Plackett-Burman Design based Optimization of Tannase production using *Pseudomonas aeruginosa*

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Abstract

The bioconversion of tannic acid is catalysed by Tannase (tannin acyl hydrolase) to produce gallic acid (Tri-hydroxyl benzoic acid). Gallic acid is an essential food antioxidant which is also used as a substrate for the production of food preservatives and various antibacterial compounds. It has several applications in food industries for the production of tea, beer, wines and fruit juice etc. In this research work, Plackett-Burman design was used for the optimization of process parameters to reduce the numbers of experiments as well as the cost of trials. Software MINITAB 17 was used for the Plackett-Burman method and analysis of data.

Optimum conditions obtained by analysis of variance (ANOVA) for tannase production were pH 6.5, temperature 45˚C, incubation time 96 hours, KH_2PO_4 0.05g/L, yeast extract 3g/L, inoculum age 72 hours and inoculum size 500µl. Production of tannase enzyme was doubled (6.5 units per ml) after optimization of process parameters.

Keywords: Tannase, Gallic, *Pseudomonas aeruginosa*, Plackett-Burman design, Submerged Fermentation.

Introduction

Tannase (Tannin acyl hydrolase) is an enzyme discovered by Teighem in 1867. The production of tannase was carried out by micro-organisms such as bacteria and fungi which act as a catalyst in bioconversion of tannic acid to produces gallic acid. Gallic acid is a natural astringent that has a large number of applications in pharmaceutical industries[1,2]. Tannic acid or tannin acts as a substrate to produce tannase[3], but production of synthetic tannins is very costly and it will make the tannase production very costly[4]. Natural sources of tannic acid can be used as a substitute of synthetic tannins for the tannase production. *Syzygiumcumini* (Jamun) leaves, tamarind seed powder, olive mill waste, agriculture residue and coffee husk are the natural sources of tannins[5].

These materials have been used widely as a substitute of synthetic tannic acid. Pomegranate peel is also a natural source of tannic acid[6] and the same materials has been used in present research work as a substrate for reduction of the cost of the trials. The production of tannase can be performed using submerged fermentation and another one is the solid state fermentation[7]. In submerged fermentation, maintaining pH level, inoculation and sterilization are easier than solid state fermentation that makes it preferable to use in enzyme production[8].

Tannase has major applications in the food industries like for clarification of beer, fruit juice and corn wine[9]. It is also used in detannification of food as well as in the production of gallic acid[10]. Various process parameters are used for tannase production and optimization of these process parameters is highly desirable[11]. There are many optimization methods used for optimization of process parameters like full factorial design and box behnken[12], but there is a requirement of large number of experiments to perform in these methods for optimization of various parameters[13]. In Placket-Burman design, we can optimize n-1 numbers of parameters by performing n numbers of experiments[14].

Plackett-Burman design matrix has (+) and (–) values, where (+) sign indicates the maximum range of process parameters and (-) sign indicates the minimum range of process parameters[15]. Aim of this research work was to maximize the production of tannase by performing minimum and low cost experiments. All the experiments were performed using natural source (pomegranate peel) of tannic acid and *pseudomonas aeruginosa* microorganism under submerged fermentation.

Material and Methods

Substrate: Pomegranate peel was collected from shopping complex, Deenbandhu Chhotu Ram University of Science and Technology, Murthal (Sonipat), Haryana, dried in oven at 50˚C for 24 hours and then grinded to obtain powder.

Microorganism: The bacterial strain used for tannase production was *Pseudomonas aeruginosa* MTCC 1035 which was arranged from the Institute of Microbial Technology (IMTECH), Chandigarh, India.

Chemicals: AR grade bovine serum albumin and sodium lauryal sulphate-triethanolamine were purchased from Sigma-Aldrich Pvt. Ltd. All other chemicals including K_2HPO_4, $MgSO_4$ and glucose were purchased from Hi-Media Pvt. Ltd.

Medium composition and culture condition: The medium used for tannase production contained K_2HPO_4 and KH_2PO_4 (0.05-0.5 g/L), $MgSO_4$ (0.5-1.5 g/L), yeast extract (1.5-3 g/L) and glucose as carbon source (2.5-5 g/L). The pH

adjustment of the medium was done between 5 and 6.5. The medium was inoculated with 1-2% and 24-72 hour grown inoculums and incubated for 48-96 hours at 35-45˚C.

