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Production of tannase by *Aspergillus niger* Aa-20 in submerged and solid-state fermentation: influence of glucose and tannic acid

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Tannase production by *Aspergillus niger* Aa-20 was studied in submerged (SmF) and solid-state (SSF) fermentation systems with different tannic acid and glucose concentrations. Tannase activity and productivity were at least 2.5 times higher in SSF than in SmF. Addition of high tannic acid concentrations increased total tannase activity in SSF, while in SmF it was decreased. In SmF, total tannase activity increased from 0.57 to 1.03 IU/mL, when the initial glucose concentration increased from 6.25 to 25 g/L, but a strong catabolite repression of tannase synthesis was observed in SmF when an initial glucose concentration of 50 g/L was used. In SSF, maximal values of total tannase activity decreased from 7.79 to 2.51 IU when the initial glucose concentration was increased from 6.25 to 200 g/L. Kinetic results on tannase production indicate that low tannase activity titers in SmF could be associated to an enzyme degradation process which is not present in SSF. Tannase titers produced by *A. niger* Aa-20 are fermentation system-dependent, favoring SSF over SmF. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 296–302.

Keywords: tannase; glucose; tannic acid; submerged and solid-state fermentation

Introduction

Tannase, or tannin acyl hydrolase (EC, 3.1.1.20), is an inducible enzyme produced by a variety of microorganisms. It catalyses the breakdown of hydrolysable tannins and gallic acid esters [17]. Tannase transforms tannic acid into glucose and gallic acid. This enzyme is used in the industrial processing of fruit juices and coffee-flavored soft drinks as a clarifying agent [17]. The major commercial applications of tannase are in the manufacture of instant tea and in the production of gallic acid [17,22]. A potential use of tannase is the treatment of wastewater contaminated with polyphenolic compounds, such as tannins.

Tannase production has been studied extensively in submerged fermentation (SmF) processes [2,4–6,10,16,18,21–23,26], while studies carried out in solid-state fermentation (SSF) processes are scarce [8,16]. Studies concerning regulatory aspects of tannase production by molds in SmF showed that tannases are induced by tannic acid or by some of its derivatives [5,7], but the regulatory mechanism of its production still remains unclear. There are very few studies in SSF, rendering difficult any explanation concerning profiles of tannase production. In addition, studies have been reported regarding the effect of tannic acid concentration or any other carbon source on tannase production profiles. Lekha and Lonsane [16], however, described differences in tannase activity when produced by SmF or SSF. They reported differences in activity titers and location of tannase produced in three different culture systems, showing that tannase activity was expressed as extracellular activity in SSF, while in SmF, it was exclusively intracellular during the first 48 h of culture. However, their study did not attempt to explain the patterns obtained. Differences in the effects of induction and repression ratios on tannase production by SSF and SmF suggested different sensitivities of *Aspergillus niger* Aa-20 to glucose repression in each type of culture [1].

In the present study, the effects of different tannic acid and glucose concentrations on tannase production in SmF and SSF were studied in order to determine the main differences between these systems.

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Materials and methods

Microorganism and culture medium

Spores of *A. niger* Aa-20 (UAM-IRD collection) were stored at −20°C in protect crioblocks (bead storage system; Technical Service Consultants). Inoculum was prepared by transferring spores to potato dextrose agar (PDA) medium and incubating the culture at 30°C for 5 days. Spores were then scraped into 0.01% Tween-80 and counted in a Neubauer chamber.

Medium for tannase production was as reported by Lekha and Lonsane [16] using $(NH_4)_2SO_4$ as the nitrogen source. Salt-containing medium was autoclaved at 121°C for 15 min. Tannic acid (Sigma, USA) solution was filter-sterilized and added to a final concentration of 12.5, 25, 50 or 100 g/l. The effect of glucose addition was also evaluated. Glucose concentrations of 6.25, 12.5, 25 and 50 g/l added to the base medium containing 25 g/l of tannic acid were tested in SmF. In SSF, glucose concentration ranged from 6.25 to 200 g/l (in the presence of 25 g/l tannic acid).

