ISSN Print: 2394-7500
ISSN Online: 2394-5869
Impact Factor: 5.2
IJAR2016; 2(5): 1073-1077
www.allresearchjournal.com
Received: 15-03-2016
Accepted: 16-04-2016

Neethu RS
Microbiology Division, Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, Thiruvananthapuram – 695 562, Kerala, India

Pradeep NS
Microbiology Division, Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, Thiruvananthapuram – 695 562, Kerala, India

Correspondence
Neethu RS
Microbiology Division, Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, Thiruvananthapuram – 695 562, Kerala, India

Rapid screening of process parameters for tannase production in solid state fermentation by *Aspergillus japonicus Asp* TBG22 (d) using Plackett-Burman statistical design

Neethu RS and Pradeep NS

Abstract
The culture components influencing the tannase production by *Aspergillus japonicus Asp* TBG22 (d) using wheat bran as solid substrate were identified using a two-level fractional Plackett–Burman design. Among eleven variables screened, four comprising pH, temperature, inoculum concentration and moisture content positively influence tannase production with maximum tannase production of 3.17U/gds. From the study it was clear that statistical approach allows rapid identification of culture parameters which are significant for tannase production under solid state fermentation. These parameters can further be explored for the production of optimum culture conditions for tannase production.

Keywords: Tannase, *Aspergillus japonicus*, Plackett–Burman design, screening

1. Introduction
Enzymes of microbial origin are having important applications in many areas of bio-based industries. Tannase (tannin acyl hydrolyse, E.C.3.1.1.20) is an inducible enzyme of high commercial value involved in the hydrolysis of tannins.Tannase catalyses the hydrolysis of ester and depside bonds of hydrolysable tannins (tannicacid, methygallate, ethylgallate, n-propyl gallate and isoamyl gallate) releasing glucose and gallicacid [1].
Tannase has wide applications in the food, feed, pharmaceutical and chemical industries [2, 3]. It is used to hydrolyze tea cream during tea processing [4]. The enzymatic treatment of tea beverage improves its quality, taste and antioxidant activity [5-7]. The hydrolysis product, gallic acid is required for the synthesis of trimethoprim, an antifolic antibacterial drug [8]; propyl gallate, a potent antioxidant in fats and oils; pyrogallol and as a photosensitive resin in semiconductor production [3]. Other applications include the manufacture of acorn wine [2]; clarification of beer, fruit juices and coffee-flavored soft drinks [9]; to reduce the antinutritional effects of poultry and animal feed; in food detanification and industrial effluent treatment [10]. Considering its immense industrial applications the search for better sources of tannase is still in progress. Filamentous fungi especially *Aspergillus* spp. are the major producers of tannase with wide range industrial applications [11-14].
Large scale production of tannase with low production cost is necessary to meet its growing demand now-a-days. Using energy-rich inexpensive resources like agricultural waste as substrate for the production of industrially important products are gaining importance [15]. Most commonly used media formulation approach is the one factor at a time method, i.e. by changing one medium component and keeping the others at a constant level [16]. The disadvantage of this is that the interaction effect between the parameters which influence the overall process for production is ignored [17]. Considering this, the present study uses Plackett–Burman statistical approach to determine the influence of various culture parameters on tannase production by *Aspergillus japonicus Asp* TBG22 (d) using wheat bran as substrate under solid state fermentation. The advantage of this design over the one factor at a time method is the reduced requirement of raw materials, time and man power due to the less number of experimental runs. Another advantage of the design is that all the factor effects can be estimated independently.

2. Materials and Methods

All the chemicals and bio-chemicals used in this study are of analytical grade. Wheat bran was obtained from the local market.

2.1 Microorganism

The *Aspergillus japonicus Asp* TBG22 (d) used in this study was isolated from the mangrove soil of Kannur district, Kerala, India. The fungal strain was maintained on Czapek Dox minimal media agar slants supplemented with 1% (w/v) tannic acid as the sole carbon source.

2.2 Preparation of spore suspension: To the ten day old fungal culture 10ml of sterile distilled water containing 0.1% (v/v) Tween 80 was added. The spores were scraped into it using a sterile inoculation loop and vortexed and counted using a Neubaur counting chamber. The suspension containing 1×10^7 spores/ml was used as inoculum.

