor was mixed with distilled water (1:10 w/v) and vortexed for 1 min. Solid particles were filtered (Whatman 41) and the clear filtrate assayed for extracellular tannase activity. The remaining solids were washed three times with 50 ml of distilled water. Intracellular enzyme was recovered by freezing the cells in liquid nitrogen and macerating in a chilled mortar. The recovery process was similar to that described for the SmC system.

2.4. Role of gallic acid and glucose in tannase production

The effect of gallic acid as sole carbon source on tannase synthesis was investigated in SmC, using a single concentration (25 g l^{-1}), following the same protocol reported above. In order to determine the basal levels of tannase activity from *A. niger* Aa-20, glucose was used as sole carbon source (30 g l^{-1}) in both culture systems. Experiments were conducted in triplicate and the average values were reported as mean values.

2.5. Analytical methods

Tannase activity was assayed using the HPLC-methodology proposed by Beverini and Metche [11]. One unit of enzyme (IU) was defined as the amount of enzyme able to release 1 μmol of gallic acid per min. For biomass determination in SmC, the mycelium obtained after filtration was dried at 70°C to constant weight. In SSC, biomass could not be measured accurately by gravimetry. Thus, it was determined by measuring the protein concentration with a Bradford-microassay (Bio-Rad®) following the technique reported by Córdova et al. [12], which evaluates the lag and exponential growth phases only. It was confirmed [12] (data not shown) that the protein fraction of mould biomass was nearly constant in surface cultures of *A. niger* Aa-20 grown, during the lag and exponential phases, on agar plates inoculated by

spreading mould spores over the plate (lawn technique) and harvesting the mycelia by careful separation from the residual agar substrate.

2.6. Induction and repression ratios

Induction and repression ratios were calculated using the following equation:

$$\text{I.R.} = \frac{\text{I.TA}}{\text{B.TA}} \quad (1)$$

where I.R. is the induction ratio in cultures with tannic acid as sole carbon source; I.TA corresponds to induced tannase activity and B.TA is the basal tannase activity obtained from a culture using glucose as a sole carbon source. In the case of repression studies, I.R. was calculated in the presence of tannic acid and glucose. SmC and SSC systems were conducted in triplicate. The average values reported are mean values.

2.7. Tannase/biomass yields ($Y_{E/X}$)

Tannase/biomass yields were estimated from the linear correlation between tannase activities (IU l^{-1}) and biomass concentration, X(g l^{-1}). The yield coefficient was defined as $Y_{E/X}$ (IU of tannase per gram X). Regression coefficients were high ($R^2 > 0.9$) for at least four data points.

2.8. Growth kinetic coefficients

Growth curves were fitted by a Marquardt 'Solver' computer program (Excel, Microsoft) using logistic equation as follows:

$$\frac{dX}{dt} = \mu_m\left(1 - \frac{X}{X_{max}}\right)X, \quad (2)$$

where μ_m (h^{-1}) was the maximal growth rate and, X and X_{max}, were the biomass concentrations at times, t, and $t \to \infty$, respectively. The algorithm minimized the sum of least square errors, comparing experimental data with the theoretical values given by Eq. (3).

$$X(t) = \frac{X_{max}}{1 + \frac{X_{max} - X_0}{X_0} e^{-\mu_m t}}. \quad (3)$$

X_0 is the biomass concentration at time, $t = 0$.

3. Results and discussion

Specific growth rates, μ_m, were affected in a different way by the initial level of tannic acid (S_o) in both types of culture systems. In SmC, they followed a Monod curve with substrate inhibition: increasing values for μ in the range $0 < S_o < 50$ g l^{-1} and decreasing for $S_o > 50$ g l^{-1}. In SSC, μ_m, was close to the average value 0.25 h^{-1} in the range, $12.5 < S_o < 100$ g l^{-1}. The parameter, X_{max}, in SmC, followed a similar trend to, μ_m: increasing values for X_{max} in the range $0 < S_o < 50$ g l^{-1} and decreasing for $S_o > 50$ g l^{-1}. In SSC, X_{max}, increased as a function of S_o values (see Table 1). Tannase/biomass yield ($Y_{E/X}$) was found in SSC to increase proportional to S_o ($R = +0.99$) and to have a low negative trend ($R = -0.74$) in SmC. A similar trend was found for the combined effect (μ_m $Y_{E/X}$) which seems to explain why, maximum enzyme titres (TTA = IU l^{-1}) were several times higher in SSC as compared to SmC (Table 1). In addition, the pH of maximum tannase production was lower in SmC than in SSC (Table 1) independent of tannic acid concentration.

