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# STATISTICAL MODELING AND OPTIMIZATION OF KERATINASE PRODUCTION FROM NEWLY ISOLATED *BACILLUS SUBTILIS* RSE163

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## ABSTRACT

A newly isolated Bacillus subtilis RSE163 was selected to study the effect of physico-chemical parameters enhancing keratinase production. These parameters were optimized statistically in two steps. Preliminary screening selects the three most significant variables glucose, peptone and time for the second level optimization by Response Surface Methodology. According to the responses from the experimental design, the effects of each variable were calculated and the interaction between the three factors and its effect on enzyme production was clearly demonstrated by 3D contour plots. The final optimized medium gave a maximum yield of 1960 U/ml of keratinase at 48 h. The validation of the results was done in the bench scale experiments in lab.

**Keywords**: keratinase, optimization, physicochemical parameters, response surface methodology, plackett–burman

## [I] INTRODUCTION

Keratinases are the versatile, extraordinary proteases, have capability to hydrolyze insoluble, hard to debase keratin and keratinous waste obtained from poultry and leather industries [1] into valuable products like rare amino acids (serine, cysteine, and proline) [2] peptides, fertilizers, glues, films, foils [3] it is also used in detergent formulations for eliminating horny epithelial cells adhering to textile fibers pharmaceutical and cosmetic industries [4] and become a alternative resourceful low-cost, ecofreindly option for biotechnological applications[5]. Generally keratinases are produced by microorganisms, when it acts on the keratin substrate in nature [6]. There are various studies have been reported including keratinases from fungi *Microsporum* [7], *Trichophyton* [8] as well as from bacteria, *Bacillus Fervidobacterium, bacillus licheniformis, bacillus pumilus* [9] *Chryseobacterium sp., Streptomyces* [10].*Bacillus* sp. are the predominant one.

Nurturing conditions are crucial in successful production of an enzyme, physicochemical parameters such as pH, temperature and media compositions are imperative in developing this process. Medium Optimization by the traditional method is immensely time

consuming and costly when a large number of variables are examine, it involves changing one independent variable while all others variables remain constant at a fixed level [2]. To overcome this complexity, experimental factorial design and response surface methodology can be employed to optimize the medium components. The statistical approaches like placket burman method and central composite design have been previously used by several researchers for media ingredient optimization.

## [II] MATERIALS AND METHODS

### 2.1 Isolation, identification and screening of keratinase producing strain.

Soil samples have been taken from different local poultry farms and about 12 bacterial strains have been isolated and their primary screening was done by streaking the cultures on milk agar plate, incubated at 37˚C for overnight, strain showed maximum clear zone on milk agar plate were selected for further experimentation. Identification of selected strain was done using 16S rDNA typing from total genomic DNA and DNA sequence was submitted to NCBI Gene bank. Stock culture of the organism was maintained at -20°C in 50% glycerol.

### 2.2 Keratinase production and Enzyme assay

To stimulate the keratinase production, basic media were used consists of glucose 1%, peptone 1%, $KH_2PO_4$ 0.9%, $K_2HPO_4$ 0.3% Feather 0.5%. This production media was inoculated with 1% selected bacterial culture and incubated at 37 ˚C at 180 rpm for 3 to 5 days, keratinase activity was determined by using the cell free extract of feather media and feather as substrate. An increase of 0.01 absorbance was considered as 1 Unit of keratinase per ml in 1 hour at 280 nm under the assay conditions.

### 2.3 Factorial design and model

Media for keratinase production by *Bacillus subtilis* RSE163 was optimized by statistical design experiments in two steps (Design Expert® 8.0.2.0 Stat-Ease, Inc., Minneapolis, MN, USA). In first step the screening of variables was done by Plackett- Burman which is a two-level design for examining n parameters in k = n+1 runs. In this part, the selected carbon (glucose), nitrogen source (peptone) and substrate source feather were optimized together with eight variables: yeast $K_2HPO_4$, NaCl, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, $KH_2PO_4$, $NaNO_3$, $NH_4Cl$ and three physical parameters including agitation, inoculum size and time. The effect of each variable was determined by the following equation: $E(x_i) = 2(\sum M_i^+ - M_i^-) / N$

Where $E(x_i)$ is the concentration effect of the tested variable, $M_i^+$ and $M_i^-$ are the total production from the trials where the measured variable ($x_i$) was present at high and low concentrations, respectively; and N is the number of trials.

