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Optimization of Lipase Production Using *Bacillus subtilis* by Response Surface Methodology

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Abstract**—**A total of 6 isolates of *Bacillus subtilis* were isolated from oil mill waste collected in Namakkal district, Tamilnadu, India. The isolated bacteria were screened using lipase screening medium containing Tween 80. BS-3 isolate exhibited a greater clear zone than the others, indicating higher lipase activity. Therefore, this isolate was selected for media optimization studies. Ten process variables were screened using Plackett–Burman design and were further optimized by central composite design of response surface methodology for lipase production in submerged fermentation. Maximum lipase production of 16.627 U/min/ml were predicted in medium containing yeast extract (9.3636g), $CaCl_2$ (0.8986g) and incubation periods (1.813 days). A mean value of 16.98 ± 0.2286 U/min/ml of lipase was acquired from real experiments.

Keywords**—***Bacillus subtilis*, extracellular lipase, Plackett–Burman design, response surface methodology.

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I. Introduction

LIPASES are ubiquitous enzymes synthesized by the majority biological systems including animals, plants and microorganisms. Lipases are produced by many microorganisms include bacteria [1], fungi [2], yeast [3], and actinomycetes [4]. Among the lipase producing bacteria, several species of *Bacillus* such as *B. subtilis, B. pumilis, B. licheniformis, B. thermoleovorans, B. stearothermophilus,* and *B. sphaericus* possess lipases suitable for biotechnological appliance [5]-[7].

The existence of vegetable oil and its wastes or their hydrolysis products in the culture medium have an inducible effect on lipase production [8]. Extracellular lipase productions by bacteria are influenced by many physico-chemical (pH and temperature) and nutritional factors (carbon, nitrogen and lipid sources) [9], [10].

Statistical experimental designs reduce the error in determining the effect of variables and also enhances finding out the optimal conditions by establishing the relationship between factors and predicted responses [11]. In the first screening, it is recommended to assess the result and approximation the main effects according to a linear model through Plackett–Burman design which design can be used to find the significant variables in a system and allow them to be ranked in order of importance and to choose which one is to be investigated additional so as to determine the optimum values [12].

Response surface methods (RSM) experimental design is an efficient approach to deal with a large number of variables and there are several reports on application of RSM for the production of primary and secondary metabolites through microbial fermentation [13].

The main aim of the present work is to improve the yield of extracellular lipase activity by *Bacillus subtilis* using waste cooking oil by statistical methods like Plackett-Burman designs (PBD) and response surface method (RSM).

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II. Materials and Methods

A. Source of Collected Samples

The sample was collected from oil mill waste in Namakkal at January 2012. All the samples (500g each) were transported in plastic bags to the laboratory.

B. Isolation of Lipase Producing Microorganisms

One gram of sample was suspended in 10ml of sterile normal saline and it was kept at 80ºC for 10min. and subjected to serial dilution technique. The diluted samples were spread on sterile olive oil medium containing 20% of olive oil as lipid source and in addition it also contained urea, a protein source to prevent the growth of other organisms. The plates were incubated at 37ºC for 2 days.

C. Screening of Lipase Producing Microorganisms

The colonies grown on medium were patched on the lipase screening medium. The plates were incubated at 37ºC for 3-4 days. Colonies which produced lipase formed clear zones around itself in the medium due to hydrolysis of Tween 80, the only carbon source in the medium [14]. The diameter of the zones around the colonies was measured in terms of millimeter.

D. Production of Lipase

The production media was prepared in 100ml of Erlenmeyer flask containing 50ml of broth prepared based on design with 5ml of waste cooking palm oil. The medium was sterilized autoclaving at 121ºC for 20min. and then it was inoculated with bacterial suspension. Medium optimization for lipase production was done in the submerged fermentation and waste palm oil as a carbon and energy source. The nutritional components and operating conditions such as glucose, yeast extract, NaCl, $CaCl_2$, KH_2PO_4, K_2HPO_4, incubation periods, pH and temperature were designed according to the statistical

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designs.

E. Plackett-Burman Design for Selection of Process Variable for Extracellular Lipase Production

The Plackett–Burman statistical experimental 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 [15].

To evaluate the effect of 10 factors of medium components and operating conditions on lipase activity PB factorial design in 12 experimental run was carried out. Ten 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 glucose, yeast extract, NaCl, $CaCl_2$, KH_2PO_4, K_2HPO_4, incubation periods, pH and temperature and 1 unassigned variables at same level were studied (Tables I and II). The media prepared as per design and inoculated with equal volume of 24h culture suspension (3% v/v) and incubated prescribed periods.

