Biosynthesis of tannase and hydrolysis of tannins to gallic acid by *Aspergillus awamori* — optimisation of process parameters

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Abstract

Tannin acyl hydrolase (tannase) is an industrially important enzyme produced by a large number of fungi. Tannic acid concentration, agitation speed and pH during the fermentation were identified as important process parameters effecting cell growth and enzyme synthesis by *Aspergillus awamori*. These parameters were optimised in a laboratory bioreactor by response surface methodology using Box and Behnken factorial design to determine the optimum conditions for enzyme production and gallic acid accumulation. Under optimum process conditions for enzyme synthesis, the fermentation run lasted 60 h with an initial tannic acid concentration of 35.0 g l^{-1}, yielding biomass concentration of 7.13 g l^{-1} containing 771 IU of intracellular tannase per gram dry cell weight and 19 g l^{-1} of gallic acid. However, maximum gallic acid accumulation (40.3 g l^{-1}) was obtained in 24 h with an initial substrate concentration of 45 g l^{-1}.

Keywords: Tannase biosynthesis; Gallic acid accumulation; Optimisation; *Aspergillus awamori*; Response surface method

1. Introduction

Tannin acyl hydrolase (tannase E.C. 3.1.1.20) hydrolyses the ester and depside bonds of gallotannins and gallic acid esters [1]. It is used widely in the manufacture of instant tea, acorn wine and gallic acid [2–4]. Gallic acid is an important substrate for the synthesis of propyl gallate in the food industry and trimethoprim in the pharmaceutical industry [5]. Tannase also has potential applications in the clarification of beer and fruit juices, manufacture of coffee flavoured soft drinks, improvement in the flavour of grape wine and as an analytical probe for determining the structures of naturally occurring gallic acid esters [6,7].

The industrial applications of tannase have not been fully exploited because of its high cost, although there are a large number of reports on the production of tannase by submerged fermentation [8–11]. Most of these do not involve the identification of critical parameters for enzyme biosynthesis and their optimisation. There is only one report on the optimisation of process parameters for tannase production by *Aspergillus niger* in solid state fermentation [12].

This enzyme is synthesised by a number of fungi [13]. Based on a preliminary screening of various isolates and other available fungal cultures, one of these organisms, *Aspergillus awamori*, was selected for further studies. The present studies were aimed at identifying key environmental parameters that play important roles in enzyme synthesis by *A. awamori* and their optimisation by response surface methodology.

2. Materials and methods

2.1. Microbial culture and development of inoculum

A. awamori obtained from the Department of Microbiology, University of Delhi, South Campus was selected as the potential intracellular tannase producer. The culture was maintained on Honey–Peptone–Barley medium. To 50 g of Pearl barley, 9 ml of solution containing honey (10%w/v) and peptone (1%) was mixed in a 500 ml Erlenmeyer flask and autoclaved at

121°C and 15 psi for 20 min and after cooling to room temperature, it was inoculated with a spore suspension from the stock culture and incubated at 37°C for 6–7 days. Spores were separated from the solid state growth by suspending the fermented medium in normal saline to bring to a concentration of 5×10^7 spores ml^{-1}. The spore suspension was used to inoculate (to bring to an initial spore concentration of 5×10^6 per ml) 500 ml shake flasks containing 100 ml of growth medium (of the same composition as mentioned in Section 2.2, but containing 2.0% tannic acid). The shake flasks were incubated in a Orbital shaker at 37°C and 250 rpm for 24 h.

2.2. Fermentation medium and experimental parameters

The medium contained tannic acid (varying concentrations), $NaNO_3$ (0.3%), KCl (0.05%), $MgSO_4 \cdot 5H_2O$ (0.05%), KH_2PO_4 (0.1%). One millilitre of solution containing $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$ and $ZnSO_4 \cdot 2H_2O$ was added to the mixture to bring the final level of these salts to 0.001% each. The concentration of tannic acid was varied from 25 to 45 g l^{-1}. The fermentation was carried out in a 3-l bioreactor (B. Braun, Biostat B) equipped with sterilizable pH probe (Ingold), temperature sensor (Pt 100) and dissolved oxygen probe (Ingold). The experiments were carried out at controlled pH of 4.0, 5.0 and 6.0, agitation speeds of 300, 350 and 400 rpm under constant aeration rate of 0.75 vvm at 37°C. The pH was controlled with 5 N aqueous NH_3 and 5 N HCl. The fermentation in each case was started using 5% (v/v) of 24-h old inoculum.