SmF

Erlenmeyer flasks (250 ml) with 50 ml of sterile liquid medium at an initial pH of 5.5 were inoculated with 2.5 ml of the spore suspension (2×10^7 spores/ml) and incubated at 30°C on a rotary shaker (220 rpm) for up to 48 h. Samples were removed at 8 h intervals. Biomass was separated by filtration through Whatman no.

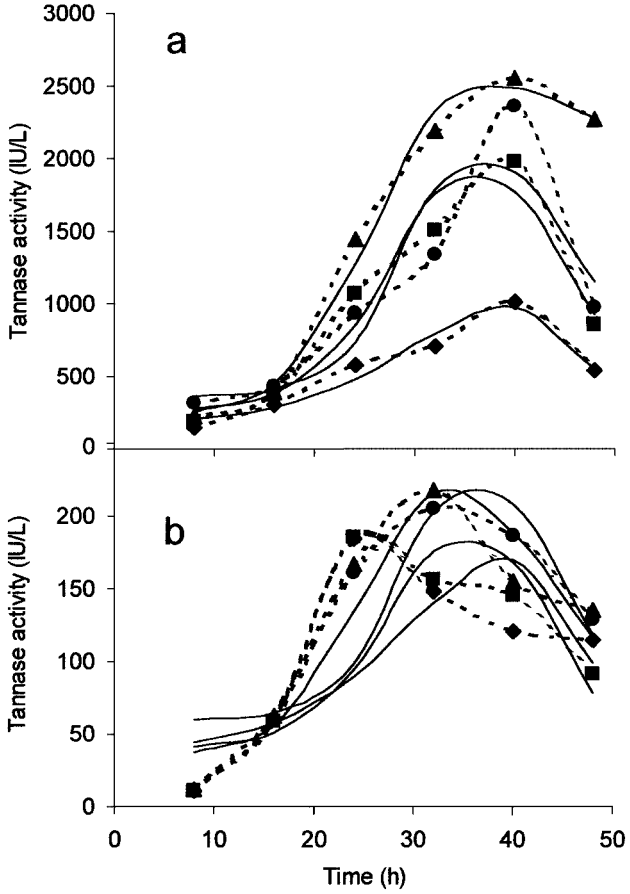

Figure 1 Extracellular (a) and intracellular (b) tannase production by *A. niger* Aa-20 in SmF with tannic acid concentrations of 12.5 (◆), 25.0 (■), 50.0 (▲) and 100.0 (●) g/l. Luedeking and Piret model curve (———) and polynomial curve (– – –).

41 filter paper. The cell-free culture broth was assayed for extracellular tannase activity and evaluated for tannic acid content. Cells were washed three times with physiological saline solution, frozen in liquid nitrogen, macerated in a chilled mortar and suspended in acetate buffer (200 mM, pH 5.5). Fungal debris was removed by centrifugation and the resulting supernatants were assayed for tannase activity.

SSF

SSF involved polyurethane foam (PUF) (Expomex, México) as a support to absorb the liquid medium. PUF was washed as reported by Zhu *et al.* [27] and then pulverized in a plastic mill. Column reactors (25×180 mm) were packed with 10 g of dry inert support inoculated (2×10^7 spores/g of dry inert support) PUF. Culture conditions were: 30°C, aeration rate, 20 ml air/g support min, initial pH 5.5, initial moisture content of 65% and an incubation time of 48 h with a sampling time of 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 no. 41 paper) and the clear filtrate was assayed for extracellular through 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 by macerating them in a chilled mortar. The recovery process was similar to that described for the SmF system.

Analytical methods

Tannase assays were carried out using an HPLC methodology developed by Beverini and Metche [6]. One unit of enzyme (IU) was defined as the amount of enzyme able to release 1 mol gallic acid/ml min.

Tannic acid concentration was evaluated by spectrophotometry (λ=420 nm) using the phenol–sulfuric acid method.

Glucose concentration was evaluated with the GOD-PAP enzymatic kit from Spinreact (1001191, Cat. N., Spinreact, Spain).

For biomass determination in SmF, the mycelium obtained after filtration was dried at 70°C to a constant weight. In SSF, biomass could not be measured accurately by gravimetry, so it was assayed by measuring protein concentration with the Bradford microassay (Bio-Rad®) following the technique reported by Córdova-López *et al.* [9]. Mycelium grown on agar plates was used as standard. Sampling in SmF and SSF systems was done in triplicate.