2.3 Production of Tannase by Solid State Fermentation

Five grams of wheat bran was taken in of 250 ml Erlenmeyer flasks and moistened with 5 ml of mineral salt solution containing 0.5% w/v of NH_4Cl, 0.1% w/v $MgSO_4.7H_2O$, and 0.1% w/v $NaNO_3$, pH 5.0. The moisture content was adjusted to 50% with distilled water. The flasks were then sterilized at 121 °C for 15 min at 15lbs pressure and inoculated with 1 ml of spore suspension ($1x10^7$spores/ml). The contents were mixed and incubated at 30 °C for 96h. After incubation 50ml of sterile distilled water containing 0.01% Tween 80 was added to the flasks and kept in a rotary shaker at 130 rpm for 1h. The contents were then filtered using Whatman No.1 filter paper and the filtrate was regarded as crude enzyme extract and preserved at 4 °C for further analysis.

2.4 Tannase activity assay

Tannase activity was determined using the colorimetric method described by Sharma *et al.* [18]. This method is based on the formation of a pink chromogen after reaction of gallic acid (released by the esterase activity of tannase) and rhodanine (2-thio-4-ketothiazolidine) under alkaline conditions. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1µmol of gallic acid per minute under specific conditions, expressed as U/gds (units per gram of dry substrate).

2.5 Screening of significant variables for tannase production using Plackett–Burman design (PBD)

Design-Expert software (version 10, Stat-Ease Corporation, USA) was used for identifying significant parameters for tannase production. The number of experiments needed to identify the significant variables which influence tannase production can be reduced while using this design. In the present study, effect of 11 physical and nutritional parameters such as incubation time, pH, temperature, inoculum concentration, moisture content, tannic acid concentration, sodium nitrate, ammonium chloride, urea, magnesium sulphate and peptone were investigated as variables using PB design to identify the components that significantly affected tannase production. In this design each selected variable was tested at two levels, low (−1) and high (+1) (Table 1). Low and high experimental levels for the 11 variables were decided based on certain preliminary studies conducted earlier under solid state fermentation.

Table 1: Variables representing culture components used in Plackett–Burman design

Variable code	Culture components	Units	Low (−1) value	High (−1) Value
A	Incubation Time	H	72	120
B	pH	-	3	6
C	Incubation Temperature	°C	25	35
D	Inoculum concentation	Spores/ml	1×10^5	1×10^7
E	Moisture Content	(%)	50	65
F	Tannic acid Concentation	(%)	1	5
G	Sodium nitrate	(%)	0.5	3
H	Ammonium chloride	(%)	0.5	2.5
J	Urea	(%)	0.5	2.5
K	Magnesium sulphate	(%)	0.1	1
L	Peptone	(%)	0.1	1

In Plackett-Burman design, a total of 12 experiments were generated for 11 variables and enzyme activities were measured as shown in Table 2. From the regression analysis, variables having a P-value of <0.05 were considered to have a significant effect on tannase production.

Based on results obtained from Plackett-Burman design the fitted first order model is

$$Y = \beta o + {}^{k}\Sigma_{i=1}\, \beta i Xi$$

where Y-Predicted response, βo, βi -Constant coefficients and Xi-Coded independent factors. The model is used to screen and evaluate the important factors that influence the tannase production. Effect of each variable on production was determined from their respective E-values [19].

$$E = \frac{\text{(Total response at high level)} - \text{(Total response at low level)}}{\text{(Number of trials)}}$$