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

Process variables	Glucose (g)	Yeast extract (g)	NaCl (g)	$MgSO_4$ (g)	$CaCl_2$ (g)	KH_2PO_4 (g)	K_2HPO_4 (g)	Incubation periods	pH	Temperature
Low level (-1)	1	4	1	0.2	0.5	0.1	0.1	1	6	30
High Level (+1)	3	8	3	0.6	1	0.5	0.5	3	8	40

TABLE II
PLACKETT-BURMAN DESIGN OF 12 RUNS FOR CODED VALUES OF 11 VARIABLES OF LIPASE PRODUCTION IN FERMENTATION BROTH

Run No.	Glucose (g)	Yeast extract (g)	NaCl (g)	$MgSO_4$ (g)	$CaCl_2$ (g)	KH_2PO_4 (g)	K_2HPO_4 (g)	Incubation periods	pH	Temperature	DV-1	Experimental Lipase activity (U /ml /min)	Predicted Lipase activity (U /ml /min)
1	+1	-1	+1	-1	-1	-1	+1	+1	+1	-1	+1	11.00	11.02083
2	+1	+1	-1	+1	-1	-1	-1	+1	+1	+1	-1	12.75	12.72917
3	-1	+1	+1	-1	1	-1	-1	-1	+1	+1	+1	14.50	14.52083
4	+1	-1	+1	+1	-1	+1	-1	-1	-1	+1	+1	12.25	12.27083
5	+1	+1	-1	+1	+1	-1	+1	-1	-1	-1	+1	14.25	14.27083
6	+1	+1	+1	-1	+1	+1	-1	+1	-1	-1	-1	13.50	13.47917
7	-1	+1	+1	+1	-1	+1	+1	-1	+1	-1	-1	12.75	12.72917
8	-1	-1	+1	+1	+1	-1	+1	+1	-1	+1	-1	12.25	12.22917
9	-1	-1	-1	+1	+1	+1	-1	+1	+1	-1	+1	11.50	11.52083
10	+1	-1	-1	-1	+1	+1	+1	-1	+1	+1	-1	12.50	12.47917
11	-1	+1	-1	-1	-1	+1	+1	+1	-1	+1	+1	11.50	11.52083
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	11.50	11.47917

F. Separation of Lipase

The samples were taken after incubation periods and the culture broth was centrifuged at 10,000rpm for 10min. The supernatant or crude enzyme extract was filtered and stored at 4ºC till the assay for lipolytic activity is performed.

G. Determination of Enzyme Activity by Emulsified Free System Method

The 250ml of Erlenmeyer flask containing 2ml of 0.1M of phosphorus buffer, 1ml of olive oil and 1ml of crude enzyme extract was incubated at 40ºC for 10 min. The reaction was stopped by the addition of 5ml of ethanol and it was titrated against 0.1N NaOH using phenolphthalein as indicator. The appearance of pale pink color was end point [16].

Lipase activity was calculated using the following formula,

Lipase activity (μg/ml/min) = Volume of alkali consumed x Normality of NaOH / Time of incubation x Volume of enzyme solution

H. Central Composite Design for Optimization of Media for Extracellular Lipase Production

Once critical factor were identified via screening, the central composite design (CCD) was proceeded to obtain a quadratic model, consisting of factorial trails and star points to estimate quadratic effects and central points to estimate the pure process variability with lipase production as response. The effect of the parameters yeast extract, $CaCl_2$ and incubation periods on the production of extracellular lipase was studied at five experimental levels: -α, -1, 0, +1, +α, where $\alpha = 2^{n/3}$, here n was the number of variables and 0 corresponded to the central point. The remaining parameters always maintained at middle level of PB design. The levels of factors used for experimental design are given in Table III and design of factorial, axial and center point was noted in Table IV.

TABLE III
THE CODED AND ACTUAL VALUES OF THE VARIABLES IN CENTRAL COMPOSITE DESIGN

Name	Unit	-α	-1	0	+1	+α
Yeast extract	g	2.636414	4	6	8	9.363586
$CaCl_2$	g	0.329552	0.5	0.75	1	1.170448
Incubation periods	days	0.318207	1	2	3	3.681793

