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Optimization of process parameters through plackett-burman design by *Paenibacillus polymyxa* G7 isolated from resins of *Pinus roxburghii*

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Abstract
Lipases are ubiquitous in nature and high demanded industrial catalysts. In the present study, the cultural conditions of extracellular lipase production from a bacterial strain *Paenibacillus polymyxa* G7 were statistically optimized by using Plackett-Burman design under submerged fermentation conditions. Maximum enzyme activity was noted in a medium containing; galactose (0.2%), ammonium sulphate (0.5%), Tragacanth gum (1.5%), yeast extract (0.25%), incubation time (4 days), temperature (40 ºC), pH 10.0 with an inoculum size 10%. As witnessed from Pareto chart; variables that were most important for lipase production were temperature and incubation time (days) whereas other parameters showed minute effect for lipase production.

Keywords: Extracellular lipase, *Paenibacillus polymyxa* G7, plackett-burman design, pareto chart

Introduction
In modern industry, microorganisms had larger impact and contribution in production of various potential enzymes. Currently, lipases denote one of the major groups of enzyme in the biotechnology field [1]. Sources of lipases represent plants, animals and microorganisms. Lipases from microorganisms have been widely employed based on their interesting characteristics viz. working under mild conditions, good stability in organic solvents and high specificity for substrate [2, 3]. Lipases have been applied in catalyzing various reactions such as enhancement of flavor, treatment of fatty waste materials, detergent formulation, biosurfactants production, biopharmaceutical formulations and production of biodiesel [4, 5].
For the bioprocess development, study of factors contributing to the yield of the desired metabolic product is an important strategy. The metabolic product accumulation and growth of cell are severely influenced by various process parameters such as pH, media, temperature, inoculums size, incubation time, substrates, surfactants, divalent ions, carbon sources and nitrogen sources. Thus, it is hard to find the main influencing factors and optimize it further for biotechnological processes as many factors are present [6]. The one factor approach or classical method of process parameter optimization includes each factor study at one time and keeping the others at constant levels. This method has many drawbacks as it is time consuming, laborious, one dimensional and does not give any sure confirmation of optimal conditions whereas it is impractical to perform experiments in consideration to every possible factorial combination due to huge number of experiments. Hence to resolve this issue, experimental design techniques represents a more uniform replacement to classical approach. The statistical approach minimizes the error in evaluation of effect of different variable factors which includes designing of a specific technique. The Plackett-Burman design short lists the large number of experimental combinations into small numbers and is a reliable technique for further optimization. It gives unbiased data of the linear effects of all factors with good accuracy for a mentioned number of observations. This design is useful in screening of desired process variables [7]. It is a factorial design in which huge numbers of independent variables (N) are studied in less number of experiments (N+1) [8, 9].
In the present study, six selected process parameters were screened for their significance on lipase production by *P. polymyxa* G7 isolated from oil contaminated soil of Trans Himalayas under submerged fermentation using Plackett-Burman design and Response Surface Methodology (RSM).

Material and Methods

Bacterial culture screening and identification

The sample was collected from resins of *Pinus roxburghii* of Trans Himalayas, India. The isolation was done on tributyrin agar medium by serial dilution technique and incubated for 72 h at 37 ºC. Through the qualitative analysis, selection of hyper potential bacterial isolate i.e. G7 was done. The bacterial isolate was further observed for its quantitative assay. The potential bacterial isolate viz. G7 was identified for morphological, biochemical and molecular identification.

Plackett-Burman design for selection of process variables for extracellular lipase production

The Plackett-Burman Design (PBD) is a special variation of a two level fraction design based on the incomplete equilibrium piece principle. It can pick up the main factors with the least number of experiments from a list of candidate factors. To evaluate the effect of 6 factors on lipase activity, PB factorial design in 12 experimental runs was carried out. Six factors consisting of incubation time, pH, temperature, inoculums size, substrate concentration and galactose concentration were prepared at two levels,-1 for low level and +1 for high level (Table 1). Total combinations were obtained. The media was prepared and incubated as per design. The culture broth was centrifuged at 10,000 x g for 15 min at 4 ºC. The supernatant was taken and assayed for enzyme activity. Design Expert was used for Plackett-Burman Design and regression analysis.

