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Screening of Process Variables for the Production of Extracellular Lipase from Palm Oil by Trichoderma Viride using Plackett-Burman Design

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***Abstract*—**Plackett-Burman statistical screening of media constituents and operational conditions for extracellular lipase production from isolate *Trichoderma viride* has been carried out in submerged fermentation. This statistical design is used in the early stages of experimentation to screen out unimportant factors from a large number of possible factors. This design involves screening of up to 'n-1' variables in just 'n' number of experiments. Regression coefficients and t-values were calculated by subjecting the experimental data to statistical analysis using Minitab version 15. The effects of nine process variables were studied in twelve experimental trials. Maximum lipase activity of 7.83 μmol /ml /min was obtained in the 6th trail. Pareto chart illustrates the order of significance of the variables affecting the lipase production. The present study concludes that the most significant variables affecting lipase production were found to be palm oil, yeast extract, K2HPO4, MgSO4 and CaCl2.

***Keywords*—**lipase, submerged fermentation, statistical optimization, *Trichoderma viride*

I. Introduction

LIPASES (Triacylglycerol acylhydrolases E.C.3.1.13) are found in animals, plants and microorganisms [1], but only microbial lipases are commercially significant [2]. Lipases have been extensively studied because of their actual and potential applications in the detergent, oil, food, pharmaceutical and chemical industries [3].

Among the lipase producing microorganisms, fungi are preferable lipase sources because fungal enzymes are usually excreted extracellularly, facilitating extraction from fermentation media [4]. Many fungi as *Rhizopus oryzae* [5], *Geotrichum* sp., [6], *Aspergillus terreus*, [7], *Trichoderma viride* [8], *Trichoderma reeshi* [9] and *Fusarium solani* [10] have been noted as producers of lipases using vegetable oil for carbon and energy source for lipase production. But, by considering the rate, all vegetable oils were highly expensive except palm oil. So, in industries increasing the contents of palm oil, the lipase yields also become increasing with low cost, because they were the cheapest substrate and available in higher volume. A cheap and effective medium can decrease the production cost, so it is crucial to the industrialization of the enzyme method [11].

The application of statistically based experimental designs to optimize fermentation media is an efficient approach to studying the effects of several factors and to improve product yields. The Plackett-Burman design provides an efficient way of a large number of variables and identifying the most important ones [123]. Numerous reports have proved the applicability of Plackett-Burman design in the optimization of media components for various enzyme production such as the optimization of Xylanase by *Aspergillus niger* LPB 326 [13] and lipase production by *Yarrowia lipolytica* NCIM 3589 [14].

Hence an attempt is made in this paper to utilize the palm oil as a substrate for the production of lipase using indigenous isolate of *Trichoderma viride* by submerged fermentation. It was under taken to optimize the key process variables, including incubation time, pH, carbon level, nitrogen level and salt level of the medium for the production of lipase using Plackett- Burman design.

II. Materials And Methods

Isolation of lipolytic fungi

Lipase producing fungal isolate of *Trcichoderma viride* was isolated from oil mill waste which was collected from December 2010 to February 2011 at Namakkal, Tamil Nadu, and India by serial dilution technique [15]. After isolation, the fungus was subcultured and the pure culture was stored in PDA at 4°C.

Lipase activity on Rhodamine-B agar plates

The fungal strain was tested for lipase production on agar plates that contained 15g, olive oil 10 ml, Rhodamine-B 0.01g and Tween 80- 0.001% in 1000 ml of distilled water. After 7 days of incubation at 28°C, the plate was irradiated with UV light (350 nm), with a bright pink fluorescent halo confirming lipolytic activity [16].

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A.Development of Inoculums

The culture was grown on Potato Dextrose Agar (PDA) plates for 5 days, which were wetted by adding 10 ml of sterilized distilled water. With the help of inoculating needle, conidia were scratched and the tubes were shaken vigorously to break the clumps of conidia and to obtain homogeneous suspension. Conidial suspension was used as inoculums and the number of spores was counted in a neubauer hemocytometer and the concentration of the spores was adjusted to 1 X 10^9 with sterile distilled water.

B.Lipase production

Medium optimization for maximum lipase production was done in the basal liquid medium (Table 1 and 2). The nutritional components and operating conditions such as palm oil, Yeast extract, KH_2PO_4, K_2HPO_4, $MgSO_4$, $CaCl_2$, $NaNO_3$, pH and incubation periods were varied according to the statistical designs and the optimum components were incorporated in the basal medium. Erlenmeyer flask with 100 ml liquid medium was inoculated with 2 ml spore suspension and incubated at 30±2°C and 120 rpm. After the fermentation periods, the culture filtrate was collected aseptically by passing through Whatman No. 1 filter paper and filtrate was assayed for lipase productivity.