The second step RSM includes the optimization of major variables glucose, peptone and time screened by placket burman design. RSM is basically a two factor experimental design where each factor is studied at three levels. The general model equation of

$$Y = \beta o + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

The statistical analysis of the data obtained from RSM for keratinase production was subjected to analysis of variance (ANOVA).

## [III] RESULTS AND DISCUSSION

### 3.1 Keratinase production and Enzyme assay

The selected isolate was identified as *Bacillus subtilis* RS E163 (NCBI Accession No. JQ887983). The maximum keratinase production in the basal media was 488 U/ml at 5 days of incubation, which is too low as no

other ingredients was added besides carbon and nitrogen source, to enhance the keratinase production and study the effect of physicochemical parameters, some other new factors were added and optimization was done through statistically.

### 3.2 Screening of significant variables by Plackett–Burman design

First step of optimization using placket burman design identified the significant effect of 14 variables on keratinase production given in table 1a, and table 1b represents the $E(x_i)$ value with ranking of investigated variables. Maximum value of $E(x_i)$ coefficient, either positive or negative, point to great impact on response; whereas a coefficient close to zero indicates little or no effect [11]. Accept agitation, inoculums and $KH_2PO_4$ all the factors had positive effect on keratinase production. The proficiency of the model was calculated, and the variables statistically significant effects were screened via Student's t-test with