Table 1: Variables selected for 2-level full factorial design

Factors	Units	Coded levels	
		-1	+1
Incubation time	Days	2	6
pH		8	12
Temperature	ºC	30	50
Inoculums size	%	5	15
Substrate concentration	%	1	2
Galactose concentration	%	0.1	0.3

Optimization of selected variables using Central Composite Design (CCD)

The concentration of different independent variables, which affect lipase production significantly (p<0.05) or showed positive effects on the lipase activity (incubation time and temperature) were further optimized by central composite design (CCD) with a total of 13 experimental runs. The experiments were conducted and enzyme activity (IU/ml) was taken as the response (Y). The statistical software "Design-Expert" (Stat Ease) was used to analyze the experimental results.

Validation of the experimental model

To validate the model equation, experiment were conducted for enzyme production under optimum conditions predicted by the model and values (predicted v/s actual) were compared and analyzed.

Results and discussions

Bacterial culture isolation, screening and identification

The hyper lipase producing bacterial isolate viz. G7 was isolated from oil contaminated soil of Trans Himalayas of Himachal Pradesh. The bacterial isolate i.e. G7 was preliminarily characterized based on their morphological and cultural characteristics having irregular, undulated, raised, cream and rough morphological characters on tributyrin agar medium (Plate 1). The biochemical analysis showed positive results for endospore staining, VP, citrate, oxidase, catalase and H_2S production whereas negative for indole, MR, urease and glucose utilization. G7 showed 18.90 mm zone of clearance during qualitative analysis whereas in quantitative analysis it revealed 430 IU/ml activity of lipase. The selected bacteria i.e. G7 was identified by using 16S rRNA molecular technique. Partial 16S rRNA gene sequence was quire to NCBI BLAST (http:// www.ncbi.nlm.nih.gov/Blast) and the nearest neighbor of the isolate was determined. Sequence similarity search (mBLAST, NCBI) for G7 showed 99% homology with nucleotide sequence of *Paenibacillus polymyxa* G7.

Plate 1: Morphological characteristics of G7

Response Surface Methodology (RSM) for enhanced production of lipase from *P. polymyxa* G7

In order to improve the growth/productivity/yield of the product may be biomass or enzyme or metabolite, various strategies are required to be adopted. The conventional optimization strategy of varying one factor at a time does not consider interactions among different variables/parameters (Alikunju *et al.,* 2018; You *et al*., 2017). In contrast, Response Surface Methodology (RSM), an effective statistical technique, can examine interference of more than one factor at a time at different levels. RSM uses quantitative data to evaluate multiple parameters and their interactions by establishing a mathematical model (Ong *et al*., 2016).

Plackett-Burman (PB) design for selection of process variables for increased lipase production

Plackett-Burman design was successfully applied to investigate the effect of 6 independent variables viz. incubation time, pH, temperature, inoculums size, substrate concentration and galactose concentration. Total 12 experiments were run. The levels of the variables and their effects on the response (enzyme activity) have been shown in Table 2. Plackett-Burman results were performed and first-order polynomial equation was derived to explain the effect of various variables on lipase production.

R1 (G7) = -70.00000 + 3.33333* temperature + 31.66667* incubation time

Table 2: The factors response with plackett-burman program and corresponding results (the response) by *Paenibacillus polymyxa* G7

Run	Substrate concentration (%)	pH	Temperature (ºC)	Incubation time (days)	Inoculum size (%)	Galactose concentration (%)	Lipase activity (IU/ml)
1	1	8	30	2	5	0.1	210
2	1	8	30	6	15	0.3	170
3	2	12	30	6	5	0.1	190
4	2	8	50	2	5	0.1	230
5	1	8	50	6	15	0.1	370
6	2	8	50	6	5	0.3	490
7	2	12	50	2	15	0.3	50
8	2	8	30	2	15	0.3	70
9	2	12	30	6	15	0.1	170
10	1	12	30	2	5	0.3	130
11	1	12	50	2	15	0.1	70
12	1	12	50	6	5	0.3	130