Tannase enzyme assay: Colorimetric Method was used to access the tannase activity[12]. 0.3 ml tannic acid and 0.1 ml of the enzyme were mixed and incubated at a temperature of 60°C for half an hour. Then 3 ml of B.S.A. (Bovine serum albumin) solution was added to stop enzymatic reactions. Thereafter, reference tubes were prepared with denatured enzyme followed by centrifugation of all the tubes at 5000 rpm for 10 min. The pellets of precipitates were obtained which were further dissolved in 3 ml STE (Sodium lauryl sulfate-triethanolamine). In this solution, 1 ml of $FeCl_3$ reagent was added and placed undisturbed for 15 min.

The absorbance of both the tubes was measured at the UV wavelength 530 nm against the blank or solution (without any concentration of tannic acid). The specific extinction coefficient of tannic acid at the wavelength of 530 nm was calculated and found to be 0.577.

By the help of this coefficient, tannase activity (one unit) can be stated as the amount of enzyme which is capable to hydrolyze 1 milli mole of the substrate (tannic acid) within 1 minute under specific assay conditions.

Plackett–Burman design: The optimization of parameters (having a significant effect on the production of tannase) was conducted by Plackett–Burman design. The design matrix for 11 process parameters (including 12 runs) was selected for performing experiments in software Minitab as in table 1 and 2, then analysed the responses of experiments to determine the effect of individual variables. Now find the estimated tannase production and optimize ranges or concentrations of process parameters in Software MINITAB 17.

Results and Discussion

We performed 12 experiments multiple times according to Plackett-Burman design as in table 3 and enzyme assay was prepared according to the Colorimetric Assay Method[12] for calculation of tannase Activity. The UV absorbance of enzyme assay was determined using the UV spectrometer RIGOL ultra-3660. According to Mondal assay method, tannase activity was calculated. Analysis of the design was performed to determine the effect of parameters using software Minitab for each variable as in figure 1.

Table 1
Parameters required for the tannase production

S.N.	Name of the parameters	Low level (-)	High level (+)
1	pH	005.00	006.50
2	Temperature (°c)	030.00	040.00
3	Incubation time (hours)	048.00	096.00
4	Inoculums size (ml)	000.25	000.50
5	Carbon source (gram/litre)	002.50	005.00
6	Yeast extract (gram/litre)	001.50	003.00
7	$MgSO_4$ (gram/litre)	001.50	003.00
8	KH_2PO_4 (gram/litre)	000.05	000.50
9	K_2HPO_4 (gram/litre)	000.05	000.50
10	Agitation (rpm)	125.00	175.00
11	Inoculums age (hours)	024.00	072.00

Table 2
Plackett-Burman design in 12 runs for up to 11 factors

Run	y_1	y_2	y_3	y_4	y_5	y_6	y_7	y_8	y_9	y_{10}	y_{11}
1	+	-	+	+	-	+	-	-	-	+	+
2	-	-	-	+	+	+	-	+	+	-	+
3	+	+	-	+	-	-	-	+	+	+	-
4	-	-	+	-	-	-	+	+	+	-	+
5	-	+	-	+	+	-	+	-	-	-	+
6	-	+	-	-	-	+	+	+	-	+	+
7	+	+	+	-	+	+	-	+	-	-	-
8	+	-	+	+	+	-	+	+	-	+	-
9	+	-	-	-	-	-	-	-	-	-	-
10	-	+	+	-	+	-	-	-	+	+	+
11	+	-	-	-	+	+	+	-	+	+	-
12	-	+	+	+	-	+	+	-	+	-	-

