Research Article

Application of Plackett-Burman Design for Screening the Media Components for Tannase Production from Redgram Husk using Submerged Fermentation

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ABSTRACT

In the present study, effect of medium components on tannase enzyme production from redgram husk using *Aspergillus foetidus* (MTCC 3557) was studied. The substrate employed in this research was collected from agriculture waste which is abundantly available in southern part of India. Placket-Burman Design (PBD) was used to evaluate the significant parameters that have large effect on the fermentation and with this experimental design a successful results were obtained. Plackett–Burman experimental design assumed that there were no interactions between the different variables in the range under consideration. A linear approach was considered to be sufficient for screening. Plackett–Burman experimental design was a fractional factorial design and the main effects of such a design may be simply calculated as the difference between the average of measurements made at the high level (+1) of the factor and the average of measurements at the low level (–1). The twelve variables namely concentrations of eleven nutrients (ammonium nitrate, ammonium chloride, sodium nitrate, ammonium sulphate, magnesium sulphate, ferrous sulphate, potassium chloride, potassium di-hydrogen phosphate, yeast extract, urea and peptone) and inducer tannic acid were screened in twenty experimental trials. From standard Plackett-Burman data analysis it was conformed that, concentration of tannic acid, concentration of potassium di-hydrogen phosphate, concentration of magnesium sulphate and concentration of ferrous sulphate were found to be the most significant for tannase enzyme production.

Keywords: *Aspergillus foetidus*, media components, plackett-burman design, redgram husk, submerged fermentation, tannase.

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INTRODUCTION

Tannin acyl hydrolase, also known as Tannase (E.C.3.1.1.20) is an inducible enzyme that catalyses the breakdown of ester linkages in hydrolysable tannins resulting in the production of gallic acid and glucose [1]. It is used widely in the manufacture of instant tea, acorn wine and gallic acid [2, 3]. Gallic acid is an important substrate for the synthesis of propyl gallate in the food industry and trimethoprim in the pharmaceutical industry [4]. Tannase also has potential applications in the clarification of beer and fruit juices, manufacture of coffee flavoured soft drinks [5]. In addition, tannase has been extensively used for the treatment of leather industry waste water containing tannic acid [6]. Although it has several industrial applications, only a few filamentous fungi, chiefly *Aspergillus* and *Penicillium* have been reported to produce tannase [7, 8].

In Industrial level tannase is mainly produced by *Aspergillus* species under submerged fermentation (SmF). The SmF is widely used for enzyme production because it offers many advantages like uniform process conditions namely concentration, Temperature, pH, aeration and agitation in the bioreactors [1]. Improvement in productivity of tannases by *Aspergillus foetidus* (MTCC 3557) is done by manipulating the nutritional and physical parameters in submerged fermentation.

Therefore the development of an economical production medium requires

selection of carbon, nitrogen, phosphorus, potassium and trace element sources. Medium optimization by single dimensional search is laborious and time consuming, especially for a large number of variables and it does not ensure desirable conditions. Plackett-Burman design is widely used in screening experiment as the number of experiment run required are very few, leading to saving of time, chemicals and man power [9].

The aim of this research was to apply the Plackett-Burman design for screening the media components to enhance the tannase enzyme production. No data on tannase production by *Aspergillus foetidus* (MTCC 3557) in submerged fermentation for optimization of medium nutrients has been published yet. In addition, the utilization of agro-industrial wastes, on one hand, provides alternative substrates and, on the other hand helps to solve pollution problems by eliminating the need for disposal of the wastes. Redgram husk is a cheap substrate which is abundantly available in southern part of India. Hence, it was selected as substrate for the production of tannase.

MATERIALS AND METHODS

Chemicals

Chemicals used in the experiments were purchased from Hi-Media, Mumbai and were of the highest purity.

