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Statistical optimization for tannase production by *Aspergillus tubingensis* in solid-state fermentation using tea stalks

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ABSTRACT

Background: A sequential statistical strategy was used to optimize tannase production from *Aspergillus tubingensis* using tea stalks by solid-state fermentation.
Results: First, using a Plackett–Burman design, inoculum size and incubation time (among seven tested variables) were identified as the most significant factors for tannase yield. The effects of significant variables were further evaluated through a single steepest ascent experiment and central composite design with response surface analysis. Under optimal conditions, the experimental value of 84.24 units per gram of dry substrate (U/gds) closely matched the predicted value of 87.26 U/gds.
Conclusions: The result of the statistical approach was 2.09 times higher than the basal medium (40.22 U/gds). The results were fitted onto a second-order polynomial model with a correlation coefficient (R^2) of 0.9340, which implied an adequate credibility of the model.

1. Introduction

Tannin acyl hydrolase, normally known as tannase (EC 3.1.1.20) [1], catalyzes the hydrolysis of tannic acid by specifically breaking its ester and depside bonds to release glucose and gallic acid [2]. Tannase is an extracellular inducible enzyme produced in the presence of tannic acid by fungi, bacteria, and yeasts [3,4]. Recently, interest in tannase production from agricultural residues has remarkably increased because of its low cost and wide application. Tannase has been proven to have important applications in various industries [5], particularly in the food and pharmaceutical sectors, including the elaboration of instantaneous tea [6], production of wine [7], coffee-flavored soft drinks, clarification of beer and fruit juices [8,9], as well as gallic acid [10]. Moreover, tannase can also be used in feed preparation to increase the bioavailability of nutrients by hydrolyzing phenolic anti-nutritional factors [11].

Given the immense potential of tannase, optimizing its production for maximum yield is a worthwhile endeavor. The optimization of fermentation parameters is an important issue in the development of economically feasible bioprocesses [12]. To the best of our knowledge, only a few reports focused on the optimization of tannase production using statistical methods. The use of the statistical approach has recently gained considerable attention for achieving medium optimization and for understanding the interactions among various physicochemical parameters using a minimum number of experiments [13]. Compared with conventional methods, the statistical approach saves more time and cost, thus making it highly practical. Thus, the use of appropriate statistical experiment design tools for tannase optimization is essential. The Plackett–Burman design (PBD) is widely used to determine the independent variables with significant effects on the response [14], whereas response surface methodology (RSM) has long been used to solve process optimization problems in the fields of chemical engineering and agro-biotechnology [15]. These methods can help to evaluate effective factors and simultaneously solve multivariate equations to study the interactions between variables, as well as to determine the optimum combination of variables for a desirable response.

In this work, statistical tools were used to optimize tannase production from *Aspergillus tubingensis* CICC 2651 using tea stalks as an economical solid substrate. The effects of medium components and

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culture conditions were investigated by combining different statistical experimental designs.

2. Materials and methods

2.1. Microorganism

A. tubingensis CICC 2651, obtained from the China Center of Industrial Collection, was used to produce tannase. The culture was maintained as spores on potato dextrose agar (PDA) slants at 4°C and subcultured every 30 d.

2.2. Inoculum preparation

Before inoculation, *A. tubingensis* CICC 2651 was cultivated on PDA slants at 30°C for 4 d. Then, the spores were scraped from the surface of slants with sterile distilled water to prepare a spore suspension (1×108 spores/mL). Thereafter, the spore solution was cultivated synchronously at 4°C for 1 h.

2.3. Production of tannase under solid-state fermentation

Tea stalk powder (5 g) was placed in 250-mL Erlenmeyer flasks and moistened with a mineral salt solution (0.1% $MgSO_4 \cdot 7H_2O$, 0.1% NaCl, and 0.1% KH_2PO_4) at an initial pH of 6.0, and then autoclaved at 121°C for 20 min. After cooling, the samples were inoculated with some spore inoculum (1×108 spores/mL). The contents were mixed thoroughly and incubated under different temperatures for a period of time. Different nutritional and growth conditions were adjusted according to the design suggested by the statistical model.

2.4. Sampling and extraction

The fermented mass was mixed with citrate buffer (0.05 N, pH 5.0) to a volume of 100 mL for each flask and agitated at 180 rpm for 1 h. The slurry was squeezed through a cheesecloth, followed by centrifugation at 10,000 × g for 10 min at 4°C. The supernatant was collected in vials and stored at 4°C for further analysis. The substrate was dried at 80°C to measure biomass.

2.5. Enzyme assay

Tannase activity was determined using the colorimetric method described by Sharma et al. [16]. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 μmol of gallic acid per minute under specific conditions, expressed as U/gds (units per gram of dry substrate).