Table 2: Experimental design and results obtained in Plackett–Burman design

Run	A	B	C	D	E	F	G	H	J	K	L	Tannase activity(U/gds)
1	(+1)	(+1)	(−1)	(−1)	(−1)	(+1)	(−1)	(+1)	(+1)	(−1)	(+1)	1.07
2	(+1)	(−1)	(−1)	(−1)	(+1)	(−1)	(+1)	(+1)	(−1)	(+1)	(+1)	0.58
3	(+1)	(−1)	(+1)	(+1)	(−1)	(+1)	(+1)	(+1)	(−1)	(−1)	(−1)	1.87
4	(+1)	(+1)	(−1)	(+1)	(+1)	(+1)	(−1)	(−1)	(−1)	(+1)	(−1)	2.58
5	(−1)	(+1)	(−1)	(+1)	(+1)	(−1)	(+1)	(+1)	(+1)	(−1)	(−1)	2.11
6	(−1)	(+1)	(+1)	(−1)	(+1)	(+1)	(+1)	(−1)	(−1)	(−1)	(+1)	2.97
7	(−1)	(−1)	(−1)	(−1)	(−1)	(−1)	(−1)	(−1)	(−1)	(−1)	(−1)	0.82
8	(−1)	(−1)	(+1)	(−1)	(+1)	(+1)	(−1)	(+1)	(+1)	(+1)	(−1)	0.56
9	(−1)	(+1)	(+1)	(+1)	(−1)	(−1)	(−1)	(+1)	(−1)	(+1)	(+1)	1.77
10	(+1)	(−1)	(+1)	(+1)	(+1)	(−1)	(−1)	(−1)	(+1)	(−1)	(+1)	3.17
11	(+1)	(+1)	(+1)	(−1)	(−1)	(−1)	(+1)	(−1)	(+1)	(+1)	(−1)	1.79
12	(−1)	(−1)	(−1)	(+1)	(−1)	(+1)	(+1)	(−1)	(+1)	(+1)	(+1)	1.37

A: Incubation Time (h); **B**: pH; **C**: Temperature (°C); **D**: Inoculum Concentration (Spores/ml); **E**: Moisture content (% v/w); **F**: Tannic acid concentration (% w/w); **G**: Sodium nitrate (% w/w); **H**: Ammonium chloride (% w/w); **J**: Urea (% w/w); **K**: Magnesium sulphate (% w/w); **L**: Peptone (% w/w)

3. Results and Discussion

Production of extracellular tannase under solid state fermentation using different substrates was carried out by several researchers due to its advantages over submerged fermentation. The present study uses wheat bran as solid substrate to identify significant factors that contribute to tannase production by *A. japonicus Asp* TBG22 (d).

Screening for significant culture parameters using PBD

Plackett-Burman screening experimental design was employed to determine the influence of independent variables on the production of extracellular tannase by *A. japonicus*. The design offers an effective screening procedure and helps to determine the significance of several variables in one experiment, which saves time and maintains convincing information on each component. Variation in tannase activities from 0.56 to 3.17U/gds demonstrates the importance of optimizing fermentation parameters to achieve higher production. The results obtained fits onto the following equation:

$$Y = 1.72 + 0.12A + 0.33B + 0.30C + 0.42D + 0.27E - 0.40H - 0.28K + 0.100L$$

where Y is the response of tannase activity and A, B, C, D, E, H, K and L represents incubation time, pH, temperature, inoculum concentration, moisture content, ammonium chloride, magnesium sulphate, peptone respectively. The Model F-value of 46.68 implies the model is significant. There is only a 0.46% chance that an F-value this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant (Table 3). In this case B, C, D, E, H, K are significant model terms. Therefore, all other insignificant variables are to be neglected and only the significant variables were selected for further optimization of tannase production.

Table 3: ANOVA for Plackett-Burman Design

Source	Squares	df	MeanSquare	f-Value	p-valueProb > F
Model	8.52	8	1.06	46.68	0.0046
A-Incubation Time	0.18	1	0.18	7.79	0.0684
B-pH	1.28	1	1.28	56.14	0.0049
C-Temperature	1.08	1	1.08	47.35	0.0063
D-Inoculum concentration	2.15	1	2.15	94.28	0.0023
E-Moisture Content	0.9	1	0.9	39.3	0.0082
H-Ammonium chloride	1.87	1	1.87	82.08	0.0028
K-Magnesium sulphate	0.94	1	0.94	41.24	0.0077
L-Peptone	0.12	1	0.12	5.26	0.1056
Residual	0.068	3	0.023		
Cor Total	8.59	11			

The effect of individual variables studied by the PBD was represented in the Pareto chart (Figure 1). The main effect was estimated based on the difference between the sum of responses obtained at the high level (+1) and at the low level (−1) of each component. From the graph it is clear that four components *viz*, pH, temperature, inoculum concentration and moisture content significantly enhanced tannase production while three components *viz*, ammonium chloride, urea and magnesium sulphate have a negative influence on tannase production. The other four components like incubation time, concentration of tannic acid, sodium nitrate and peptone has a slight influence on tannase yield.