Microorganism

Fungal strain used in this work is well preserved in the laboratory. Fungal strain *Aspergillus foetidus* (MTCC 3557) was obtained from IMTECH, Chandigarh, 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. The fungal strain was sub cultured periodically, grown at 30ºC for 7 days. The well grown culture was stored at 4°C in a refrigerator and used for further sub culturing.

Submerged fermentation (SmF)

100 ml of Czapek Dox minimal medium in 250 ml Erlenmeyer flask was inoculated with the *Aspergillus foetidus* spore suspension. The composition of the Czapek Dox medium used for tannase enzyme production was tannic acid 10 g/L, sodium nitrate - 6 g/L, potassium dihydrogen orthophosphate- 1.52 g/L, magnesium sulphate-0.52 g/L, potassium chloride- 0.52 g/L, ferrous sulphate- 0.01 g/L and zinc sulphate- 0.01 g/L. 3 gm of substrate was added separately to the Czapek Dox medium for studying their effect on the enzyme production. The cultures were grown at 30°C, 140 rpm for six days in an incubator shaker. The samples were withdrawn at regular intervals of 24 h. The biomass was separated by the filtration through Whatman No.1 filter paper. The cell free culture broth was assayed for the tannase activity.

Tannase Assay

Tannase activity was estimated by the method of Mondal and Pati [10]. 0.1 ml of enzyme solution was incubated with 0.3 ml of 1.0% (w/v) tannic acid and add 5 ml of 0.2 M acetate buffer (pH 5.0) at 40 °C for 10 min and then the enzyme production was stopped by cooling to 0°C by the addition of 2 ml Bovine Serum Albumin (BSA) (1 mg/ml), which precipitates the remaining tannic acid simultaneously A control without the enzyme was incubated and the samples were analyzed. The tubes were then centrifuged (5,000 x g, 10 min) and the precipitate was dissolved in 2 ml of Sodium Dodecyl Sulphate (SDS) – triethanolamine (1% w/v SDS in 5% v/v triethanolamine) solution and the absorbency was measured at 550 nm after addition of 1 ml of $FeCl_3$ (0.01 M $FeCl_3$ in 0.01N HCl).

One Unit of the tannase enzyme is defined as the amount of enzyme required to hydrolyse 1µmole of ester linkage of tannic acid in 1 min at specific condition.

Experimental Design

Plackett-Burman Design

The purpose of this optimization step is to identify which ingredients of the medium have significant effect on tannase enzyme production. The Plackett-Burman statistical experimental design is very useful in screening the most important factors. This design does not consider the interaction effects between the variables and is used to screen the important variables affecting tannase production [11]. It can represent by first-order polynomial equation:

$$Y= \beta_0+ \sum\beta_i x_i \quad \text{..... (1)}$$

Where Y represents the response, β_0 is the model coefficient, β_i is the linear coefficient,

x_i is the level of the independent variable. This model does not describe interaction among factors and it is used to screen and evaluate the important factors that influence the response. In the present work, twelve variables (**Table 1**) were screened in twenty experimental runs (**Table 2**) and the insignificant variables were eliminated to obtain a smaller, more manageable set of factors. The low level (– 1) and high level (+ 1) of each factor are listed in (**Table 1**). The statistical software package 'Design Expert 7.1.5', was used for analyzing the experimental data.

RESULTS AND DISCUSSION

Screening of medium nutrients by Plackett-Burman:

The effect of twelve variables namely concentrations of eleven nutrients (ammonium nitrate, ammonium chloride, sodium nitrate, ammonium sulphate, magnesium sulphate, ferrous sulphate, potassium chloride, potassium di-hydrogen phosphate, yeast extract, urea and peptone) and tannic acid as inducer on tannase enzyme production by submerged fermentation by *A. foetidus* were analysed. (**Table 1**) shows the Plackett–Burman experimental design of experiments and the results obtained from the experiments which are generated by the MINITAB 15 software. From the table, it was observed that the variation in tannase activity was 36.76 – 120.40 U/ml. The two values of each variable {maximum (+) and minimum (-)} were chosen such that the difference between the two values (+ and -) is large enough to ensure that it includes the peak area for the maximum enzyme production. The maximum and minimum values of twelve variables are given in (**Table 2**).