2.6. Determination of fungal biomass

The biomass in the fermented substrate was determined by estimating the glucosamine present in the cell wall of the culture, and this was expressed in milligrams per gram of dry substrate (mg/gds). A known quantity of fermented matter, ground to powder, was placed in a test tube and hydrolyzed with concentrated sulfuric acid (60%, w/v) for 24 h. The mixture was then diluted and autoclaved at 121°C for 1 h. After cooling, the mixture was percolated, and the filtrate was neutralized to a pH of 7 by adding 1 N NaOH. The glucosamine content was estimated using the method of Sakurai et al. [17].

2.7. Screening of significant factor using PBD

PBD [14] was employed to evaluate the relative importance of various factors relative to tannase yield. In this work, seven physical and nutritional components (independent variables) were studied, along with four "dummy variables" generated by the software. These seven factors and the selection of the range were set according to the result of our pilot study [10]. Each variable was tested at two levels, high (+1) and low (-1). In PBD, a total of 12 experiments were generated and enzyme activities were measured, which are shown in Table 1. From the regression analysis, variables having a *P*-value of <0.05 were considered to have a significant effect on tannase production.

2.8. Steepest ascent method

Generally, the initial estimate of the optimal operating conditions for the system will be far from the actual optimum. In such circumstances, the levels of significant variables are optimized with respect to the responses of a single steepest ascent experiment.

2.9. Response surface methodology

The variables with significant effects on tannase production, according to the results of PBD and steepest ascent method (SAM), were further evaluated using a central composite design (CCD). The variables were studied at five different levels, namely, -1.414 (lowest), -1 (low), 0 (middle), +1 (high), and +1.414 (highest). The full experimental plan is given in Table 3. The quadratic model for predicting the optimal point is expressed according to the following equation:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_{i2} + \sum \beta_{ij} x_i x_j, \quad I = 1,\ 2,\ 3,\ \ldots j \qquad \text{[Equation 1]}$$

where Y is the predicted response, x_i is the coded independent variable, β_0 is the intercept, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the interactive coefficient.

2.10. Data analysis

All experiments were conducted in duplicate and all values were averaged. Data were analyzed using the Microsoft Excel program and Design-Expert 8.04 statistical software package to obtain the standard deviations.

3. Results and discussion

During the course of our earlier studies, the maximum production of tannase by *A. tubingensis* CICC 265 was observed for 120 h, and the value only reached 40.22 U/gds using an initial TS medium. Thus, statistical optimization of the fermentation parameters for maximal enzyme

Table 1
Experimental design and corresponding results of PBD.

Run	Variables							Tannase yield (U/gds)	Biomass (mg/gds)
	A	*B*	*C*	*D*	*E*	*F*	*G*		
1	2	5	50	8	2.5	48	30	29.55 ± 1.36	24.22 ± 1.82
2	5	2	75	8	2.5	48	20	34.72 ± 2.27	20.12 ± 2.61
3	2	5	75	5	2.5	120	30	68.35 ± 2.56	26.43 ± 3.21
4	5	5	75	5	1	48	30	26.45 ± 2.89	35.36 ± 4.05
5	2	2	50	5	1	48	20	2.00 ± 0.24	7.14 ± 1.09
6	5	5	50	8	2.5	120	20	55.69 ± 0.44	24.63 ± 1.36
7	2	2	50	8	1	120	30	52.22 ± 4.20	23.21 ± 3.82
8	5	2	50	5	2.5	48	30	31.32 ± 2.95	22.38 ± 1.59
9	2	2	75	5	2.5	120	20	73.49 ± 2.10	33.24 ± 1.97
10	2	5	75	8	1	48	20	10.59 ± 2.39	15.56 ± 1.26
11	5	5	50	5	1	120	20	56.20 ± 3.84	23.57 ± 5.64
12	5	2	75	8	1	120	30	69.63 ± 0.42	40.75 ± 2.96

A: NH_4Cl, % (w/w); B: α-lactose, % (w/w); C: humidity, % (v/w); D: initial pH; E: inoculum size, mL; F: incubation time, h; G: temperature, °C.

Table 2
Experimental design and corresponding results of SAM.

Run	Inoculum size (mL)	Incubation time (h)	Tannase yield (U/gds)	Biomass (mg/gds)
1	1.75	84	59.31 ± 3.21	26.09 ± 3.54
2	2.55	96	70.01 ± 2.82	31.49 ± 2.08
3	3.35	108	88.29 ± 4.14	34.58 ± 2.59
4	4.15	120	80.19 ± 3.78	28.79 ± 0.46
5	4.95	132	70.93 ± 2.38	27.72 ± 1.17
6	5.75	144	62.64 ± 2.71	27.63 ± 2.34

production was conducted in three stages by employing PBD and SAM, followed by RSM with CCD.