2.3. Isolation of tannase

The culture broth was filtered and the mycelium washed with citrate buffer (0.05 M, pH 5.5) twice before suspending in buffer to bring to a concentration of 100 g wet cell mass l^{-1}. The suspension was disintegrated in a French press (SLM Amincon Instruments) at 1500 psi in two cycles of 5 min each. The entire operation was carried out after pre-cooling the suspension to 4°C, such that the temperature of the mixture does not exceed 10°C. The disrupted cell suspension was then centrifuged at 10 000 rpm and the supernatant containing the enzyme was recovered. The debris was washed with buffer to recover the enzyme bound to the residue and washing was mixed with the supernatant.

Table 1
Design of experiment-levels of various process parameters

	Levels		
	−1.0	0	1.0
pH	4	5	6
Substrate conc. (g l^{-1})	25	35	45
Agitation speed (rpm)	300	350	400

2.4. Assay of tannase activity

To 4 ml of 0.35%(w/v) tannic acid in citrate buffer (0.05 M, pH 5.5), 1 ml of the enzyme sample was added and incubated at 35°C for 45 min. The samples (40 µl) withdrawn at various intervals of time from 0 to 45 min were diluted 100 times with 90% ethanol and absorbance measured at 310 nm. One IU of tannase activity is defined as the amount of enzyme which hydrolyses 1 µmol of ester bond in 1 min [14] which is computed as:

$$\text{IU/ml} = C \times v/V \times 1/\Delta E(0-T) \times \Delta E(t_1 - t_2)/t_2 - t_1$$

where C is the µmoles of ester bonds in 0.35% tannic acid, v/V is the dilution factor of the enzyme, $\Delta E(0-T)$ is the maximum decrease in absorbance of tannic acid which corresponds to all the ester bonds hydrolysed, $\Delta E(t_1 - t_2)$ is the decrease in absorbance of tannic acid for the stipulated time, t corresponds to time in minutes.

2.5. Estimation of gallic acid

Gallic acid was estimated by HPLC (Waters 510 with UV detector 440) using 3.9×30 cm^2 Bondapak C_{18} reverse phase column. A 20-µl sample was injected and initially eluted with 5% acetic acid in distilled water until the gallic acid was eluted. The ester was then detected with methanol as the mobile phase. The flow rate was maintained at 1.80 ml min^{-1} and both the acid and the ester were monitored at 280 nm [15].

2.6. Design of experiments

The experiments were designed according to Box and Behnken's response surface design [16]. The various levels of the experimental parameters have been summarised in Table 1.

3. Results and discussion

To identify the key process variables for experimental design that influence cell growth, enzyme synthesis and gallic acid accumulation independently, shake flask experiments were carried out to study the effect of agitation rate, carbon and nitrogen sources, their concentration and pH on the above parameters. The temperature chosen in these studies was optimum

Table 2
Effect of various process parameters on enzyme activity and gallic acid accumulation