C.Assay for lipase activity

Lipase activity was determined in an emulsifier free system using olive oil as substrate [17]. Reactions were carried out in 100 ml conical flasks at 40°C by immersion in a water bath. The reaction mixture, consisting of 2 ml of 0.1 M potassium phosphate buffer, pH 7.0, 1 ml of olive oil and 1 ml of culture supernatant was incubated at 40°C for 30 min. The enzyme reaction was terminated by the addition of 5 ml of 96% ethanol and followed by titration with 0.01 N NaOH solution using phenolphthalein as the indicator. One unit of lipase activity was defined as the amount, which liberated 1 µmol of fatty acid per min at 40ºC.

D,*Plackett-Burman design*

The Plackett–Burman statistical experimental design is used for experiments confronted with two-factor interaction. This is a very economical design with the run number a multiple of four and comprises of two level screening designs. This design is extremely useful in finding importance of the factors affecting the production of the enzyme. PB design mathematically computes the significance of a large number of factors in one experiment, which is time saving and gives the effect of change in more than one factors in single experiment [18]. To evaluate the effect of 9 factors of medium components and operating conditions on lipase activity PB factorial design in 12 experimental run was carried out. Nine assigned variables and two unassigned variables (commonly referred as dummy variables) were screened in PB design of 12 experiments. Dummy variable are used to estimate experimental errors in data analysis [19]. Nine factors consisting of medium components and operating conditions prepared at two levels –1 for low level and +1 for high level. The factors (in g or ml /1000ml) such as palm oil, yeast extract, KH_2PO_4, K_2HPO_4, $MgSO_4.7H_2O$, $CaCl_2$, $NaNO_3$, pH and Incubation periods and 2 unassigned variables at same level were studied. Each variable is represented at two levels, high and low denoted by (+1) and (–1) respectively (Table 1).

TABLE I
ACTUAL VALUES OF THE PROCESS VARIABLES FOR 1000ML OF MEDIUM

Process variables	Low levels (-1)	High Level (+1)
Palm oil (ml)	5	20
Yeast extract (g)	15	40
KH_2PO_4 (g)	0.5	3.0
K_2HPO_4 (g)	0.5	3.0
$MgSO_4$ (g)	0.1	0.5
$CaCl_2$ (g)	0.1	0.5
$NaNO_3$ (g)	1	5
pH	4	8
Incubation periods	3	7

Statistical analysis

The statistical software package Minitab version 15 (Minitab Ltd., Coventry CV3 2TE, UK) was used for analyzing the experimental data. Table 2 shows the factors considered for investigation and the PB design for 12 experimental run and the experimental lipase activity. PB experimental design is based on the first order model as given in equation 1.

$$Y = \beta_0 + \Sigma \beta_i x_i \quad (1)$$

Where, Y is the response (enzyme activity), β_0 is the model intercept, i is variable number, β_i is variable estimates and X_i are independent variables. This model describes no interaction among the factors that influence lipase production and enzyme activity. All experiments were carried out in duplicate and the averages of lipase activity were taken as responses. The variables whose confidence levels were higher than 90 % were considered that significantly influences the lipase activity.

III. Results And Discussion

Trichoderma viride isolated and identified based on macroscopic and microscopic observation with descriptions and illustrations in mycological literature of Domsch et al. [20] and it showed a zone of fluorescence which indicated lipolytic activity using Rhodamine B method [16]. The data on enzyme activity in table 2 was subjected to multiple linear regression analysis to estimate t-value, p-value and confidence level of each component. The results (Table 2) indicated that there was a variation of lipase production in the twelve trials in the range from 5.67 to 7.83 µmol /ml /min. These variations reflected the importance of medium optimization to obtain higher lipase yield. On analysis of regression coefficients and t-value of 9 factors such as palm oil, yeast extract, K_2HPO_4, $MgSO_4.7H_2O$ and $CaCl_2$ showed a positive effect on lipase production, all other factors shown a negative effect on lipase production. When the sign of the concentration effect of the tested variable was positive, the influence of the variable upon the lipase production was greater at a high concentration, and when it was negative, the influence of the variable was greater

TABLE II
PLACKETT-BURMAN DESIGN OF 12 RUNS FOR 11 VARIABLES ALONG WITH OBSERVED CONCENTRATION OF LIPASE PRODUCTION IN FERMENTATION BROTH

Run No.	Palm oil	Yeast extract	KH_2PO_4	K_2HPO_4	$MgSO_4$	$CaCl_2$	$NaNO_3$	pH	Incubation periods	DV-1	DV-2	Experimental Lipase activity (µmol /ml /min)	Predicted Lipase activity (µmol /ml /min)
1	+1	-1	+1	-1	-1	-1	+1	+1	+1	-1	+1	5.67	5.558
2	+1	+1	-1	+1	-1	-1	-1	+1	+1	+1	-1	6.50	6.612
3	-1	+1	+1	-1	1	-1	-1	-1	+1	+1	+1	5.83	5.863
4	+1	-1	+1	+1	-1	+1	-1	-1	-1	+1	+1	7.30	7.333
5	+1	+1	-1	+1	+1	-1	+1	-1	-1	-1	+1	7.67	7.558
6	+1	+1	+1	-1	+1	+1	-1	+1	-1	-1	-1	7.83	7.797
7	-1	+1	+1	+1	-1	+1	+1	-1	+1	-1	-1	6.17	6.137
8	-1	-1	+1	+1	+1	-1	+1	+1	-1	+1	-1	5.67	5.782
9	-1	-1	-1	+1	+1	+1	-1	+1	+1	-1	+1	6.33	6.218
10	+1	-1	-1	-1	+1	+1	+1	-1	+1	+1	-1	6.83	6.942
11	-1	+1	-1	-1	-1	+1	+1	+1	-1	+1	+1	6.67	6.703
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	6.00	5.967