Lack of fit test

The data obtained from CCD on lipase production was subjected to analysis of variance (ANOVA) and three process orders were suggested by Design Expert 11.0 analysis (Fig 1 and Table 2). The analysis of variance of the response surface quadratic model (model F-value of 6.72) implies that the model was significant. There is only 0.01% chance that this "Model F-value' could occur due to noise. Values of 'Prob>F" less than 0.1052 indicated that model terms were significant. In this case, A, B, C, AB, AC, A^2, B^2 and C^2 are significant model terms. The "Lack of Fit F-value" of 3.35 implies the Lack of Fit is not significant relative to the pure error. There is a 10.52% chance that a "Lack of Fit F-value could occur due to noise. Non-significant lack of fit is good- we want the model to fit.

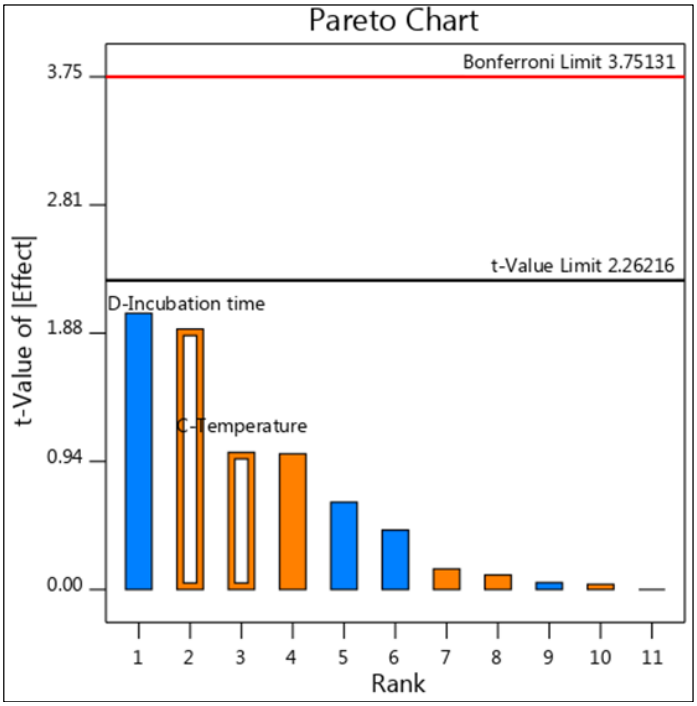

Fig 1: A pareto chart of *P. polymyxa* G7 showing the positive and negative effects of selected variables (incubation time, pH, temperature, inoculums size, substrate concentration and galactose concentration)

Table 2: Effect of positive factors of *P. polymyxa* G7 on lipase production using CCD

Run	Factor A: Temperature (ºC)	Factor B: Incubation time (Days)	Response: Lipase activity (µg/ml/min)
1	30	2	270
2	50	2	290
3	40	4	1430
4	40	4	1120
5	40	1	190
6	40	6	590
7	40	4	1540
8	40	4	1650
9	30	6	230
10	40	4	1700
11	50	6	330
12	30	4	240
13	50	4	310

Regression analysis

3D graph was generated for regression analysis of CCD design, using pair wise combination of two factors for lipase production. This 3D response surface plot described the effects of the independent variables and combined effect of each independent variables upon the response (Fig 2).