3.1. Evaluation of significant variables by PBD

A 12-run Plackett–Burman experiment (Table 1) was conducted to determine the significant factors affecting enzyme yield. As shown in Table 1, wide variation was observed in tannase activities from 2.0 to 73.49 U/gds, which demonstrated the importance of the optimization of fermentation parameters to achieve higher production. Furthermore, the results were fitted onto the following equation:

$$Y = 42.52 + 3.15A - 1.38B + 4.69C - 0.45D + 6.34E + 20.08F + 3.74G \quad \text{[Equation 2]}$$

where Y is the response of tannase activity and A, B, C, D, E, F, and G represent NH_4Cl, lactose, humidity, pH, inoculum size, incubation time, and temperature, respectively (Table 1).

The adequacy of the model was tested and the variables that were determined to have a statistically significant effect were screened via Fisher's test for analysis of variance (ANOVA). ANOVA showed that the model is significant given its F-value of 17.29 and P-value of 0.0076. We only found a 0.76% chance that a "Model F-value" could occur because of noise. Generally, factors evidencing P-values <0.05 were desirable. The lowest P-value indicates the most significant factor on enzyme production. Furthermore, P-values of E (0.0347) and F (0.0006), as well as their regression coefficients (6.34 and 20.08, respectively), implied that inoculum size and incubation time had a significant positive effect on tannase production. Meanwhile, such factors as NH_4Cl, lactose, moisture level, pH, and temperature, which had P-values >0.05, had no significant effect on tannase activities. Therefore, all other insignificant variables were neglected and only the two significant variables were selected for further optimization using SAM and RSM.

3.2. Determination of proper regions by SAM

The second optimization step was a single steepest ascent experiment to identify the proper regions of the variables by increasing inoculum size and extending culture time regularly. Table 2 lists the directions of changes for the two variables, while the other insignificant variables remained in the 0 level, that is, NH_4Cl was 2.5%, α-lactose was 2.5%, humidity was 62.5%, initial pH was 6.5, and temperature was 25°C. The tannase activities were observed to reach the peak on the third group. Therefore, this condition was selected for further research.

3.3. Optimization of significant factors by RSM

Based on the results of PBD and SAM, crucial factors and their interaction were analyzed and optimized by RSM using a CCD. A total of 13 experiments were performed in duplicate; their corresponding results are shown in Table 3. In all 13 runs, tannase activity varied from 69.83 to 89.02 U/gds. Further, the second-order polynomial [Equation 3] was used to explain the tannase production and interaction among the variables (in coded form) as follows:

$$Y = 83.42 + 4.29X_1 + 3.82X_2 + 2.36X_1X_2 - 3.05X_1^2 - 3.69X_2^2 \quad \text{[Equation 3]}$$

where Y presents the tannase activity and X_1 and X_2 are the inoculum size and incubation time, respectively.

The ANOVA of the response surface model demonstrated that [Equation 3] was highly significant, as evident from the model F-value of 19.82 and P-value of 0.0005 ($P < 0.01$). We only observed a 0.05% chance that a "Model F-Value" could occur because of noise. Values of P (Prob > F) <0.0500 indicate that model terms are significant. In this case, A (0.0006), B (0.0012), A_2 (0.0061), and B_2 (0.0023) were significant model terms.

The R^2 value for the model was 0.9340, which indicated that 93.4% of the observed variations in the tannase yield could be explained by the

Table 3
Experimental design and corresponding responses of CCD.

Run	Inoculum size (X_1)	Incubation time (X_2)	Tannase yield (U/gds)		Biomass (mg/gds)
			Actual	Predicted	
1	-1 (2.35 mL)	-1 (96 h)	69.83 ± 2.90	70.94	31.63 ± 2.11
2	1 (4.35 mL)	-1	75.60 ± 2.20	74.79	30.02 ± 2.51
3	-1	1 (120 h)	73.80 ± 3.09	73.87	32.23 ± 1.82
4	1	1	89.02 ± 2.57	87.16	44.11 ± 2.48
5	-1.414 (1.94 mL)	0 (108 h)	72.27 ± 2.16	71.27	33.66 ± 3.80
6	1.414 (4.76 mL)	0	81.67 ± 3.86	83.39	40.56 ± 1.82
7	0 (3.35 mL)	-1.414 (91 h)	71.02 ± 2.74	70.64	39.60 ± 3.82
8	0	1.414 (125 h)	80.35 ± 3.39	81.46	41.99 ± 4.50
9	0	0	83.88 ± 3.02	83.42	34.58 ± 2.92
10	0	0	80.95 ± 4.97	83.42	35.64 ± 2.82
11	0	0	84.05 ± 2.56	83.42	42.82 ± 4.15
12	0	0	81.69 ± 3.86	83.42	39.27 ± 1.99
13	0	0	86.55 ± 5.11	83.42	38.49 ± 3.39