TABLE IV
CENTRAL COMPOSITE DESIGN (CCD) OF FACTORS IN CODED VALUE FOR OPTIMIZATION OF FERMENTATION MEDIA

Trails	Type	Yeast Extract	$CaCl_2$	Incubation periods	Experimental Lipase activity (U/ml/min)	Predicted Lipase activity (U/ml/min)	Residual
1	Factorial	-1	-1	-1	13.25	13.25653	-0.00653
2	Factorial	1	-1	-1	15.25	13.77682	1.473182
3	Factorial	-1	1	-1	10.25	9.004897	1.245103
4	Factorial	1	1	-1	14.50	14.40018	0.099816
5	Factorial	-1	-1	1	14.75	14.2088	0.541202
6	Factorial	1	-1	1	12.25	12.85408	-0.60408
7	Factorial	-1	1	1	10.50	11.33216	-0.83216
8	Factorial	1	1	1	15.50	14.85245	0.647549
9	Axial	-1.68179	0	0	12.50	12.75442	-0.25442
10	Axial	1.681793	0	0	15.50	16.15212	-0.65212
11	Axial	0	-1.68179	0	12.00	12.52565	-0.52565
12	Axial	0	1.681793	0	10.25	10.63089	-0.38089
13	Axial	0	0	-1.68179	11.25	12.61273	-1.36273
14	Axial	0	0	1.681793	14.25	13.7938	0.456198
15	Center	0	0	0	15.00	15.01574	-0.01574
16	Center	0	0	0	15.25	15.01574	0.234256
17	Center	0	0	0	15.00	15.01574	-0.01574
18	Center	0	0	0	14.75	15.01574	-0.26574
19	Center	0	0	0	15.25	15.01574	0.234256
20	Center	0	0	0	15.00	15.01574	-0.01574

The response variable was fitted by a second order model in order to correlate the response variable to the independent variables. The linear quadratic model with 3 variables expressed as:

$$Y=\beta 0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X_{12} + \beta_{22}X_{22} + \beta_{33}X_{32} + \beta_{12}X_1X_2+ \beta_{13}X_1X_3 + \beta_{23}X_2X_3$$

where y is the measured response, $\beta 0$ is the intercept term, β_1, β_2, β_3 are linear coefficient, β_{11}, β_{22}, β_{33} are quadratic coefficient, β_{12}, β_{13}, β_{23} are interaction coefficient and X_1, X_2, X_3 are coded independent variables.

I. Statistical Analysis

The statistical software package Minitab version 15 was used for regression and graphical analyses of the data obtained. The optimal concentrations of the critical variables were obtained by analyzing contour plots. The statistical analysis of the model was represented in the form of analysis of variance (ANOVA).

III. RESULTS AND DISCUSSION

Six isolates of *B. subtilis* were isolated from oil mill waste collected in Namakkal district, Tamil Nadu, India and were identified as *B. subtilis* based on various biochemical tests and designated as BS-1 to BS-6. Among 6 isolates, BS-3 selected as potential lipase producing isolate based on colonies with highest clear zone of hydrolyzing lipids on the plate, indicating higher lipase activity and used for further study (Table V).

TABLE V
SCREENING OF LIPASE PRODUCING *BACILLUS SUBTILIS*

S.NO	Name of *Bacillus subtilis* isolates	Zone of hydrolysis (Diameter in mm)
1	*Bacillus subtilis* –BS-1	19
2	*Bacillus subtilis* –BS-2	18
3	*Bacillus subtilis* –BS-3	24
4	*Bacillus subtilis* –BS-4	22
5	*Bacillus subtilis* –BS-5	17
6	*Bacillus subtilis* –BS-6	15

In Table VI, yeast extract (confidence level– 98.10%), $CaCl_2$ (97.60%) and incubation period (confidence level – 97.00%) were found as most important factors influences on the lipase production at 95% confidence interval. Pam oil and yeast extract acted as carbon source and nitrogen source respectively for lipase enzyme production was reported by Treichel et al. [17]. Wang et al. [18] reported production of a highly thermostable alkaline lipase by *Bacillus* strain A 30-1 (ATCC 53841) in a medium that contained yeast extract (0.1%) and ammonium chloride (1%) as nitrogen sources.