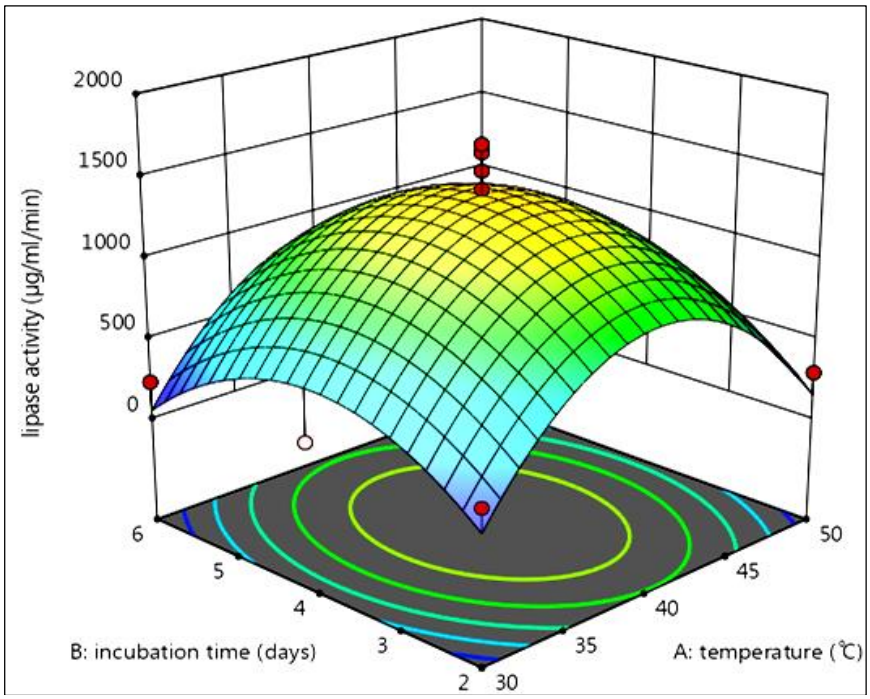

Fig 2: Representation of interaction of temperature and incubation time on production of lipase by *P. polymyxa* G7

Validation of experimental model

Validation of the predicted results was done by comparing the predicted values with the results obtained. Maximum lipase activity obtained by performing experiments was 1700 IU/ml which was very close to the predicted value as calculated by ANOVA (Table 3). The following regression equations were derived after the analysis of variance:

R1 (G7) = -13211.56491 + 636.89276*temperature + 913.03803*incubation time + 1.00000* temperature*incubation time - 7.97158*temperature^2 - 120.33669* incubation time^2

A high similarity between the predicted and experimental results reflects the accuracy and applicability of the RSM to optimize the process for enzyme production. After determining the interactions between the selected variables, the optimized conditions for hyper lipase production were observed. The pertubation plot showed the optimized conditions i.e. temperature 40 ºC and incubation time of 4 days (Fig 3 a and b). The comparative analysis of production conditions before and after the plackett-burman design given in Table 4. These conditions were selected for further hyper production of lipase.

Table 3: Analysis of variance of *P. polymyxa* G7 [Partial sum of squares-Type III]

Source	Sum of squares	Df	Mean square	F-value	p-value
Model	3.928E+06	5	7.856E+05	7.99	0.0083*
A-Temperature	6016.67	1	6016.67	0.0612	0.8117
B-Incubation time	2399.42	1	2399.42	0.0244	0.8803
AB	1600.00	1	1600.00	0.0163	0.9021
A^2	1.987E+06	1	1.987E+06	20.21	0.0028
B^2	1.216E+06	1	1.216E+06	12.37	0.0098
Residual	6.882E+05	7	98317.15		
Lack of Fit	4.755E+05	3	1.585E+05	2.98	0.1594**
Pure Error	2.127E+05	4	53170.00		
Cor Total	4.616E+06	12			