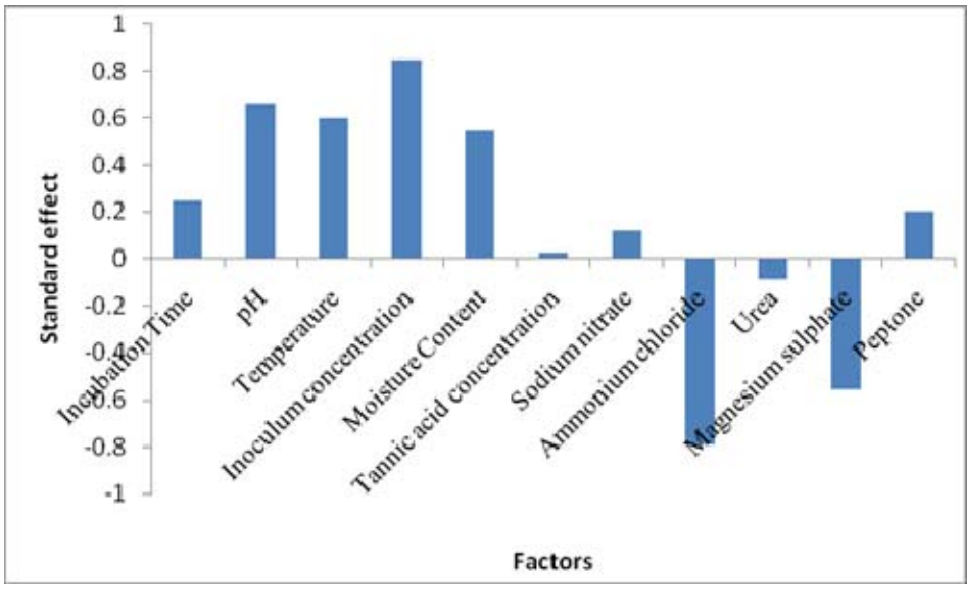

Fig 1: Pareto chart showing the effects of individual variables on tannase productionby *A. japonicus* based on PB experimental results.

Incubation time showed no significant effect on tannase production. Therefore this variable was fixed at the lowest level (72h). Souza *et al*. [20] reported a similar negative effect of incubation time on tannase production by *Aspergillus* sp. gm4 in solid state fermentation and they fixed the variable at lowest level (2 days). The results of the study depicts that tannic acid has no significant role in tannase production under SSF. Tannic acid act as an inducer for tannase production, but higher concentration do not promote an equivalent increase in enzyme synthesis [21]. Tannic acid at high concentration may produce complexes with membrane protein of the organism, thereby growth and enzyme production is inhibited [22]. Similarly ammonium chloride and magnesium sulphate showed a negative impact on tannase production. This suggests that these two parameters are required only in lower levels for tannase production.

4. Conclusion

A significant increase in the tannase production by a mangrove *A. japonicus* isolate in SSF using wheat bran was attained by manipulating the culture parameters using a two-stage statistical design. The Plackett-Burman design helped to identify the key factors that significantly affected the tannase production. The result confirmed that pH, temperature, inoculum concentration and moisture content were the most significant components for tannase enzyme production. This will serve as an efficient guide in developing an effective culture condition for enhanced tannase production using wheat bran as substrate.

5. Acknowledgement

The authors are thankful to Director, JNTBGRI for facilities and Kerala State Council for Science Technology & Environment (KSCSTE) for the financial support.