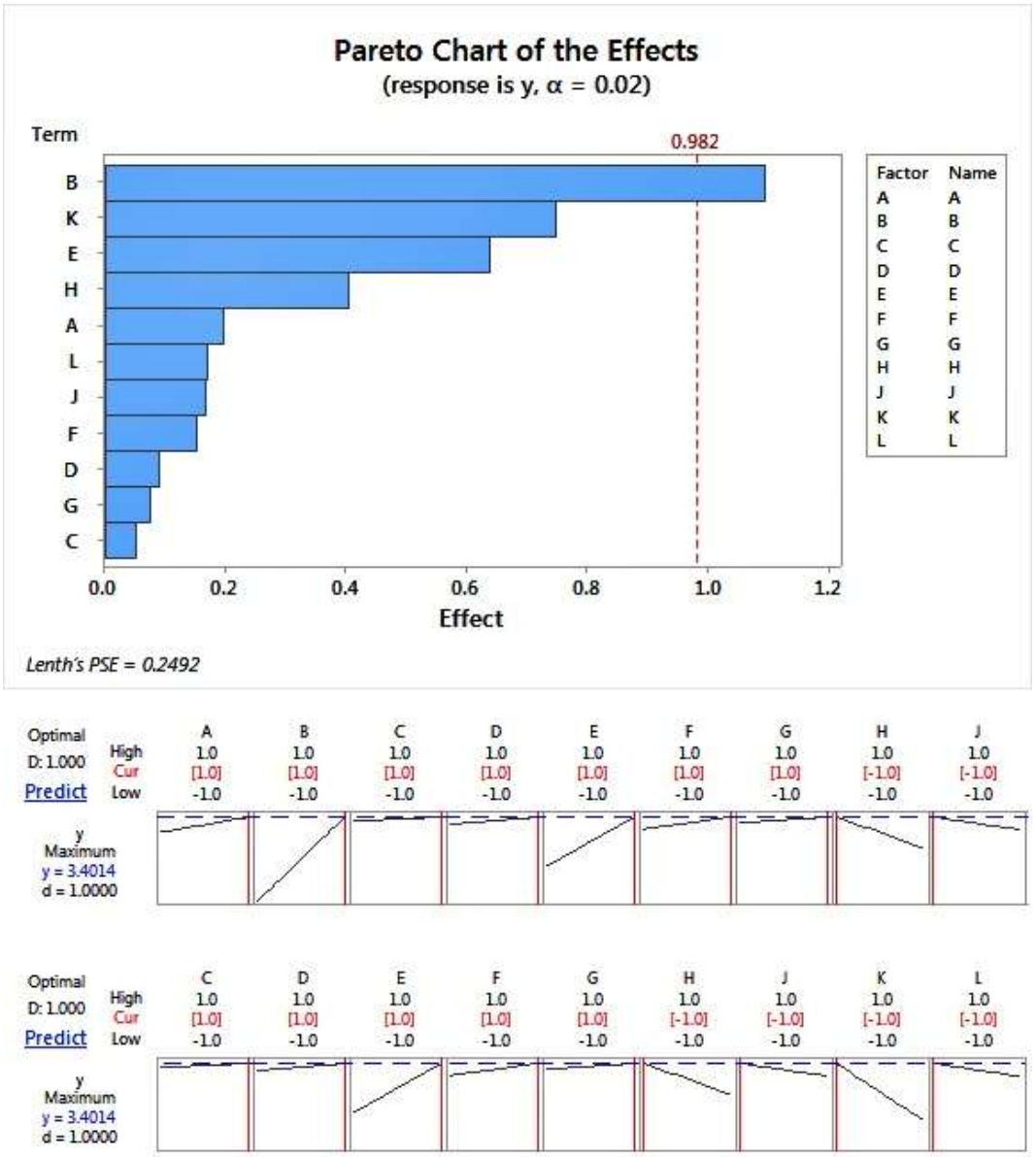

Figure 1: Pareto chart of the effect of each factor and Optimized ranges of process parameters by response optimizer

Table 3
Process parameters arrangement according to Plackett-Burman design

Run	A	B	C	D	E	F	G	H	J	I	I	Y
1	6.5	35	20	72	48	175	2.5	1.5	.025	.50	.50	2.4000±0.16
2	5.0	45	10	72	96	175	2.5	3.0	.25	.05	.50	1.8000±0.12
3	6.5	45	10	72	48	125	2.5	3.0	.25	.50	.05	3.0258±0.19
4	6.5	35	20	24	48	125	5.0	3.0	.25	.05	.50	1.2400±0.09
5	6.5	45	10	72	96	125	5.0	1.5	.025	.05	.50	2.8300±0.17
6	5.0	45	10	24	48	175	5.0	3.0	.025	.50	.50	0.6700±0.06
7	6.5	45	20	24	96	175	2.5	3.0	.025	.05	.05	1.1590±0.10
8	5.0	35	20	72	96	125	5.0	3.0	.025	.50	.05	1.1000±0.08
9	5.0	35	10	24	48	125	2.5	1.5	.025	.05	.05	0.6800±0.06
10	5.0	45	20	24	96	125	2.5	1.5	.25	.50	.50	1.1000±0.08
11	6.5	35	10	24	96	175	5.0	1.5	.25	.50	.05	0.8000±0.07
12	5.0	45	20	72	48	175	5.0	1.5	.25	.05	.05	1.2500±0.09

where, A = pH, B = temperature in Celsius**,** C = inoculums size in ml/L, D = inoculums age in hours, E = incubation time in hours, F = agitation**,** G = carbon source in g/L, H = yeast extract in g/LJ = $MgSo_4$ in g/L, K = KH_2PO_4 in g/L, L = K_2HPO_4 in g/L, Y = Enzyme activity in unit/ml

Factor B has the highest effect and factor C has the lowest effect for the production of tannase enzyme. Then using a response optimizer in Minitab, we got the optimal concentrations and ranges of the process parameters as in Figure 2 indicating 1 and -1 for minimum and maximum concentrations respectively. In this research work, the process parameters were designed in such a manner using Plackett-Burman (PB) design that maximum enzyme production can be reached.