Table 1: The effect of Variables on Tannase Enzyme Production by *A. foetidus* using Plackett-Burman Experimental Design

Run No.	A	B	C	D	E	F	G	H	I	J	K	L	Tannase Activity (U/ml) Experimental	Predicted
1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	44.35	33.35
2	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	89.09	86.97
3	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	87.98	85.79
4	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	48.49	56.01
5	1	-1	1	-1	1	1	1	1	-1	-1	1	1	78.74	88.91
6	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	88.11	80.05
7	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	70.53	77.26
8	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	49.96	55.37
9	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	120.40	108.95
10	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	105.80	103.64
11	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	100.43	103.92
12	-1	1	1	1	1	-1	-1	1	1	-1	1	1	64.70	64.94
13	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	87.40	81.16
14	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	99.65	98.06
15	1	-1	1	1	1	1	-1	-1	1	1	-1	1	47.56	38.96
16	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	77.65	85.43
17	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	53.20	55.71
18	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	89.60	91.38
19	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	69.34	70.24
20	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	36.76	43.67

Table 2: Statistical analysis of medium optimization using Plackett-Burman design for tannase production using *A. foetidus*

Nutrient Code	Nutrient	Minimum Value	Maximum Value	t-value	p-value
A	Tannic acid	1	5	-3.40	0.011
B	Yeast extract	0.1	1	-1.19	0.273
C	Magnesium sulphate	0.1	1	-3.31	0.013
D	Ferrous sulphate	0.1	1	-3.85	0.006
E	Ammonium nitrate	0.1	1	0.47	0.655
F	Ammonium chloride	0.1	1	1.89	0.101
G	Urea	0.1	1	1.08	0.317
H	Potassium chloride	0.1	1	-0.88	0.407
I	Sodium nitrate	0.1	1	-0.43	0.678
J	Potassium di-hydrogen phosphate	0.1	1	-3.96	0.005
K	Ammonium sulphate	0.1	1	1.75	0.124
L	Peptone	0.1	1	-0.11	0.918

On analysis of regression coefficient (t-value) of twelve medium components in (**Table 2**), ammonium sulphate , ammonium nitrate, ammonium chloride, urea, showed positive effect on tannase production, whereas the remaining components namely, yeast extract, potassium chloride, tannic acid, ferrous sulphate, peptone, sodium nitrate, potassium di-hydrogen phosphate and magnesium sulphate showed negative effect on tannase production. There is a close agreement between the experimental values of tannase production and theoretical values predicted by PB design model equation for all the medium components.

The maximum tannase activity was obtained with the medium having the following composition (run No.9) namely tannic acid -1%, yeast extract- 1%, magnesium sulphate -1%, ferrous sulphate- 0.1%, ammonium nitrate - 1%, ammonium chloride- 1%, urea- 0.1%, potassium chloride- 0.1%, Sodium nitrate- 0.1%, potassium di-hydrogen phosphate- 0.1%, ammonium sulphate-1% and peptone- 0.1%. The minimum tannase activity was obtained with the medium having the following composition (run No.20) namely tannic acid - 5%, yeast extract- 1%, magnesium sulphate -0.1%, ferrous sulphate- 1%, ammonium nitrate - 1%, ammonium chloride- 0.1%, urea- 0.1%, potassium chloride- 0.1%, Sodium nitrate- 0.1%, potassium di-hydrogen phosphate- 1%, ammonium sulphate-0.1% and peptone- 1%. The variables namely concentration of tannic acid, concentration of potassium di-hydrogen phosphate, concentration of magnesium sulphate and concentration of ferrous sulphate were found to be the most significant for tannase enzyme production as indicated by p-value < 0.05. The statistical design of experiments offers efficient methodology to identify the significant variable and to optimize the factors with minimum number of experiments for tannase production by *A. foetidus.*