Results and discussion

Effect of tannic acid on tannase production in SmF and SSF

Tannase has been reported to be an inducible enzyme, having tannic acid as an inducer as well as a sole carbon source [2,16,26]. In SmF, the concentration of tannic acid adequate for tannase

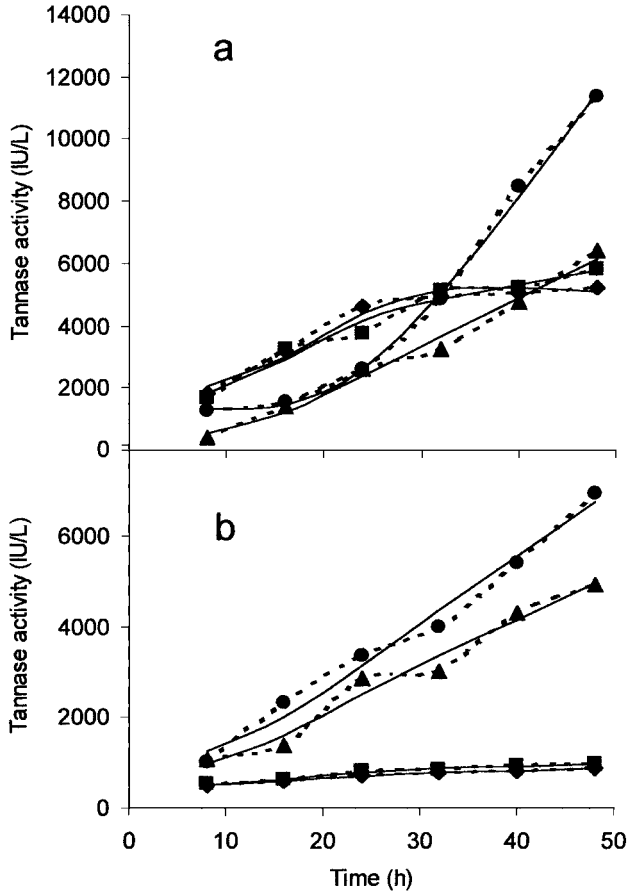

Figure 2 Extracellular (a) and intracellular (b) tannase production by *A. niger* Aa-20 in SSF with tannic acid concentrations of 12.5 (◆), 25.0 (■), 50.0 (▲) and 100.0 (●) g/l. Luedeking and Piret model curve (———) and polynomial curve (– – –).

Table 1 Reference and maximal tannase productivity at different initial tannic acid concentrations

S_0 (g/l)	$\mathcal{P}_R = q_P Y_{E/X}$ (IU/h l)*		$\mathcal{P}_{max}$ (observed) (IU/h l)*	
	SmF	SSF	SmF	SSF
12.5	491	1751	28	127
25	934	1959	52	141
50	1234	2424	68	235
100	607	4107	63	381
Average	816	2560	52	221
DS	335	1069	17	116
P	<0.05	<0.05	<0.05	<0.05

*See Appendix A.

production ranged from 0.1% to 10% [2,17], while in SSF, a concentration of up to 20% tannic acid has been used [1]. Enzyme production by *A. niger* Aa-20 was therefore studied using SmF and SSF with different initial tannic acid concentrations (S_0=12.5, 25, 50 and 100 g/l). Kinetics of extra- and intracellular activities in SmF are shown in Figure 1. Extracellular tannase activities (Figure 1a) as well as intracellular activities (Figure 1b) followed the model of Luedeking and Piret [19] with negative values of k (see Figure 5 and Appendix). They had well-defined peak values before the end of the fermentation. Peak values increased for extra- and intracellular tannase activities with increasing amounts of tannic acid and the maximal extracellular peak value was nearly 10 times (2500 IU/l) as compared to the maximal peak value of intracellular activity (*ca.* 250 IU/l). Negative values of k are associated with decaying rates of tannase. The effect of increasing levels of tannic acid on the peak values of intracellular activity was not as pronounced compared to the effect found for extracellular tannase. The extra/intracellular tannase activity ratio ranged from 8 to 16 and maximal values of extracellular activity were reached after those obtained for intracellular activity, which is consistent with a sequential model of synthesis and excretion of this enzyme. Lekha and Lonsane [17] reported that the initial tannic acid concentration affected the levels of enzyme and, depending on the microorganism and the amount of tannase produced, the fermentation took 1–10 days. In the present case, peak tannase activity was reached with 50 g/l after 40 h of incubation.