Among the 11 process parameters, incubation time (E), temperature (E) and KH_2PO_4 (K) were identified as the most effective parameters having a significant effect on the fabrication of tannase using submerged fermentation. The effect of temperature (B) and incubation time (E) on tannase production is shown in Figure 3. Tannase production is high when the temperature (B) was at 40° C and incubated (E) for 96 hours. Tannase production will be low if temperature and incubation time will decrease. The effects of temperature (B) and KH_2PO_4 (K) on tannase production is shown in fig. 4.

Tannase production is high when KH_2PO_4 concentration was 0.05 gram/liter and the temperature was set on a higher level at 40˚C. The effects of incubation time and KH_2PO_4 on tannase production are shown. Tannase production is high when KH_2PO_4 concentration was 0.05 gram/liter and was incubated for 72-96 hours. Increasing temperature and incubation time to their higher levels, 40˚C and 96 hours respectively will increase the production of tannase. In submerged fermentation, the microorganism produced maximum tannase at temperature 30-40˚C and according to Plackett-Burman design, temperature has a significant effect on tannase production with an optimum range of 45˚C.

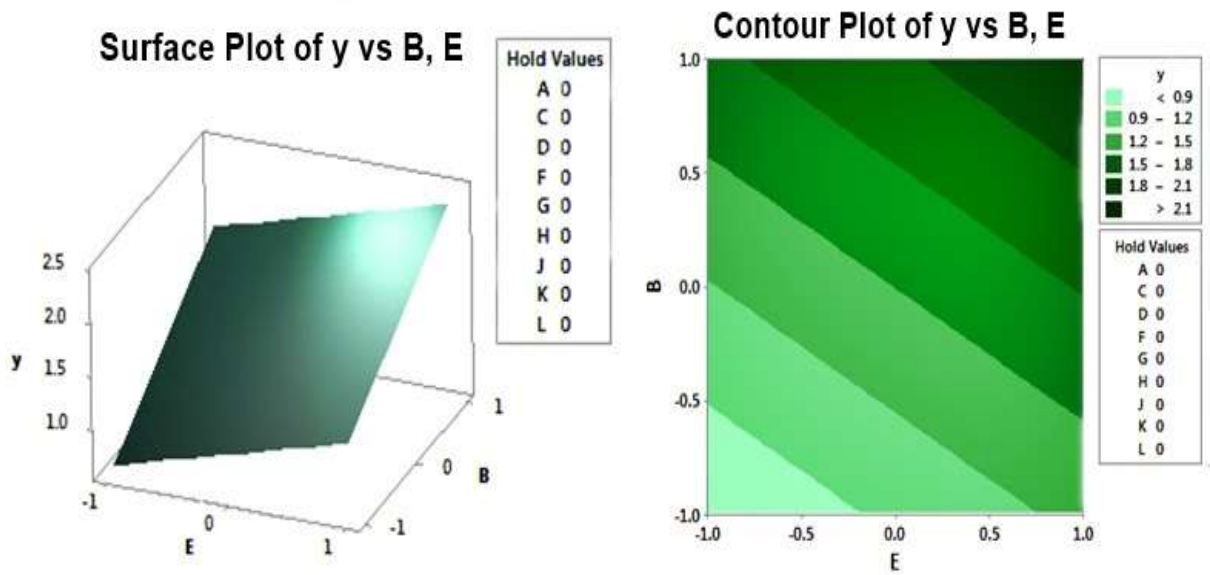

Figure 2: Contour plots showing y response variation with temperature (B) and incubation time (E)