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TABLE VI
STATISTICAL ANALYSIS OF PLACKETT-BURMAN DESIGN ON LIPASE ACTIVITY BY *BACILLUS SUBTILIS* –BS-3

Term	Effect	Coef	T	P	Confidence interval at 95%
Constant		12.5208	601.00	0.001	99.90*
Glucose	0.3750	0.1875	9.00	0.070	93.00
Yeast Extract	1.3750	0.6875	33.00	0.019	98.10*
NaCl	0.3750	0.1875	9.00	0.070	93.00
$MgSO_4$	0.2083	0.1042	5.00	0.126	87.40
$CaCl_2$	1.1250	0.5625	27.00	0.024	97.60*
KH_2PO_4	-0.3750	-0.1875	-9.00	0.070	93.00
K_2HPO_4	-0.2917	-0.1458	-7.00	0.090	91.00
Incubation periods	-0.8750	-0.4375	-21.00	0.030	97.00*
pH	-0.0417	-0.0208	-1.00	0.500	50.00
Temperature	0.2083	0.1042	5.00	0.126	87.40
R-Sq = 99.96% ; R-Sq(adj) = 99.58%					

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

The low incubation periods in this experiment model coincide with the data presented for Hasan and Hameed [19] who reported that the *Bacillus* culture supernatant obtained after 48h of incubation showed the maximum lipase activity but Selva Mohan et al. [20] studied that *Bacillus* strains showed maximum activity during 24h of the culture period. The enzyme production was reasonably improved in the presence of Ca^{2+} which is significant in maintaining cell wall rigidity, stabilizing oligomeric proteins and covalently bounding protein peptidoglycan complexes in the outer membrane [21]. Lipase production by various *Bacillus* sp. was stimulated in the presence of Ca^{2+} [22]. The goodness of fit model was checked by the determination of coefficient (R^2) which indicated that the model could explain up to 99.96% variation of the data.

Hymavathi et al. [23] 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 (Fig. 1)

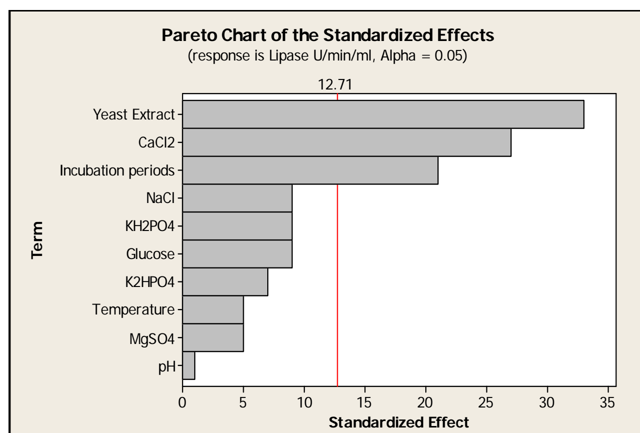

Fig. 1 Pareto Chart of Effects of Process Variables for Extra-Cellular Lipase Production

A central composite design (CCD) was employed within a range of -1.68179 to + 1.68179 in relation to production of lipase production. The results indicated that there was a variation of lipase production in the thirty one trials in the range from 10.25 to 15.50U/ml/min (Table V). The coefficients t and p values for linear, quadratic and combined effects are given at 95% significance level.

In Table VII, revealed that the coefficient for overall effect of the variables had high significance (p=0.000) on extracellular lipase production. The individual effect of yeast extract (p=0.002) and $CaCl_2$ (p=0.049), quadratic effect of $CaCl_2$ (p=0.001) and interaction effect of yeast extract * $CaCl_2$ (p=0.004) are found the most significant factor on extracellular lipase production.

TABLE VII
ESTIMATED REGRESSION COEFFICIENTS FOR LIPASE PRODUCTION

Term	Coef	T	P
Constant	15.0157	39.568	0.000*
Yeast Extract (X_1)	1.0101	4.012	0.002*
$CaCl_2$(X_2)	-0.5633	-2.237	0.049*
Incubation periods(X_3)	0.3511	1.395	0.193
Yeast Extract*Yeast Extract (X_1^2)	-0.1989	-0.811	0.436
$CaCl_2$*$CaCl_2$ (X_2^2)	-1.2153	-4.958	0.001*
Incubation periods* Incubation periods (X_3^2)	-0.6408	-2.614	0.026*
Yeast Extract*$CaCl_2$ (X_1*X_2)	1.2187	3.705	0.004*
Yeast Extract*Incubation periods(X_1*X_3)	-0.4688	-1.425	0.185
$CaCl_2$*Incubation periods (X_2*X_3)	0.3437	1.045	0.321
R-Sq = 87.35% ; R-Sq(adj) = 75.96%			

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

The correlation measures for testing the goodness of fit of the regression equation is the coefficient of determination (R^2). When R^2 is closer to 1, correlation is better between the experimental values and the values predicted by the second order polynomial model [24], [25]. In the present experiments, the high R^2 (0.8735) implies high degree of correlation between the observed and predicted values. The adjusted R^2 in this study was 0.7596, which is close to the R^2 value. Hence the model was well fitted to represent the effect of these three variables on lipase production using CCD statistical optimization.