Kinetics of extra- and intracellular activities in SSF are shown in Figure 2. The production pattern by SSF was sharply different from the production pattern of SmF since no definite peak values were observed for extra- (Figure 2a) or intracellular (Figure 2b) tannase activities. This coincides with the plot of parameter, k *vs.* S_0 (Figure 5), where positive values were obtained. This is evidence of a much higher stability of tannase obtained by the SSF system. Maximal tannase activities found at the end of each fermentation were at least 2.5 times higher in SSF when compared to SmF. Maximal extra- (Figure 2a) and intracellular (Figure 2b) tannase activities were obtained by SSF with the highest concentration of tannic acid (100 g/l). Extracellular tannase activity profiles were similar for tannic acid concentrations from 12.5 to 50 g/l, but intracellular tannase activity increased considerably from 50 to 100 g/l of tannic acid. At tannic acid concentrations below 50 g/l, the extra/intracellular tannase activity ratio was around 6, whereas above 50 g/l of initial tannic acid, this ratio was around 1–1.5. Although extracellular tannase activity was higher than intracellular activity for all the tannic acid concentrations tested, it appeared that tannase secretion in SSF was favored at low tannic acid concentrations. The opposite was observed in SmF, where the higher extra/intracellular tannase activity ratio was obtained with an initial tannic acid concentration of 50 g/l. Furthermore, in SSF experiments, tannase activity did not decrease at the end of culture as in SmF.

Differences in tannase production profiles between SmF and SSF could be partially attributed to the presence of protease activity

Table 2 Growth of *A. niger* Aa-20 in SmF and SSF with tannic acid as sole carbon source

System	Tannic acid (g/l)	$\mathcal{P}^a_{max}$	q_s[b]	q_P[c]	Tannic acid uptake (%)
SmF	12.5	28	0.37	100	92
	25.0	52	0.68	110	94
	50.0	68	0.59	60	93
	100.0	63	0.52	50	54
SSF	12.5	127	0.94	460	100
	25.0	141	1.81	490	100
	50.0	235	2.44	550	100
	100.0	381	2.93	730	73

Summary of kinetic parameters obtained as maximal values.
[a]Tannase productivity $\mathcal{P}^a_{max}$ = IU/l h.
[b]Specific substrate uptake rate $q_s = \mu / Y_{X/S}$, g S/g h.
[c]Specific product formation rate $q_P = \mu Y_{E/X}$, IU/g h.