Substrate conc. (g l^{-1})	Agitation rate (rpm)	pH	Activity (U g^{-1} DCW)		Gallic acid conc. (g l^{-1})		$Y_{p/s}$ gallic acid
			Experimental	Predicted	Experimental	Predicted	
25	300	5	485.43	540.07	16.92	18.67	0.85
45	300	5	701.74	731.98	37.60	37.94	0.97
35	300	6	580.00	531.29	23.10	22.17	0.68
35	300	4	532.17	495.92	25.12	23.96	0.96
45	350	6	546.74	565.21	37.60	38.19	0.97
25	350	6	34.78	28.85	18.80	17.98	0.45
45	350	4	444.35	425.88	37.30	38.12	0.93
25	350	4	444.35	450.28	18.73	18.14	0.81
35	400	4	391.80	440.51	18.52	19.47	0.59
35	400	6	86.96	123.13	20.00	21.16	0.50
45	400	5	643.26	588.62	37.76	36.01	0.98
25	400	5	250.00	219.76	15.43	15.09	0.69
35	350	5	760.87	760.87	27.30	27.30	0.86
35	350	5	760.86	760.87	27.30	27.30	0.86
35	350	5	760.85	760.87	27.30	27.30	0.86
25	300	4	–	392.58	–	17.41	–
25	300	6	–	171.88	–	15.50	–
25	350	5	–	485.20	–	20.28	–
25	400	4	–	248.61	–	12.08	–
25	400	6	–	0	–	13.67	–
35	300	5	–	771.49	–	25.28	–
35	350	4	–	573.54	–	25.11	–
35	350	6	–	432.50	–	25.06	–
35	400	5	–	539.66	–	22.53	–
45	300	4	–	328.50	–	36.56	–
45	300	6	–	619.77	–	34.89	–
45	350	5	–	765.59	–	40.37	–
45	400	6	–	300.08	–	34.70	–
45	400	4	–	361.48	–	32.88	–

(37°C) for cell growth. The agitation rate, carbon source concentration and pH significantly affected intracellular enzyme activity (IU g^{-1} dry cell mass), cell growth and gallic acid accumulation. These were therefore, chosen as the parameters for further study in a 3-l mechanically agitated bioreactor with 2 l working volume. In the bioreactor, the agitation speed was varied from 300–400 rpm and the air flow-rate was kept at 0.75 vvm. The agitation and aeration rate was kept constant throughout the fermentation run because it was experimentally noted that allowing the dissolved oxygen level fall along with the cell growth resulted in better enzyme synthesis than varying the aeration and agitation rate to maintain a minimum pre-set dissolved oxygen level. Moreover, increasing the aeration rate beyond 0.75 vvm resulted in the oxidation of tannins. The oxidation of tannins at higher aeration rates was also observed by Barthomeuf et al. [11]. The increase in agitation rate beyond 400 rpm resulted in a drastic fall in specific enzyme activity largely due to shearing effects on the mycelium. The agitation speed below 300 rpm resulted in inadequate mixing of the broth towards the later stages of growth. The substrate concentration was varied from 25 to 45 g l^{-1}, as increasing it further resulted in reduced growth due to the accumulation of gallic acid on the surface of the mycelia. The pH was varied from 4.0 to 6.0, as the growth rate of the organism decreased drastically beyond a pH of 6.0 and the specific enzyme activity was very low below a pH of 4.0.

The individual and the interactive effect of the three parameters (pH, substrate concentration and agitation speed) was studied by conducting the fermentation runs at randomly selected different levels of the three parameters (excluding the set of terminal values). Data were collected for maximum enzyme activity (IU g^{-1} DCW) and gallic acid concentration accumulated in each run. These data were fitted into a multiple non-linear regression model using DESIGN EXPERT (ver. 5.0), as indicated in Eqs. (1) and (2), and correlation coefficients along with other statistical parameters were estimated. The results of the experimental data and the simulated values are listed in Table 2. Detailed statistical analysis, based on the model is presented in Table 3.

$$\text{Enzyme activity (IU g}^{-1}\text{DCW)} = 760.87 + 140.27A - 115.91B - 70.52C - 135.47A^2 - 105.29B^2 - 257.84C^2 + 44.24AB - 88.17BC + 127.99AC \quad (1)$$

$$\text{Gallic acid concentration (g l}^{-1}) = 27.30 - 10.05A - 1.38B - 0.02C + 3.02A^2 - 3.40B^2 + 2.22C^2 + 0.41AB + 0.88BC - 0.88AC \quad (2)$$

where A, B and C represent the substrate (tannic acid) concentration in g l^{-1}, agitation speed in rpm and pH respectively.

As evident from Eqs. (1) and (2), the initial substrate concentration has a positive interaction with agitation speed with respect to tannase synthesis whereas pH has a negative interactive effect with agitation speed for enzyme production. The substrate concentration and pH interact positively for the enzyme synthesis. The optimum conditions for the production of tannase as evident from Table 2 are: 35g l^{-1} substrate concentration, 300 rpm and pH of 5.0; while for gallic acid accumulation, the optimum parameters are: 45g l^{-1} substrate concentration, 350 rpm and pH 5.0. The effect of pair wise interaction of the parameters is depicted in the three-dimensional graphs (Figs. 1 and 2) when the third parameter is kept constant.