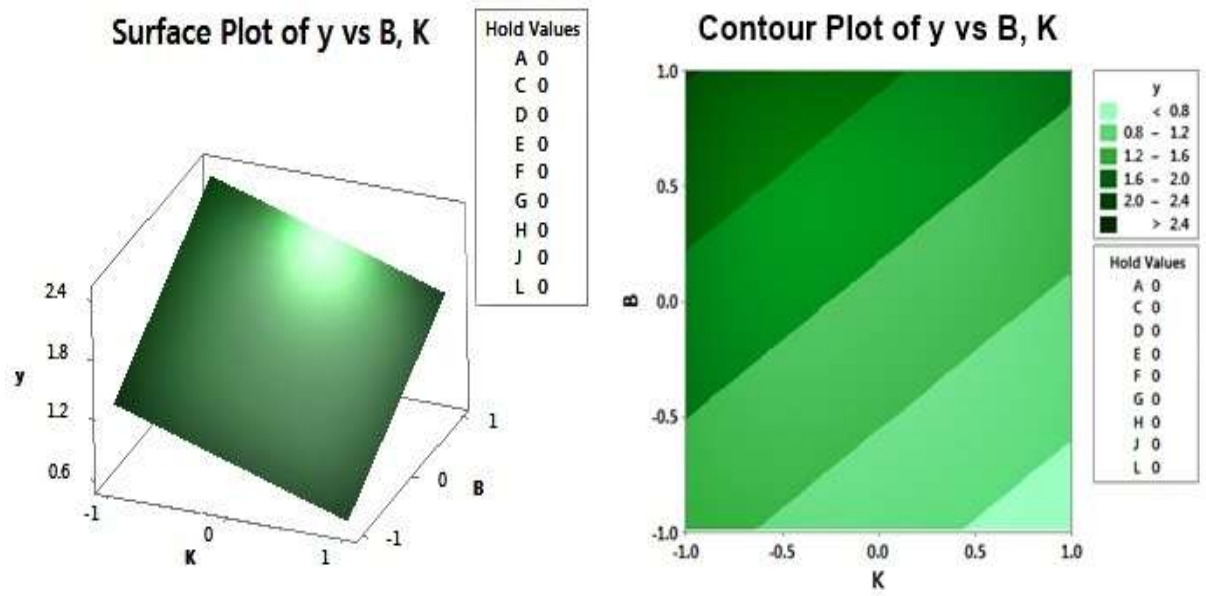

Figure 3: Contour plots showing y response variation with temperature (B) and KH_2PO_4 (K)

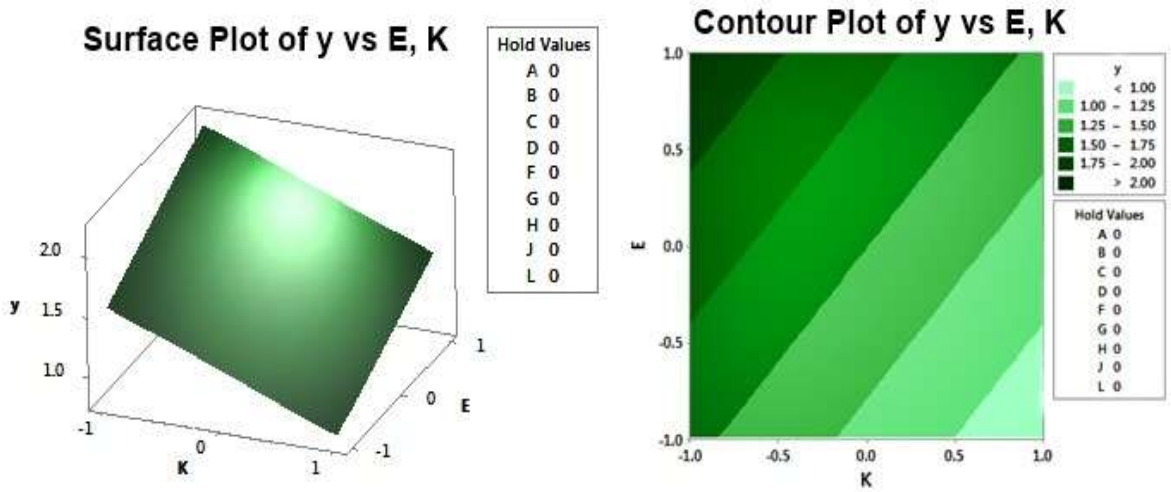

Figure 4: Contour plots showing y response variation with incubation time (E) and KH_2PO_4 (K)

The results determined (based on Plackett–Burman model design) that temperature, incubation time, yeast extract and KH_2PO_4 have a higher effect but among all these, the temperature has a most substantial influence on the production of tannase.

Conclusion

In this research work, we optimized the process parameters by performing minimum experimental runs to get the maximum tannase production and reduce the cost of the experiments. Using pomegranate peel powder as a natural substrate also reduces the cost of production for tannase using *Pseudomonas aeruginosa* with submerged fermentation. Plackett-Burman design was used to reduce the experimental runs and cost for the optimization of process parameters.

Optimum conditions obtained by analysis of variance for tannase production were pH 6.5, temperature 45˚ C, incubation time 96 hours, KH_2PO_4 0.05g/L, yeast extract 3g/L, inoculum age 72 hours and inoculum size 500µl. Optimization of process parameters minimized the experimental runs and maximized the tannase production, hence reduced the cost of experiments

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