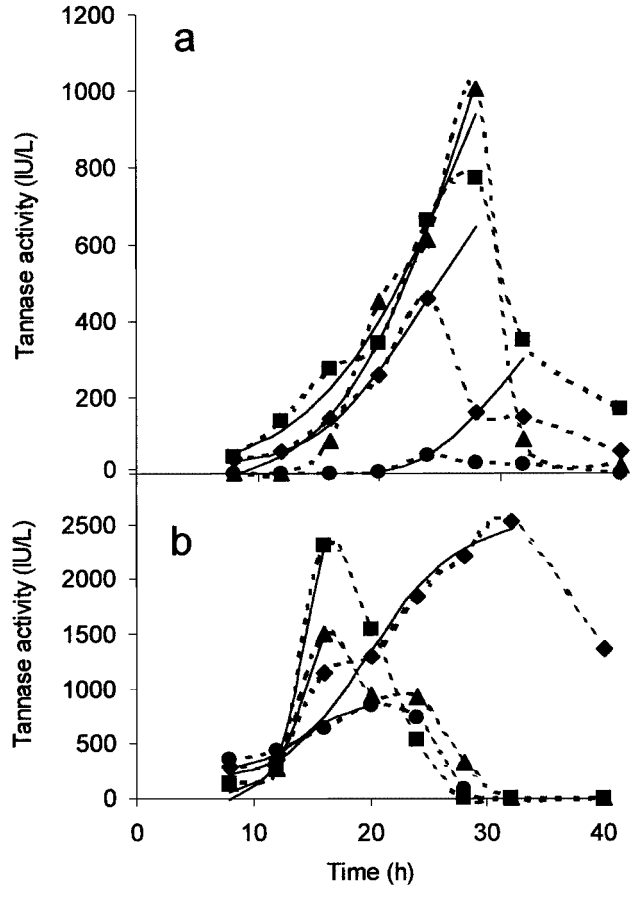

Figure 3 Extracellular (a) and intracellular (b) tannase production by *A. niger* Aa-20 in SmF with 25 g/l tannic acid and glucose concentrations of 6.25 (◆), 12.5 (■), 25 (▲) and 50 (●) g/l. Luedeking and Piret model curve (———) and polynomial curve (– – –).

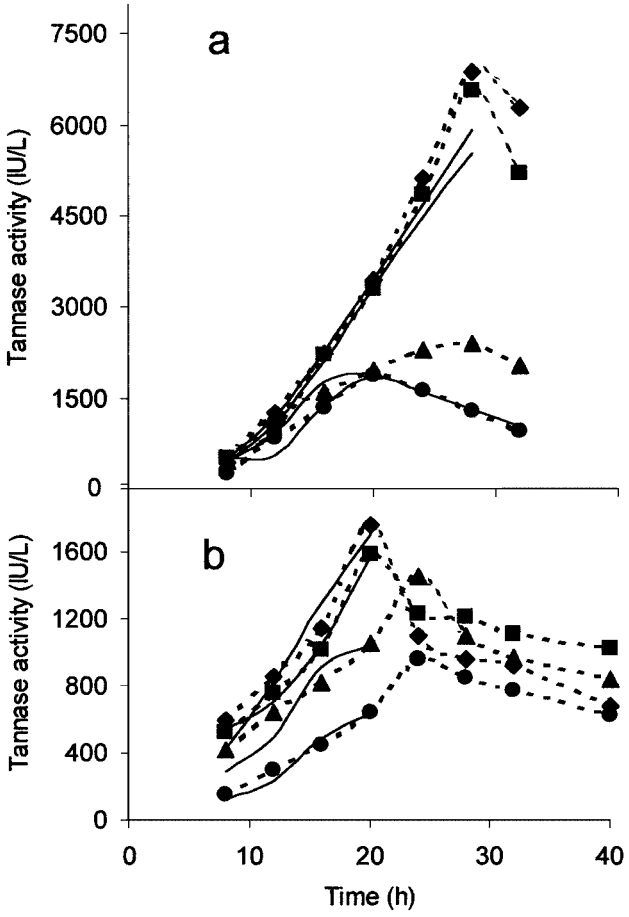

Figure 4 Extracellular (a) and intracellular (b) tannase production by *A. niger* Aa-20 in SSF with 25 g/l tannic acid and glucose concentrations of 6.25 (◆), 12.5 (■), 50.0 (▲) and 200.0 (●) g/l. Luedeking and Piret model curve (———) and polynomial curve (– – –).

in SmF. Bradoo *et al.* [7] showed that tannase activity decreased at the end of the culture. Hadi *et al.* [14] also observed such a decrease of activity. They assumed that it was due to substrate limitation, to the presence of toxic substances in the culture medium or to low pH values at the end of culture. Beverini and Metche [6] showed that tannase activity was stable at pH values between 3.5 and 5.5. In our case, the lowest pH of the culture was 3.9. Thus, decrease in tannase activity in the SmF system (Figure 1) might be related to protease production. These results support the suggestion by Hadi *et al.* [14] that protease production is favored by lack of substrate at the end of culture. This is also consistent with the fact that intracellular tannase activity decreased earlier than the extracellular activity.

Maximal tannase productivity, $\mathcal{P}_{max}$, estimated per unit of liquid broth (IU/h l), as defined in the Appendix, was several times higher for the SSF system than for the SmF system (Table 1). The reference value for productivity ($\mathcal{P}_R = q_P X_{max}$) showed a similar trend. This supports an intrinsic higher productivity of tannase by the SSF system. Such a higher level of productivity cannot be attributed solely to the fact that tannase is more stable because it is also produced at a faster specific rate, q_P, by the SSF system (Table 2) although maximal biomass levels, X_{max}, were similar in both types of systems (see Appendix).