The analysis of variance of regression for enzyme production and gallic acid accumulation has been summarised in Table 3. As evident from Table 3, the observed data for enzyme activity and gallic acid production had a correlation coefficient of 0.982 and 0.986 with their respective calculated models, respectively. The calculated models are also able to explain 98% of the results in the case of enzyme synthesis and 98.5%

Table 3
Analysis of variance of the calculated model

	Intracellular activity (IU g^{-1} DCW)	Gallic acid conc. (g l^{-1})
Regression		
Sum of squares	851 100	931
DF	9	9
Mean squares	94 569	103
F-ratio	41.60	56.40
P	0.0001	0.0001
Residual		
Sum of squares	15 913	12.84
DF	7	7
Mean squares	2273	1.83
F-ratio	–	–
P	–	–
Correlation coefficient (R^2)	0.982	0.986

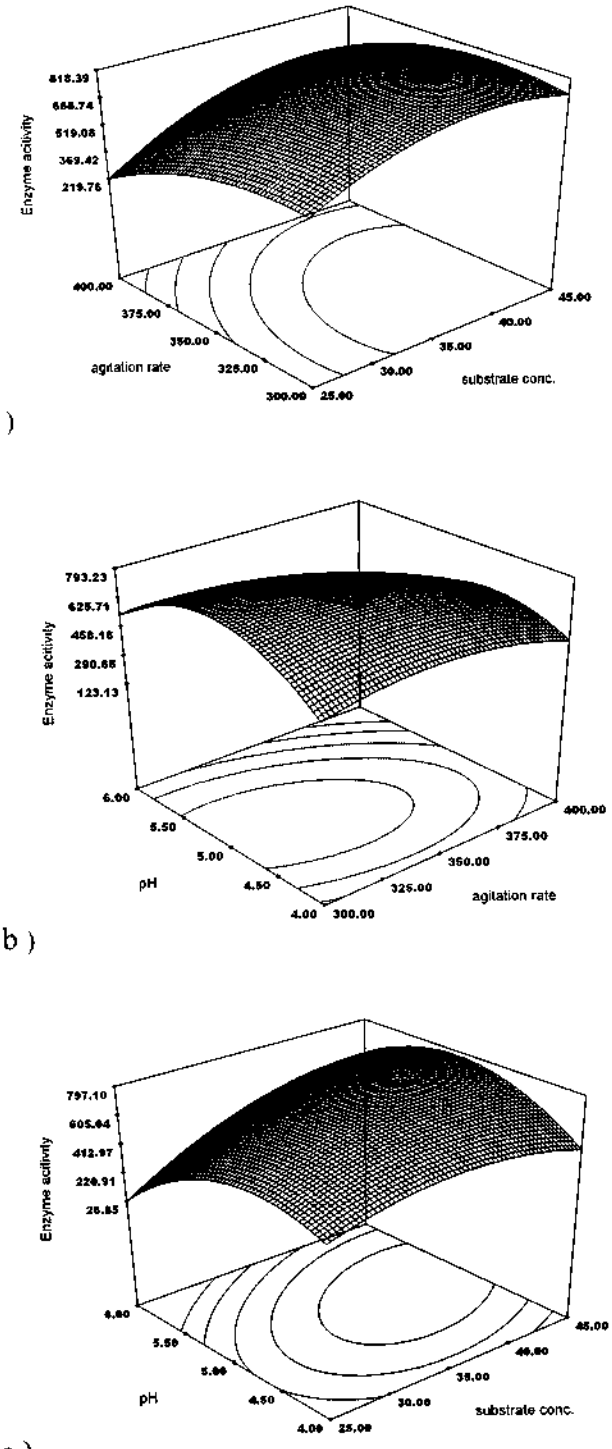

Fig. 1. (a) Effect of substrate concentration and agitation rate on enzyme activity at a pH of 5.0. (b) Effect of agitation rate and pH on enzyme activity on 35 g l^{-1} tannic acid. (c) Effect of substrate concentration and pH on enzyme activity at 350 rpm.

for gallic acid production which shows it to be a good fit for the data. The model also confirms that the selected process parameters significantly influence the enzyme synthesis and gallic acid accumulation.