*indicates model is significant

**indicates lack of fit is non-significant

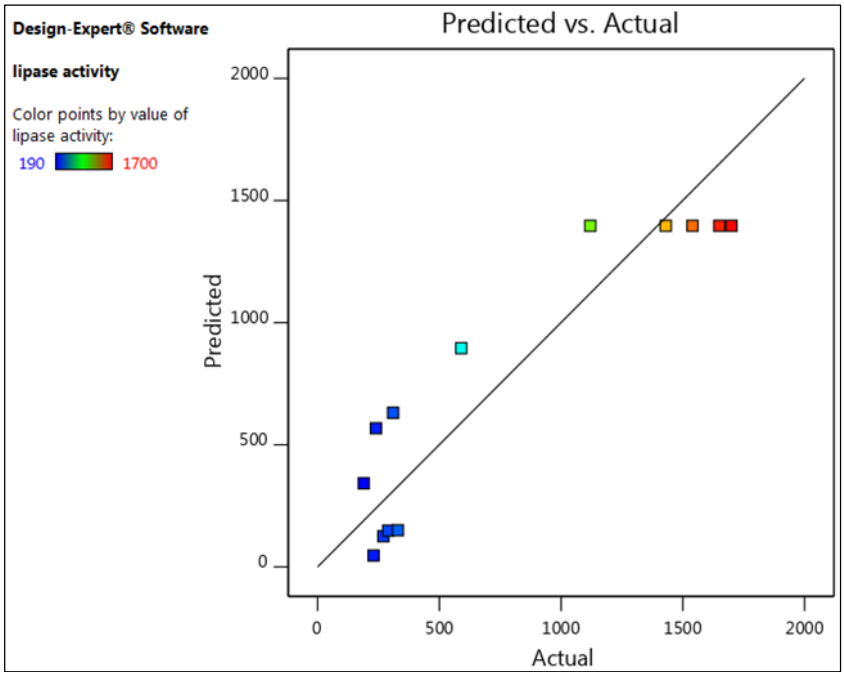

Fig 3a: Comparison between predicted and actual values in CCD of *P. polymyxa* G7

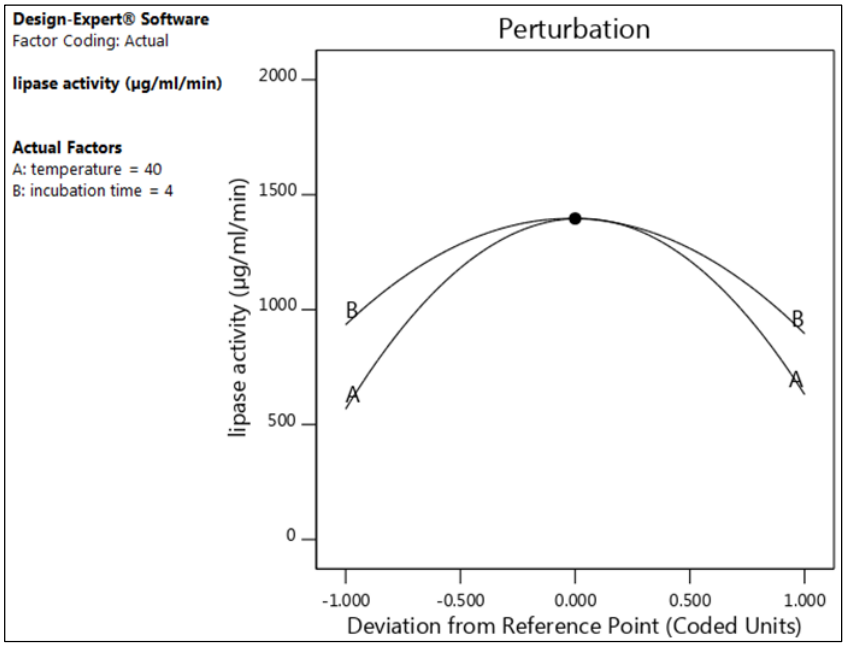

Fig 3b: Perturbation plot chart of *P. polymyxa* G7 showing the deviation from reference point of selected variables

Table 4: Comparative analysis of production conditions before and after Plackett-Burman design (PBD) from *Paenibacillus polymyxa* G7

Culture Conditions	Before PBD	After PBD
Temperature (ºC)	40	40
Incubation time (Days)	4	4
Lipase activity (IU/ml)	1730	1740

Conclusion

The Plackett-Burman design is an efficient and economical technique in which the major/positive variables were screened out. These major/positive factors screened through Plackett-Burman design are employed for the consecutive stage of optimization of process parameters using response surface methodology. Maximum lipase activity of 1740 IU/ml was obtained from the optimized conditions viz. temperature 40 ºC and incubation time of 4 days.

Conflict of interest

There is no conflict of interest.

Acknowledgements

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