Enzymic induction ratios (I.R., in Fig. 1) were studied in a medium with tannic acid as sole carbon source in SmC (6.25 g $l^{-1} \le S_o \le 100$ g l^{-1}) and SSC (6.25 g $l^{-1} \le S_o \le 160$ g l^{-1}). *A. niger* Aa-20 showed different tannase activities whether extracellular (empty symbols in Fig. 1) or intracellular (solid symbols in Fig. 1). Clearly, I.R., values in SmC (squares) were much lower than, I.R., values in SSC (circles) system (Fig. 1) but in both cases, I.R., curve was convex with respect to S_o, showing a maximum value in SmC (I.R. = 4) and an asymptotic value in SCC (I.R. ≈ 17). Extracellular, I.R. values were parallel to intracellular values both in SmC

Table 1
Total tannase activity (TTA), maximum biomass production (X_{max}) pH at 48 h, specific growth rate (μ_m) and tannase/biomass yield ($Y_{E/X}$) of culture using several inducer (tannic acid) concentrations in SmC and SSC[a]

S_o (g l^{-1})	SmC					SSC				
	TTA (IU l^{-1})	X_{max} (g l^{-1})	μ_m (h^{-1})	$Y_{E/X}$ (IU g^{-1})	pH	TTA (IU l^{-1})	X_{max} (g l^{-1})	μ_m (h^{-1})	$Y_{E/X}$ (IU g^{-1})	pH
12.5	1100	3.46	0.13	739	2.40	6110	3.89	0.288	1599	4.44
25.0	2100	7.86	0.226	507	2.80	6800	4.12	0.295	1665	4.12
50.0	2700	19.72	0.248	250	2.80	11310	4.70	0.223	2475	3.95
100.0	2570	12.51	0.146	320	2.92	18300	5.48	0.217	3382	3.31
160.0	ND	ND	ND	ND	ND	9645	5.94	ND	1624	3.14

[a] ND: not determined.

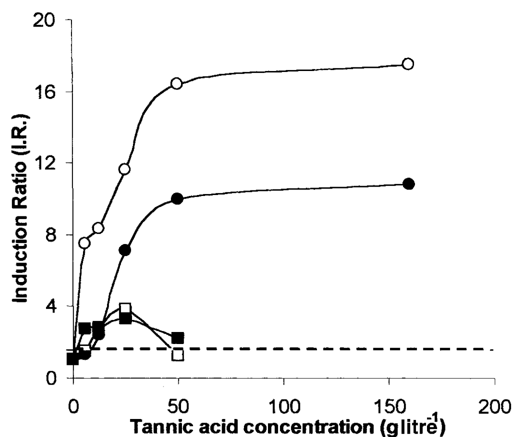

Fig. 1. Tannase induction patterns. Intracellular (●) and extracellular (○) tannase produced in SSC. Intracellular (■) and extracellular (□) tannase produced in SmC. The dotted line represents basal tannase activity.

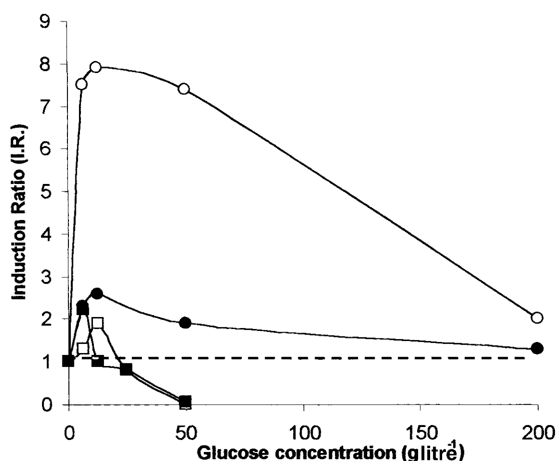

Fig. 2. Effect of glucose concentration on the induction ratio of tannase. Intracellular (●) and extracellular (○) tannase produced in SSC. Intracellular (■) and extracellular (□) tannase produced in SmC. The dotted line represents basal tannase activity.

and SSC systems which is a pattern quite different to that previously reported [6,13]. This could be due to differences in harvesting time since extracellular tannase is broken down in the SmC system by proteases. This does not occur in the SSC system (unpublished results). In SmC, enzyme yields ($Y_{E/X}$) with tannic as a sole carbon source, shown in Table 1, were low (740 IU $g^{-1} < Y_{E/X} < 320$ IU g^{-1}) with a low correlation coefficient vs. S_o ($R^2 = 0.54$). In SSC (see also Table 1), the trend was proportional to S_o, with a high correlation coefficient ($R^2 = 0.98$) and much higher values (1600 IU $g^{-1} < Y_{E/X} < 3380$ IU g^{-1}). Thus, the higher TTA levels observed in SSC as compared to SmC, were related mainly to a much higher enzyme yield ($Y_{E/X}$) and a steady growth rate (μ_m) although maximal biomass levels, X_{max}, were much higher in SmC than SSC.