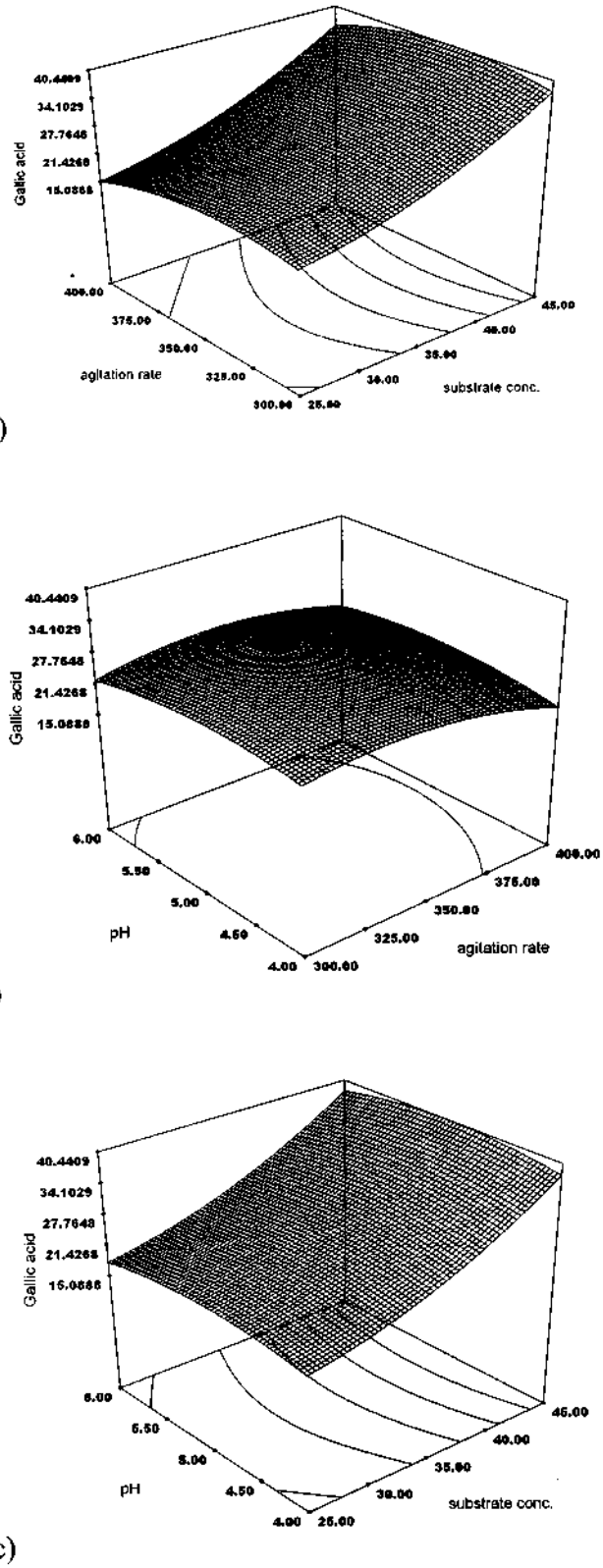

Fig. 2. (a) Effect of substrate concentration and agitation rate on gallic acid production at a pH of 5.0. (b) Effect of agitation rate and pH on gallic acid production at 35 g l^{-1}. (c) Effect of substrate concentration and pH on gallic acid production at 350 rpm.

3.1. Effect of process parameters on enzyme synthesis

The response surface profiles of the calculated model for the enzyme synthesis is shown in Fig. 1a–c. Increases in substrate concentration at higher agitation speed enhanced enzyme synthesis. An increase in agitation rate at lower substrate concentration decreased enzyme activity, while at tannic acid concentration of 45 g l^{-1}, the activity initially increased followed by a decrease. Enzyme synthesis seems to be affected by deposition of gallic acid on the cell surface (at higher substrate concentration and lower agitation speed) and the increased shearing effect on the cells as a result of higher agitation speed. The optimum substrate concentration and agitation speed was therefore a compromise between these two phenomena.