In the present study, special attention was paid to regulatory mechanisms of tannase synthesis because high levels of tannic acid lead to a significant supply of the breakdown products namely glucose and gallic acid. In addition, such compounds may be closely related to the control of enzyme expression. Glucose is involved in catabolite repression and gallic acid may be involved in end-product repression. Bajpai and Patil [5] reported that gallic acid is not involved in the regulatory mechanism, whereas Bradoo *et al.* [7] reported that it acts as a repressor by end-product regulation either in constitutive or induced conditions. The exact role(s) played by glucose (from tannic acid) is(are) unknown. In SSF, maximal tannase productivity, $\mathcal{P}_{max}$ or $\mathcal{P}_R$, was from 2.7 to 6.0 times higher than those obtained by SmF. Such differences were increased with increments of initial tannic acid concentration (Table 2). When results obtained in SSF and SmF were compared, it was clear that the use of high tannic acid concentrations resulted in a higher efficiency of conversion of substrate to product, reaching a higher productivity (at least 3.7 times) of tannase. This was also associated with a higher tannic acid uptake rate (Table 2). Such behavior indicates that SSF is a more productive process for tannase activity, in comparison with SmF. Furthermore, in SSF, the degradation capacity of tannic acid by *A. niger* Aa-20 was high for all initial tannic acid concentrations tested (higher than 70%). However, in SmF, the total tannic acid breakdown was higher than 90% for initial tannic acid concentrations ranging from 6.25 to 50 g/l. Such a breakdown was nearly 50% for an initial substrate concentration of 100 g/l. Those features of the SSF system could be very important if tannase was to be used for the removal of polyphenolic compounds, such as tannins, from wastewater.

Effect of glucose concentration on tannase production in SmF and SSF

Lekha and Lonsane [17] reported that tannase produced by *A. niger* PKL104 was not affected by the presence of another readily utilizable carbon source. Fumihiko and Kiyoshi [11] reported the use of additional carbon sources, such as glucose (1%) and sucrose (3%), along with tannic acid for tannase production by *A. oryzae*. In this work, enzyme production by *A. niger* Aa-20 was studied in SmF and SSF with different initial glucose concentrations (G_0=6.25, 12.5, 25, 50 and 200 g/l) in the presence of a fixed initial amount, S_0=25 g/l, of tannic acid. Kinetics of extra- and

Table 3 Growth of *A. niger* Aa-20 in SmF and SSF with glucose in the presence of tannic acid

System	Glucose (g/l)	$\mathcal{P}$[a]	q_s[b]	q_P[c]	Tannic acid uptake (%)
SmF	6.25	83	0.95	160	67
	12.5	161	0.48	80	62
	25.0	99	0.86	90	46
	50.0	42	1.63	120	3
SSF	6.25	280	0.55	130	61
	12.5	277	0.43	70	46
	50.0	156	0.39	30	27
	200.0	108	0.41	20	0

Summary of kinetic parameters obtained as maximal values.
[a]Tannase productivity $\mathcal{P}$=IU/L h.
[b]Specific substrate uptake rate $q_s = \mu / Y_{X/S}$, g S/g h.
[c]Specific product formation rate $q_P = \mu Y_{E/X}$, IU/g h.

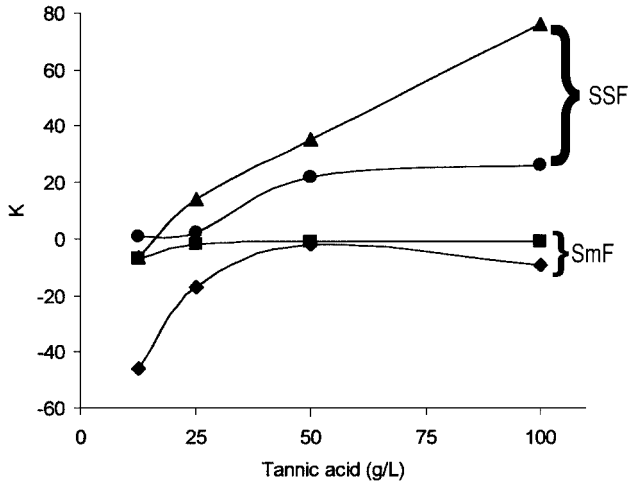

Figure 5 Extra- and intracellular tannase decomposition rate (k) production by *A. niger* Aa-20 in SSF and SmF at different initial tannic acid concentrations [extra- (◆) and intracellular activity (■) in SmF and extra- (▲) and intracellular activity (●) in SSF].