ANOVA of CCD

Source	Model	X_1X_1	X_2	X_1X_2	X_1^2	X_2^2	Residual	Lack of fit	Pure error	Cor total
Sum of squares	427.24	146.90	116.99	22.27	64.53	94.53	30.18	10.67	19.50	457.41
DF	5	1	1	1	1	1	7	3	4	12
Mean square	85.45	146.90	116.99	22.27	64.53	94.53	4.31	3.56	4.88	–
F-value	19.82	34.08	27.14	5.17	14.97	21.93	–	0.73	–	–
P-value	0.0005	0.0006	0.0012	0.0573	0.0061	0.0023	–	0.5857	–	–

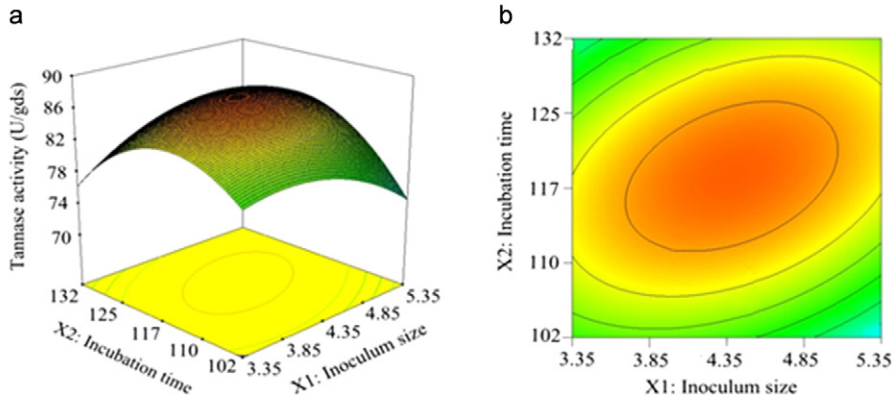

Fig. 1. (a) Response surface curves and (b) contour plots of interaction effect between inoculum size and incubation time on tannase activity.

model. This result also suggested that the prediction of experimental data was satisfactory. The lack-of-fit *F*-value of 0.73 implied that the lack of fit was not significant relative to the pure error. The "Pred R^2" value of 0.7674 was in reasonable agreement with the "Adj R^2" value of 0.8869. "Adeq Precision" measures the signal to noise ratio; a value >4 is generally desirable. In the present study, the ratio of 11.710 indicated that an adequate signal was detected.

3.4. Effect of significant factors on tannase production

To investigate the interaction and to visualize the effects of the two factors on tannase activity better, a graphical representation of RSM (a) and contour plots (b) are presented in Fig. 1. The circular shape of the curve indicates that no interaction occurred, whereas the elliptical shape indicated good variation of the two variables [18]. The response surface obtained in this study was convex in nature, which suggested that the optimum conditions were well defined. Meanwhile, the shapes of the contour plots indicated the nature and extent of the interactions. Fig. 1a indicated that the interaction between the inoculum size and incubation time was not significant. Furthermore, increasing the inoculum size and prolonging the incubation time have a positive effect on the response until a certain optimum value, whereas extremely high values of these variables will inhibit tannase production (Fig. 1b). Similar results were reported by Sabu et al. [19], when *Aspergillus niger* ATCC 16620 was used to ferment palm kernel cake (Fig. 1).

3.5. Experimental validation of the model

The response surface model predicted a maximum tannase content of 87.26 U/gds when the regression equation was solved by the numerical optimization function in the Design-Expert software. The optimum levels of significant variables were an inoculum size of 4.35 mL after 118 h of incubation. To confirm the predicted model, four replicate experiments were performed and the tannase activities of each were determined. Finally, the average maximum yield reached 84.24 U/gds, which was comparable to the predicted value (87.26 U/gds) and was 1.09-fold higher than that of the basal medium (40.22 U/gds). Consequently, the proposed model was considered to be accurate and reliable for predicting the production of tannase from *A. tubingensis*.

4. Conclusions

Tannase production from *A. tubingensis* with tea stalks as substrates was optimized through a series of statistical methodologies. Among the variables studied, the inoculum size and incubation time were found to affect tannase activity significantly. The optimal conditions of variables for maximum tannase production were an inoculum size of 4.35 mL and 118 h of incubation. The actual tannase activity of 84.24 U/gds was consistent with the predicted value of 87.26 U/gds. The optimization process doubled the tannase yield (from 40.22 U/gds to 84.24 U/gds) as compared with the basal medium.

Conflict of interest

None.

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