The interaction between pH and agitation speed for enzyme synthesis was a result of the influence of pH on the morphology and thus the shear resistance of the fungal mycelium. It may be noted that lower pH favoured cell growth and enzyme synthesis was maximal at a pH of 5.0 at all three agitation speeds studied. pH and substrate concentration directly influenced cell growth rate and specific enzyme synthesis rate. In general the increase in tannic acid concentration increased enzyme synthesis but at a pH of 4.0, the cell growth rate increased faster than enzyme synthesis, thereby exhibiting a maxima.

3.2. Effect of process parameters on gallic acid accumulation

The response surface profiles of the calculated model for gallic acid production are shown in Fig. 2a–c. Gallic acid accumulation increased with substrate concentration at all agitation speeds and at different pH. The increase in agitation speed and pH at different initial tannic acid concentrations resulted in maxima for gallic acid concentration, although the variation was not too steep. A similar effect was noted for the interaction of agitation speed with pH.

The fermentation profiles of the run conducted under the optimised conditions for tannase synthesis and gallic acid production have been shown in Figs. 3 and 4, respectively. The total tannase synthesis reached a maximum (771 IU g^{-1} DCW and 5497.2 IU l^{-1}) at 60 h of fermentation with a gallic acid concentration of 19 g l^{-1}. However, under optimum conditions for gallic acid production the total tannase activity obtained was 2970.5 IU l^{-1} broth at 72 h and the gallic acid concentration reached a maximum of 40.1 g l^{-1} at 30 h of fermentation run. The experimental profiles provide a good fit to the predicted values from the model and the system can be used either for maximising tannase activity or gallic acid accumulation depending on the application.

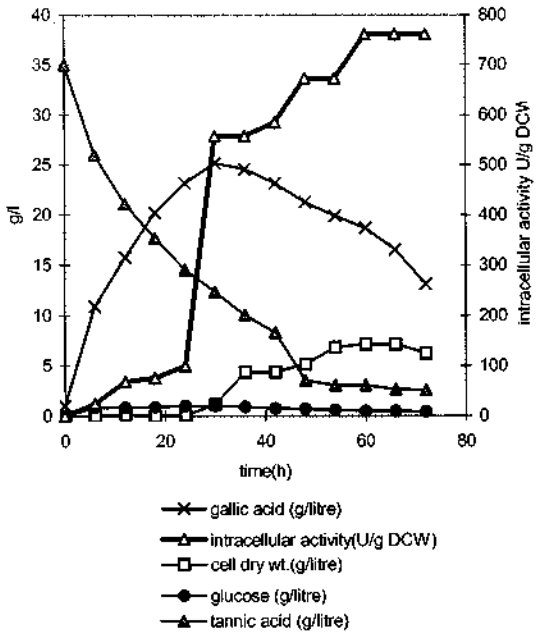

Fig. 3. Fermentation profile of *A. awamori* under optimised conditions for tannase biosynthesis.

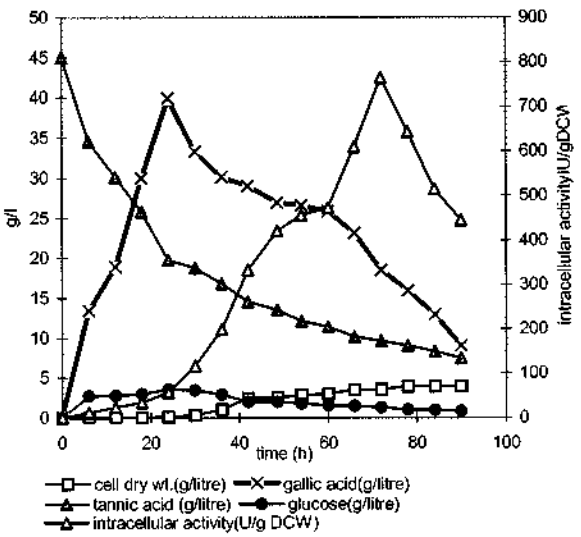

Fig. 4. Fermentation profile of *A. awamori* under optimised conditions for gallic acid production.

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