The interaction effect of each variables revealed that the lipase activity would increase as yeast extract and $CaCl_2$ increase, but further increases in $CaCl_2$ after the optimal point would reverse the trend (Fig. 2).

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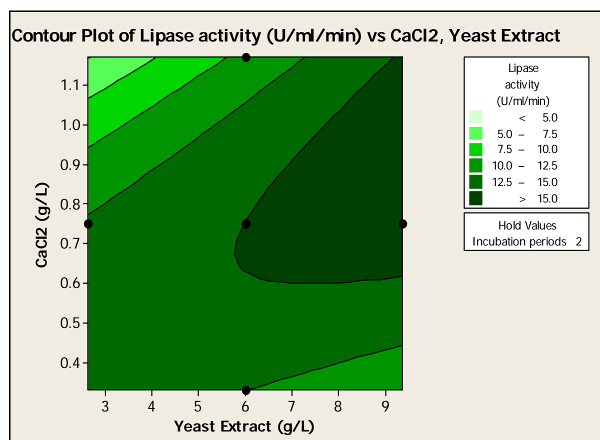

Fig. 2 Interaction Effect of Yeast Extract and $CaCl_2$ on Extracellular Lipase Production

Similar effects on lipase activity were observed for yeast extract and incubation periods (Fig. 3). The interaction effects of incubation periods and $CaCl_2$ showed that lipase activity increased with increase of these factors but beyond optimal level lipase activity decreased (Fig. 4).

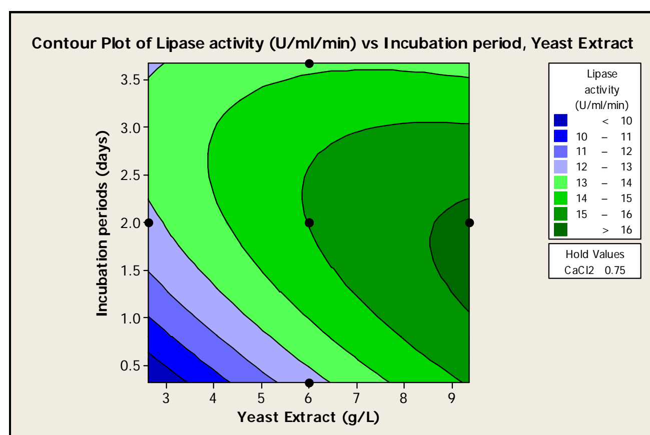

Fig. 3 Interaction Effect of Yeast Extract and Incubation Periods on Extracellular Lipase Production

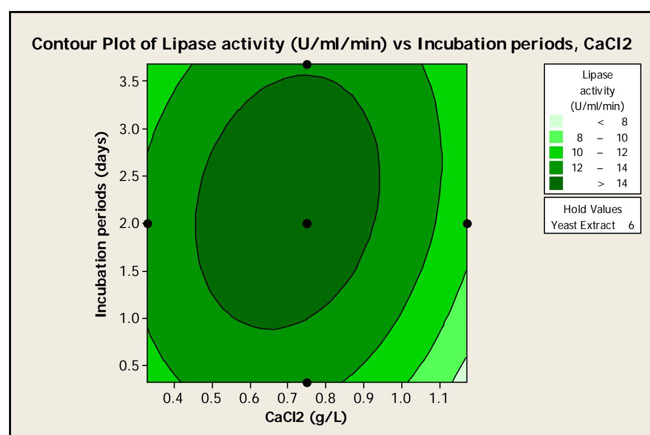

Fig. 4 Interaction Effect of $CaCl_2$ and Incubation Periods on Extracellular Lipase Production

The regression equation was solved by optimizer in MINITAB 15.0 software for optimum value of the variables for maximum extracellular lipase production. The optimum value of the variables in actual unit was predicted as yeast extract (9.3636g), $CaCl_2$ (0.8986g) and incubation periods (1.813 days) with the predicted maximum extracellular lipase production of 16.627U/min/ml of fermented media. A mean value of 16.98 ± 0.2286 U/min/ml of lipase was acquired from real experiments was represented in the form of analysis of variance (ANOVA).

V. Conclusion

The use of an experimental design where the main point was to reveal the influence of concentrations of macro-nutrients on extracellular lipase production allowed the rapid screening of large experimental domain in search of the best culture conditions for optimization of extracellular lipase production by *Bacillus subtilis* BS-3. The chosen method of optimization of medium composition and environmental factors was efficient, relatively simple, and time and material saving.

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