Sign '+1' is for high concentration of variables and '-1' is for low concentration of variables; DV- Dummy variables

TABLE III
STATISTICAL ANALYSIS OF PLACKETT-BURMAN DESIGN ON LIPASE ACTIVITY BY *Trichoderma viride*

Variable/ Term	Main Effect	Coefficients	SE Coefficients	t-value	p- value	Confidence level ,%
Constant		6.5392	0.05827	112.23	0.000	100
Palm oil	0.8550	0.4275	0.05827	7.34	0.018*	98.2
Yeast Extract	0.4783	0.2392	0.05827	4.10	0.055*	94.5
KH_2PO_4	-0.2550	-0.1275	0.05827	-2.19	0.160	84
K_2HPO_4	0.1350	0.0675	0.05827	1.16	0.366	63.4
$MgSO_4$	0.3083	0.1542	0.05827	2.65	0.118	88.2
$CaCl_2$	0.6317	0.3158	0.05827	5.42	0.032*	96.8
$NaNO_3$	-0.1850	-0.0925	0.05827	-1.59	0.253	74.7
pH	-0.1883	-0.0942	0.05827	-1.62	0.247	75.3
Incubation Period	-0.6350	-0.3175	0.05827	-5.45	0.032*	96.8
R-Sq = 98.67%, R-Sq(adj) = 92.67%						

SE- Standard error; t – student's test; p – corresponding level of significance; * Significant.

at a low concentration [21].

In the present work, variables with confidence levels greater than 90% were considered as significant. Palm oil, incubation period, $CaCl_2$ and yeast extract were found to be highly significant factor for lipase production (Table 3). Palm oil and yeast extract acted as carbon source and nitrogen source respectively for lipase enzyme production was reported by Treichel et al. [22]. The low incubation periods in this experiment model coincide with the data presented for Nadia et al. [23] in which the maximum lipase production was observed at 3 days of incubation periods after that it was reduced. This may be attributed to the fact that submerged technique allowed better emulsification of the lipids and also good aeration. The enzyme production was moderately enhanced in the presence of Ca^{2+} which is important in maintaining cell wall rigidity, stabilizing oligomeric proteins and covalently bounding protein peptidoglycan complexes in the outer membrane [24]. The model in equation 2 gives the effect of significant and most important variables on lipase activity.

$$Y = 6.5392 + 0.4275 * \text{Plam oil} + 0.2392 * \text{Yeast extract} - 0.1275 * KH_2PO_4 \ldots\ldots \quad (2)$$

The goodness of fit model was checked by the determination of coefficient (R^2) which indicated that the model could explain up to 98.67% variation of the data.

TABLE IV
ANALYSIS OF VARIANCE (ANOVA) FOR LINEAR MODEL ON EFFECT OF INDEPENDENT VARIABLES ON LIPASE PRODUCTION

Source	DF	SS	ASS	MSS	F	P
Main Effects	9	6.03021	6.03021	0.67002	16.45	0.059
Residual Error	2	0.08148	0.08148	0.04074		
Total	11	6.11169				

DF- Degree of freedom; SS- Sum of squares; ASS- Adjusted sum of squares; MSS- Mean sum of squares; F – Fishers's function; p – Corresponding level of significance

Table 4 shows the Analysis of Variance (ANOVA) for linear model on effect of independent variables on lipase production. A similar result was obtained by Baskar and Renganathan [25] in the optimization of L-asparaginase by *Aspergillus terreus* MTCC 1782.

Hymavathi et al. [26] reported that the Pareto chart of effects was plotted for identifying the factors that are important for enzyme production and shows the factors main effect estimates on the horizontal axis. The selected factors main effects are rank ordered according to their significance. The chart also shows a vertical line indicating statistical

significance (p = 0.10). It is evident from the Pareto chart of process variables (Fig. 1) that the most important for lipase production is palm oil, followed by incubation period, $CaCl_2$ and yeast extract.

Fig. I Effect of medium components and operating conditions on lipase activity

IV. Conclusion

The evaluation of the medium components and operating conditions for lipase production was done using the Plackett-Burman statistical method which offers efficient methodology to identify the significant variables with minimum number of experiments for extracellular lipase production by *Trichoderma viride* using palm oil as a substrate. The effect of nine medium components and operating conditions were studied and among them palm oil, incubation period, $CaCl_2$ and yeast extract were found to be the significant variables for lipase production by *Trichoderma viride* as the percentage confidence level was more than 90%. The significant factors identified by Plackett-Burman design are considered for the next stage of the medium optimization using response surface optimization technique for the future study.

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