intracellular activities in SmF are presented in Figure 3. The SmF pattern of extra- and intracellular tannase activities with small amounts of glucose (G_0=6.25 g/l) is shown in Figure 3a and b. The pattern is similar to that observed without glucose (Figure 1a and b). For example, with sharp peaks of tannase activity, peak values decrease with increasing G_0 levels, but with a reversal on the ratio of peak intra/extra activities, since now the intracellular level was nearly 2.5 times that of the extracellular level. It is worth noting that the peak value for extracellular activity with G_0=100 g/l was lower than 100 IU/l. In the SSF system, glucose addition produced definite peaks of intra- and extracellular tannase activities (compare Figures 2 and 4), but the extracellular tannase titer with G_0=100 g/l was above 1500 IU/l, showing a remarkable resistance to catabolite repression (Figure 4). Altogether, these findings are consistent with the idea that the SSF system is more robust than the SmF system with regard to catabolic repression and, for that mater, can be more productive with a wider variety of substrate mixtures.

In SSF with tannic acid as sole carbon source, tannase production did not decrease at the end of culture. However, when glucose was added to the medium, tannase production decreased. In this case, the results could be partially explained by the fact that the medium changed considerably in its carbon/nitrogen ratio with increasing glucose concentrations. Higher enzymatic activity titers were observed in SSF compared to SmF. In several studies in which enzymatic activity titers were compared between SmF and SSF, similar results were obtained [13,16,20,25]. In all cases, enzymatic activity titers were higher in SSF than in SmF. Some theories have been proposed to explain this behavior; however, at present, only one has been experimentally tested [20]. This study demonstrated changes in the composition of membrane fatty acids, but more research is needed to explain such behavior.

When glucose was added at different concentrations, the maximal tannase productivity ($\mathcal{P}_{max}$) in SSF was from 1.5 to 3.4 times higher than that obtained in SmF, but it decreased with increments of initial tannic acid concentration (Table 3). The q_s values were enhanced in comparison to those obtained without glucose, as this molecule is more easily taken up by the fungus than tannic acid. Thus, tannic acid uptake percent decreased sharply (from 67% to 3%) with increases in initial glucose concentration (Table 3).

In SSF, the addition of glucose significantly decreased all kinetic parameters with respect to those obtained without glucose. Furthermore, the degradation capacity of tannic acid was seriously affected. Results from experiments with glucose and tannic acid show that substrate uptake rate is three to five times higher in SSF than in SmF. The concentration of tannic acid required to inhibit fungal growth was much higher in SSF as compared to SmF. This could be attributed to differences in the diffusion of nutrients present in the medium, thereby provoking changes in regulatory mechanisms of enzyme biosynthesis. A comparison of kinetic parameters obtained in both fermentation systems showed SSF to be a more attractive process for tannase production, but only when glucose was absent from the culture medium. In SmF, glucose concentrations higher than 25 g/l resulted in strong catabolite repression.