| | Factor 1 | Factor 2 | Factor 3 | Factor 4 | Factor 5 | Factor 6 | Factor 7 | Factor 8 | Factor 9 | Factor 10 | Factor 11 | Factor 12 | Factor 13 | Factor 14 | Response 1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Std** | **A: Glucose** | **B: Peptone** | **C: Yeast** | **D: CaCl2** | **E :K2HPO4** | **F: KH2PO4** | **G :NaCl** | **H: NaNo3** | **J: MgSo4** | **K: NH4Cl** | **L: Feather** | **M: Agitation** | **N: Inoculum** | **O:time** | **R1** |
| | % | % | % | % | % | % | % | % | % | % | % | Hrs | % | rpm | |
| 1 | 2 | 1 | 0.01 | 0 | 0.5 | 0.4 | 0.1 | 2 | 0.01 | 0.05 | 0.5 | 200 | 1 | 24 | 384 |
| 2 | 0.5 | 1 | 0.5 | 0 | 0 | 0.4 | 0.1 | 2 | 0.1 | 0.01 | 1 | 140 | 3 | 24 | 400 |
| 3 | 2 | 0.02 | 0.5 | 0.02 | 0 | 0 | 0.1 | 2 | 0.1 | 0.05 | 0.5 | 200 | 1 | 72 | 403 |
| 4 | 2 | 1 | 0.01 | 0.02 | 0.5 | 0 | 0.05 | 2 | 0.1 | 0.05 | 1 | 140 | 3 | 24 | 550 |
| 5 | 0.5 | 1 | 0.5 | 0 | 0.5 | 0.4 | 0.05 | 0.5 | 0.1 | 0.05 | 1 | 200 | 1 | 72 | 347 |
| 6 | 0.5 | 0.02 | 0.5 | 0.02 | 0 | 0.4 | 0.1 | 0.5 | 0.01 | 0.05 | 1 | 200 | 3 | 24 | 202 |
| 7 | 0.5 | 0.02 | 0.01 | 0.02 | 0.5 | 0 | 0.1 | 2 | 0.01 | 0.01 | 1 | 200 | 3 | 72 | 250 |
| 8 | 0.5 | 0.02 | 0.01 | 0 | 0.5 | 0.4 | 0.05 | 2 | 0.1 | 0.01 | 0.5 | 200 | 3 | 72 | 208 |
| 9 | 2 | 0.02 | 0.01 | 0 | 0 | 0.4 | 0.1 | 0.5 | 0.1 | 0.05 | 0.5 | 140 | 3 | 72 | 278 |
| 10 | 0.5 | 1 | 0.01 | 0 | 0 | 0 | 0.1 | 2 | 0.01 | 0.05 | 1 | 140 | 1 | 72 | 387 |
| 11 | 2 | 0.02 | 0.5 | 0 | 0 | 0 | 0.05 | 2 | 0.1 | 0.01 | 1 | 200 | 1 | 24 | 286 |
| 12 | 0.5 | 1 | 0.01 | 0.02 | 0 | 0 | 0.05 | 0.5 | 0.1 | 0.05 | 0.5 | 200 | 3 | 24 | 152 |
| 13 | 2 | 0.02 | 0.5 | 0 | 0.5 | 0 | 0.05 | 0.5 | 0.01 | 0.05 | 1 | 140 | 3 | 72 | 500 |
| 14 | 2 | 1 | 0.01 | 0.02 | 0 | 0.4 | 0.05 | 0.5 | 0.01 | 0.01 | 1 | 200 | 1 | 72 | 206 |
| 15 | 2 | 1 | 0.5 | 0 | 0.5 | 0 | 0.1 | 0.5 | 0.01 | 0.01 | 0.5 | 200 | 3 | 24 | 316 |
| 16 | 2 | 1 | 0.5 | 0.02 | 0 | 0.4 | 0.05 | 2 | 0.01 | 0.01 | 0.5 | 140 | 3 | 72 | 450 |
| 17 | 0.5 | 1 | 0.5 | 0.02 | 0.5 | 0 | 0.1 | 0.5 | 0.1 | 0.01 | 0.5 | 140 | 1 | 72 | 438 |
| 18 | 0.5 | 0.02 | 0.5 | 0.02 | 0.5 | 0.4 | 0.05 | 2 | 0.01 | 0.05 | 0.5 | 140 | 1 | 24 | 504 |
| 19 | 2 | 0.02 | 0.01 | 0.02 | 0.5 | 0.4 | 0.1 | 0.5 | 0.1 | 0.01 | 1 | 140 | 1 | 24 | 395 |
| 20 | 0.5 | 0.02 | 0.01 | 0 | 0 | 0 | 0.05 | 0.5 | 0.01 | 0.01 | 0.5 | 140 | 1 | 24 | 134 |

**Table: 1a.** Design Placket Burman

| Variable | Component | $M_i^+$– | $M_i^-$ | $E(x_i)$ | Absolute $E(x_i)$ | Ranking |
|---|---|---|---|---|---|---|
| A | Gluose | 376.8 | 302.2 | 74.6 | 74.6 | 4 |
| B | Peptone | 363 | 316 | 47 | 47 | 6 |
| C | Yeast | 384 | 294.4 | 89.6 | 89.6 | 2 |
| D | CaCl2 | 355 | 324 | 31 | 31 | 7 |
| E | K2HPO4 | 389.2 | 289.8 | 99.4 | 99.4 | 1 |
| F | KH2PO4 | 337.4 | 341.6 | -4.2 | 4.2 | 12 |

| | | | | | | |
|---|---|---|---|---|---|---|
| G | NaCl | 345.3 | 333.7 | 11.6 | 11.6 | 11 |
| H | NaNO3 | 382.2 | 296.8 | 85.4 | 85.4 | 3 |
| I | MgSo4 | 345.7 | 333.3 | 12.4 | 12.4 | 10 |
| J | NH4Cl | 370.7 | 308.3 | 62.4 | 62.4 | 5 |
| K | Feather | 352.3 | 326.7 | 25.6 | 25.6 | 8 |
| L | Agitation | 257.4 | 403.6 | -146.2 | 146.2 | 14 |
| M | Inoculum | 330.9 | 384.4 | -53.5 | 53.5 | 13 |
| N | Time | 346.7 | 332.3 | 14.4 | 14.4 | 9 |