Tannase repression experiments (Fig. 2), were carried out using, $S_o = 25$ g l^{-1}, of tannic acid as inducer and several glucose concentrations (G_o) as a potential catabolic repressor. The value of S_o was selected because at this concentration I.R. values were higher than unity for SmC and SSC systems (Fig. 1). In addition, similar tannic acid concentrations have been used in previous studies [6].

Fig. 2 shows that addition of small amounts of glucose addition ($G_o = 12.5$ g l^{-1}) increased tannase production in SSC but had very little effect in SmC. Higher levels of, G_o, led to a definite repression pattern in SmC as compared to smaller repression effect on SSC. Fig. 3 shows biomass production in both systems (Fig. 3a for SmC, and Fig. 3b for SSC), indicating that initial mould growth was very similar in both systems and depended on glucose addition. Maximal biomass levels were very similar for each system (Fig. 3a and Fig. 3b) although, apparent biomass, estimated by protein content, seemed to decrease after 20 h. It is not known if such a decrease was due to intracellular protein translocation (cytoplasmic into spores) or actual biomass dissapearance [12].

Fig. 4, presents the effect of tannic acid ($S_o = 25$ g l^{-1}) and glucose ($G_o \geq 6.25$ g l^{-1}) concentrations on tannase synthesis measured as tannase/biomass yield coefficients ($Y_{E/X}$). Small additions of glucose ($G_o \geq 6.25$ g l^{-1}), led in SmC, to a very small enzyme yield ($Y_{E/X} \approx 4 \times 10^{-3}$ IU g^{-1}), as compared to a much

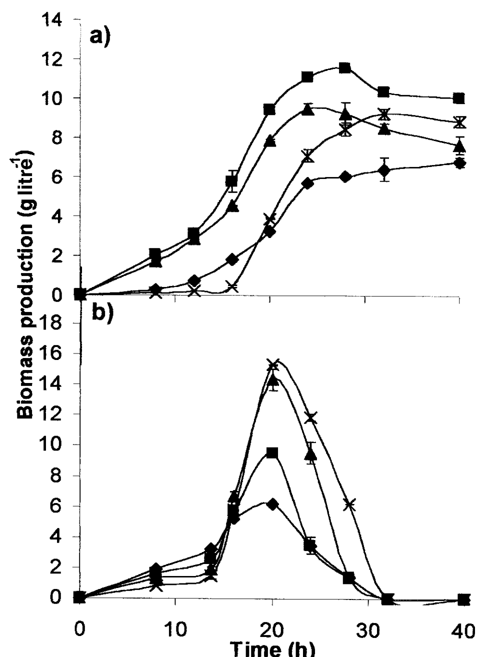

Fig. 3. Biomass production by *A. niger* Aa-20 in SmC (a) and SSC (b) using different glucose concentrations and tannic acid (25 g l^{-1}) as inducer of tannase activity. In SmC the glucose concentrations were: 6.25 (◆), 12.5 (■), 25 (▲) and 50 (×) g l^{-1} and in SSC: 6.25 (◆), 12.5 (■), 50 (▲) and 200 (×) g l^{-1}.

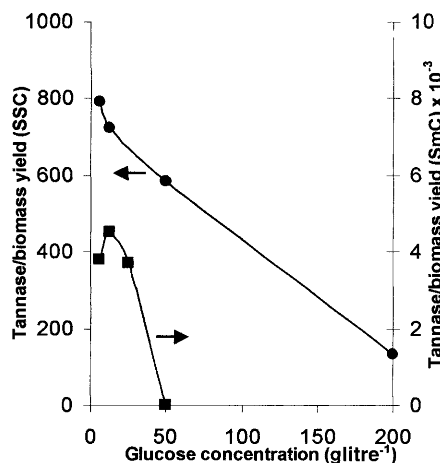

Fig. 4. Tannase/biomass yield coefficients in SmC and SSC as a function of glucose (as repressor) concentration.