6. Reference

1. Barthomeuf C, Regerat F, Pourrat H. Production, purification and characterization of a tannase from *Aspergillus niger* LCF 8. Journal of Fermentation and Bioengineering. 1994; 77 (3):320-323.
2. Mondal KC, Pati BR. Studies on the extracellular tannase from newly isolated *Bacillus licheniformis* KBR 6. Journal of Basic Microbiology. 2000; 40:223-232.
3. Rodríguez-Durán LV, Valdivia-Urdiales B, Contreras-Esquivel JC, Rodríguez-Herrera R, Aguilar CN. Novel Strategies for Upstream and Downstream Processing of Tannin Acyl Hydrolase. Enzyme Research. 2011; 823-619.
4. Su E, Xia T, Gao L, Dai Q, Zhang Z. Immobilization and characterization of tannase and its haze-removing. Food Science and Technology International. 2009; 15(6):545-552.
5. Lu MJ, Chen C. Enzymatic tannase treatment of green tea increases in vitro inhibitory activity against N-nitrosation of dimethylamine. Process Biochemistry. 2007; 42:1285-1290.
6. Lu MJ, Chen C. Enzymatic modification by tannase increases the antioxidant activity of green tea.Food Research International. 2008; 41(2):130-137.
7. Lu MJ, Chu SC, Yan L, Chen C. Effect of tannase treatment on protein-tannin aggregation and sensory attributes of green tea infusion.LWT - Food Science and Technology. 2009; 42(1):338-342.
8. Sittig M. Trimethoprim. In Pharmaceutical Manufacturing Encyclopedia, New Jersey: Noyes Publication, 1988.
9. Aguilar CN, Augur C, Favela-Torres E, Viniegra-González G. Induction and repression patterns of fungal tannase in solid-state and submerged cultures. Process Biochemistry. 2001; 6(6):565-570.
10. Garcý´a-Conesa MT, Ostergaard P, Kauppinen S, Williamson G. Hydrolysis of diethyl diferulates by a tannase from *Aspergillus oryzae*. Carbohydrate Polymers. 2001; 5(2):319-324.
11. Srivastava A, Kar R. Biosynthesis of tannase and hydrolysis of tannins to gallic acid by *Aspergillus awamori* — Optimization of process parameters. Process Biochemistry. 2000; 36:39-44.
12. Seth M, Chand S. Characterization and Application of Tannase Produced By *Aspergillus niger* ITCC 6514.07 on Pomegranate Rind. Brazilian Journal of Microbiology. 2009; 40:782-789.
13. Paranthaman R, Vidyalakshmi R, Kumaravel S. Production, Purification and Characterization of Tannin

Acyl Hydrolase enzyme from *Aspergillus oryzae* by Submerged Fermentation. Electronic Journal of Environmental, Agricultural and Food chemistry. 2011; 10(7).
14. Lal D, Gardner JJ. Production, Characterization and Purification of Tannase from *Aspergillus niger*. European Journal of Experimental Biology. 2012; 2(5):1430-1438.
15. Ramachandran S, Singh S, Larroche C, Soccol CR, Pandey A. Oil cakes and their biotechnological applications: a review. Bioresource Technology. 2007; 98:2000-2009.
16. Prajapati VS, Trivedi UB, Patel KC. Optimization of glucoamylase production by *Colletotrichum* sp. KCP1 using statistical methodology. Food Science and Biotechnology. 2013; 22:31-38.
17. Silva CJSM, Roberto IC. Optimization of xylitol production by *Candida guilliermondii* FTI 20037 using response surface methodology. Process Biochemistry. 2001; 36:119-124.
18. Sharma S, Bhat TK, Dawra RK. A spectrophotometric method for assay of tannase using rhodanine Analytical Biochemistry. 2000; 279(1):85-89.
19. Gupta N, Mehra G, Gupta R. A glycerol inducible thermostable lipase from *Bacillus* sp: Medium optimization by a Plackett- Burman design and by response surface methodology. Canadian Journal of Microbiology. 2004; 50:361-368.
20. Patrícia Nirlane da Costa Souza, Natália da Costa Maia, Luís Henrique Souza Guimarães, Mário Lúcio Vilela de Resende and Patrícia Gomes Cardoso. Optimization of culture conditions for tannase production by *Aspergillus* sp. gm4 in solid state fermentation. Acta Scientiarum. Biological Science. 2015; 37(1):23-30.
21. Battestin V, Macedo GA. Tannase production by *Paecilomyces variotii*. Bioresource Technology. 2007; 98:1832-1837.
22. Banerjee D, Mondal KC, Pati BR. Tannase production by *Aspergillus aculeatus* DBF9 through solid-state fermentation. Acta Microbiologica Et Immunologica Hungarica. 2007; 54:159-166.