**Table:1b.** Ranking of the variables investigated in the Plackett–Burman design

95% confidence levels. Table 1c and 1d represents the effect of each variable along with the mean squares, F-values, p-values and regression values. The keratinase production varied from 130 to550 U/ml. The 69.91 F-value of model implies the model significance. There is only a 0.01% chance of non significance of model.Values of "Prob > F" less than 0.0500 indicate the significant effect on responses [12]. In this case A, B, C, D, E, H, K, L, M are significant model terms. P Values greater than 0.1000 indicate the model terms are not significant. This model can be used to navigate the design space and for further screening of the significant variables by response surface methodology [13].

### 3.3 Optimization of significant variables by response surface methodology

The optimal concentration of media components and incubation time were optimized by response surface methodology by choosing the three variables Glucose, peptone and time. The design matrix and associated results of RSM experiments to conclude the effects of three independent variables (glucose, peptone and time), along with the mean predicted values and the residual value are shown in Table 2a.

A maximum keratinase production was obtained about 1860 units/ml in the presence of 1.25% glucose, 1% peptone with 48 h of incubation time [14]. Analysis of variance of the quadratic regression model showed that the model is highly significant (29.16 F-value) (Table 2b). The response of keratinase production R1 by *Bacillus subtilis* RSE163. can be represents in terms of the following regression equation.

Keratinase Activity = +171.7 + 4.91 A + 3.04 B + 34.15 C + 0.75 AB + 1.00 AC + 0.000 BC - 4.56 A2 - 2.09 B2 - 23.12 C2

Where A BC belongs to glucose, peptone and time.

Based on the F-test, the predictive valueR-Squared value of 0.8086 is very high with reference to value of adjusted determination coefficient "Adj R-Squared" 0.9302 which is in reasonable agreement with the "Adeq Precision" measures the signal to noise ratio (Table 2c). The R-squared value provided a measure of how much of the variability in the observed response values could be explained by the experimental factors and their interactions. These interactions effect and variable responses were studied by response surface plots shown in fig 1a, 1b an

| | **Sum of** | | **Mean** | **F** | **p-value** | |
|---|---|---|---|---|---|---|
| **Source** | **Squares** | **df** | **Square** | **Value** | **Prob > F** | |
| Model | 2793 | 14 | 199.4972857 | 69.91074 | < 0.0001 | significant |
| A-Glucose | 278.3 | 1 | 278.258 | 97.51121 | 0.0002 | |
| B-Peptone | 110.5 | 1 | 110.45 | 38.70549 | 0.0016 | |
| C-yeast | 406.8 | 1 | 406.802 | 142.5575 | < 0.0001 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| D-CaCl2 | 48.05 | 1 | 48.05 | 16.83838 | 0.0093 | |
| E-K2HPO4 | 494 | 1 | 494.018 | 173.121 | < 0.0001 | |
| F-KH2PO4 | 0.882 | 1 | 0.882 | 0.309083 | 0.6022 | |
| G-NaCl | 6.728 | 1 | 6.728 | 2.357724 | 0.1853 | |
| H-NaNo3 | 364.7 | 1 | 364.658 | 127.7888 | < 0.0001 | |
| J-MgSo4.7H2O | 7.688 | 1 | 7.688 | 2.694141 | 0.1616 | |
| K-NH4Cl | 194.7 | 1 | 194.688 | 68.2254 | 0.0004 | |
| L-Feather | 32.77 | 1 | 32.768 | 11.48304 | 0.0195 | |
| M-agitation | 821.8 | 1 | 821.762 | 287.9738 | < 0.0001 | |
| N-Inoculum | 15.84 | 1 | 15.842 | 5.551584 | 0.0651 | |
| O-time | 10.37 | 1 | 10.368 | 3.633305 | 0.1150 | |
| Residual | 14.27 | 5 | 2.8536 | | | |
| Cor Total | 2807 | 19 | | | | |