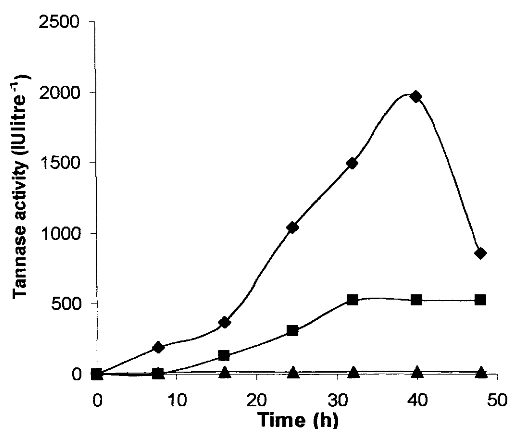

Fig. 5. Effect of carbon source on tannase production by *A. niger* Aa-20 in SmC. 30 g l^{-1} of glucose (■), 25 g l^{-1} of gallic acid (▲) and 25 g l^{-1} tannic acid (◆).

higher value for SSC ($Y_{E/X} \approx 800$ IU g^{-1}). Furthermore, in SmC, a concentration of $G_o = 50$ g l^{-1}, led to complete repression of tannase activity. These results are different to those shown in Table 1 ($S_o = 25$ g l^{-1}; $G_o = 0.0$ g l^{-1}), where $Y_{E/X} \approx 500$ IU g^{-1} for SmC and $Y_{E/X} \approx 1700$ IU g^{-1}, for SSC. Thus, Fig. 4 shows that catabolite repression is largely minimized in the SSC system since it can produce tannase with very high levels of glucose ($G_o > 50$ g l^{-1}), in the presence of a moderate level of the inducer ($S_o = 25$ g l^{-1}) which is impossible for the SmC system. This confirms the idea that the SSC system minimizes catabolite repression [2,5] and agrees with other comparative studies of titres of enzymes produced by SmC and SSC [3,4,6,14].

In the particular case of tannase, it has been reported that is not under catabolic repression in the presence of sucrose or glucose [13]. The present results show that tannase produced is regulated by glucose but in quite a different way according to the culture system. Recently, Bradoo et al. [8] reported that in SmC tannase is constitutive when produced on simple and complex substrates but activity is doubled in the presence of tannic acid as sole carbon source. In the present study, constitutive or basal levels were measured (Fig. 1 and Fig. 2) in both SSC and SmC but is more than doubled in SSC system when tannic acid is added to the culture medium.

Lekha and Lonsane [13] have questioned the inductive role of tannic acid due to its large molecular size and high reactivity. However, tannic acid and pentagalloyl glucose seem to be the best inducers of tannase activity [9,14]. In addition, Nishira and Mugibayashi [15] reported that tannase was induced either by tannic acid or gallic acid. Due to the fact that tannase is an esterase and that gallic acid contains no ester linkage, it seemed important to test the induction or repression of tannase by gallic acid in this system using *A. niger* Aa-20. Results of cultures with gallic acid as sole carbon source showed that it did not induce tannase activity (Fig. 5). Moreover, this compound repressed tannase activity produced in SmC, which was lower than basal activity (Fig. 5). This suggests an end-product repression mechanism. Lekha and Lonsane [13] in their tannase review suggested that tannase is induced following the mechanism involved in cellulase induction. However, they did not discuss the tannase repression mechanism.

This work agrees with the work of Bradoo et al. [8], strengthening the notion that tannase is not induced by gallic acid as shown in Fig. 5. Both reports showed that tannase activity is highest when higher tannic acid concentrations are used. Nevertheless, the present literature supporting constitutivity or inducibility of tannase by tannic acid is still scarce and further research on this topic is needed.

It is important to state that this is the first work that includes a detailed description of induction and repression patterns of fungal tannase activity in SmC and SSC systems both in terms of kinetic coefficients such as induction and yield ratios. In addition, the differences in activity titres, may be due to different sensitivities of the microorganism to glucose repression in each of the culture systems.

Further research is necessary to improve knowledge in this field, using techniques of molecular biology and microscopic analysis in order to uncover the details of the regulation loops of tannase induction and production between submerged and solid cultures of moulds. For example, to determine if there are special signals acting on special sensing genes related to high density cultures or perhaps the apparent resistance of SSC to catabolic repression could be related to micro gradients of substrates around the mycelial cells.

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