Concluding remarks

Kinetic analysis by the Luedeking and Piret [19] model, as shown in Appendix, helps to classify the behavior of enzyme-producing systems, namely, on the basis of the values of the secondary rate constant k. For example, when $k<0$, the system will have a convex fermentation curve indicating a secondary specific rate of enzyme decomposition that may balance the production rate associated with vegetative growth (q_P). This factor is responsible for the presence of peak values along the fermentation runs (Figures 1 and 5). Also, if there is a secondary rate of production ($k>0$), the curve will be convex with no peak values (Figures 2 and 5). From the production point of view, a non-negative value of k helps to identify fermentation conditions in which the excreted enzyme activity will be stable in the fermentation broth and may be a significant factor for better enzyme productivity. On the other hand, evaluation of the specific rate of production (q_P), together with the maximal biomass value (X_{max}), helps to distinguish whether productivity is based on a very productive strain (high q_P) or simply because biomass is produced in large quantities (high X_{max}). Apparently, in this particular case, synthesis and excretion of tannase in the SSF system is more productive than in the SmF system, both because k is positive and also because the mold is more productive in the SSF system, but not necessarily because more biomass was produced. However, recent work [24] has shown that three strains of *A. niger* grown on PUF were more productive of invertase because the organisms grew much better (higher levels of X_{max}) and not because q_P was much higher in the SSF system than in the SmF system. Thus, there is reason to assume that differences between SSF and SmF systems may be due to a variety of factors. For example, in data presented by Romero-Gómez *et al.* [24], the packing density of foam was 10 times lower than in this work and this may be a significant factor affecting steric hindrance of molds [15] and gas diffusion rates within the packed bed reactor [3]. At any rate, present results help to clarify why tannase production has been difficult to develop in SmF systems [12,16,17,22], despite the fact that tannase is a fungal enzyme known for more than a century and there are a number of patents using tannase in the food industries. Here it is shown that a significant factor is the presence of a sharp decay of tannase activity when produced by the SmF technique. This is probably due to the excretion of ancillary proteases that may be absent in the SSF system and not because tannase is produced as an intracellular enzyme in the SmF system [16].

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Appendix

Kinetic parameters used in this work were estimated as follows.

Growth kinetics

Biomass production, estimated as X (g/l), was followed by the logistic equation:

$$\frac{dX}{dt} = \mu\left[1 - \frac{X}{X_{\max}}\right]X \tag{1}$$

where μ is the maximal specific growth rate and $X_{\max}$ is the equilibrium value for X where $dX/dt=0$. The solution of Equation 1 is given below:

$$X = \frac{X_{\max}}{\left[1 + \frac{(X_{\max}-X_0)}{X_0}\mathrm{e}^{-\mu t}\right]} \tag{2}$$

with X_0 being the value of X when $t=0$. From previous work [1], the following (growth) parameters (μ, $X_{\max}$ and $Y_{E/X}$) were obtained for the indicated initial values of tannic acid

Enzyme production kinetics

Production of enzyme activity, E (U/l), was estimated according to the Luedeking and Piret [19] model:

$$\frac{dE}{dt} = Y_{E/X}\frac{dX}{dt} + kX \tag{3}$$

where $Y_{E/X}$ (U/g) is the yield production coefficient and k (U/h g) is the secondary coefficient of enzyme production ($k>0$) or destruction ($k<0$). The solution of Equation 3, $E=E(X)$, is given below:

$$E = E_0 + Y_{E/X}(X - X_0) + \frac{kX_{\max}}{\mu}\ln\left[\frac{(X_{\max} - X_0)}{(X - X_0)}\right] \tag{4}$$

with E_0 being the value of E when $X=X_0$.

Evolution of $E=E(t)$ can be estimated by the insertion of $X(t)$ obtained from Equation 2 in Equation 4.

Estimation of parameters for Equations 2 and 4 can be obtained using minimization of the sum of squared errors between experimental and calculated values for $X(t)$ and $E(t)$ using a minimization program such as the Solver routine from Microsoft Excel.

Enzyme production and substrate uptake parameters

The specific production rate of enzyme, q_{P}, was defined as follows:

$$q_{\mathrm{P}} = \mu Y_{E/X} \tag{5}$$

The specific substrate uptake rate, q_{s}, was defined as follows:

$$q_{\mathrm{S}} = \mu/Y_{X/S} \tag{6}$$

$Y_{E/X}$ and $Y_{X/S}$ were estimated from the linear correlation between tannase activities and biomass concentration, and biomass and tannic acid concentration, respectively. Regression coefficients (R^2) were above 0.9 for at least four data points before the maximum growth was achieved.

Enzyme productivity, $\mathcal{P}$, was defined for every $t>0$ as $E(t)/t$, having the following reference value, $\mathcal{P}_{\mathrm{R}}$, defined below and $\mathcal{P}_{\max}=[E(t)/t]_{\max}$ as the maximum value of productivity observed for a given fermentation run:

$$\mathcal{P}_{\mathrm{R}} = \mu\mathcal{Y}_{\mathcal{E}/\mathcal{X}}\mathcal{X}_{\max} \tag{7}$$

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