The Pareto chart as shown in (**Fig.1**) offers a convenient way to view the results obtained by P.B. Design and the order of significance of the variable affecting tannase production. Earlier studies reported that the addition of inorganic nitrogen sources such as $(NH_4)Cl$ and $(NH_4)_2SO_4$ reduced the rate of growth of *Aspergillu*s and thus inhibited the production of enzymes [12,13]. It was similar results in this study that the supplementation of $(NH_4)Cl$ and $(NH_4)_2SO_4$ reduced the synthesis of tannase enzyme. Paranthaman et al reported that the supplementation of inducer tannic acid to redgram husk substrate was sufficient to enhance the production of tannase enzyme by *A.foetidus* [14]. This is in good agreement with the current results obtained.

CONCLUSION

The Plackett-Burman Design was effectively applied for screening of nutrients for the production of tannase from *Aspergillus foetidus* (MTCC 3557) using redgram husk as a substrate in submerged fermentation.

From standard Plackett-Burman data analysis it was conformed that, concentration of tannic acid, concentration of potassium di-hydrogen phosphate, concentration of magnesium sulphate and concentration of ferrous sulphate were found to be the most significant for tannase enzyme production.

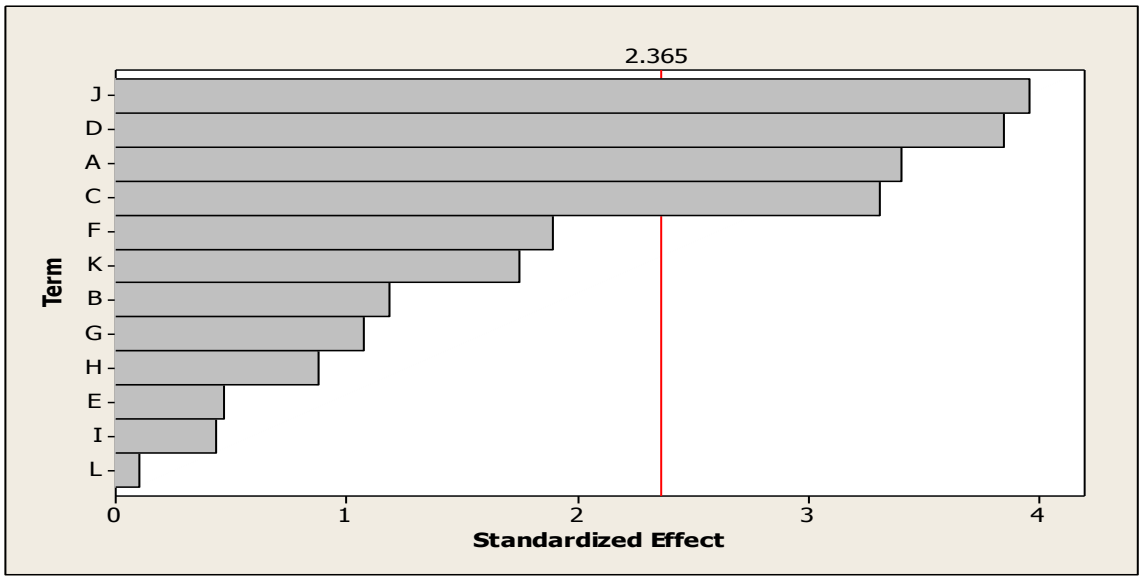

Figure 1: Pareto Plot for Plackett-Burman Design of Experiments for Tannase Production Using *A. foetidus*

A – Tannic Acid	G - Urea
B - Yeast extracts	H - Potassium chloride
C - Magnesium sulphate	I - Sodium nitrate
D - Ferrous sulphate	J - Potassium di-hydrogen phosphate
E - Ammonium nitrate	K - Ammonium sulphate
F - Ammonium chloride	L - Peptone

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