**Table: 1c.** ANOVA for Placket Burman

| | | | |
|---|---|---|---|
| **Std. Dev.** | 1.689 | **R-Squared** | 0.994917 |
| **Mean** | 33.95 | **Adj R-Squared** | 0.980686 |
| **C.V. %** | 4.976 | **Pred R-Squared** | 0.918679 |
| **PRESS** | 228.3 | **Adeq Precision** | 28.70927 |

**Table: 1d.** Regression values by Plackett burman

| **Factor 1** | **Factor 2** | **Factor 3** | **Actual value** | **Predicted value** | **Residual** |
|---|---|---|---|---|---|
| **A:Glucose** | **B:peptone** | **C:time** | | | |
| % | % | % | | | |
| 0.5 | 0.5 | 24 | 1050 | 1010.6291 | 3.370923 |
| 2 | 0.5 | 24 | 1110 | 1070.9409 | 3.059082 |
| 0.5 | 1.5 | 24 | 1100 | 1060.2132 | 3.786784 |
| 2 | 1.5 | 24 | 1150 | 1150.5251 | -0.52506 |
| 0.5 | 0.5 | 72 | 1640 | 1670.9326 | -3.9326 |
| 2 | 0.5 | 72 | 1700 | 1780.2444 | -8.24444 |
| 0.5 | 1.5 | 72 | 1650 | 1720.5167 | -7.51674 |
| 2 | 1.5 | 72 | 1780 | 1850.8286 | -7.82858 |
| -0.01 | 1 | 48 | 1510 | 1500.5972 | 0.402781 |
| 2.51 | 1 | 48 | 1730 | 1670.0987 | 5.901297 |
| 1.25 | 0.15 | 48 | 1620 | 1600.7318 | 1.268169 |
| 1.25 | 1.84 | 48 | 1760 | 1700.9641 | 5.035908 |
| 1.25 | 1 | 7.63 | 410 | 480.91177 | -7.91177 |
| 1.25 | 1 | 88.36 | 1780 | 1630.7841 | 14.21585 |
| 1.25 | 1 | 48 | 1640 | 1710.7469 | -7.74693 |
| 1.25 | 1 | 48 | 1670.4 | 1710.7469 | -4.34693 |
| 1.25 | 1 | 48 | 1650 | 1710.7469 | -6.74693 |
| 1.25 | 1 | 48 | 1700 | 1710.7469 | -1.74693 |
| 1.25 | 1 | 48 | 1860 | 1710.7469 | 14.25307 |
| 1.25 | 1 | 48 | 1770 | 1710.7469 | 5.253065 |

**Table:2a.** Central composite design matrix for the experimental design and predicted responses for keratinase activity

| | **Sum of** | | **Mean** | **F** | **p-value** | |
|---|---|---|---|---|---|---|
| **Source** | **Squares** | **Df** | **Square** | **Value** | **Prob > F** | |
| Model | 24178 | 9 | 2686.4445 | 29.15511097 | < 0.0001 | Significant |
| A-Glucose | 328.6939 | 1 | 328.69394 | 3.567208801 | 0.0882 | |
| B-peptone | 126.3831 | 1 | 126.38308 | 1.371594596 | 0.2687 | |
| C-time | 15928.57 | 1 | 15928.573 | 172.8676394 | < 0.0001 | |
| AB | 4.5 | 1 | 4.5 | 0.048837041 | 0.8295 | |
| AC | 8 | 1 | 8 | 0.086821407 | 0.7743 | |
| BC | 3.64E-12 | 1 | 3.638E-12 | 3.94818E-14 | 1.0000 | |
| A^2 | 299.7252 | 1 | 299.72516 | 3.252819949 | 0.1015 | |
| B^2 | 62.68531 | 1 | 62.685312 | 0.680303365 | 0.4287 | |
| C^2 | 7704.683 | 1 | 7704.6826 | 83.61642272 | < 0.0001 | |
| Residual | 921.4317 | 10 | 92.143174 | | | |
| Lack of Fit | 563.3984 | 5 | 112.67968 | 1.573592059 | 0.3155 | not significant |
| Pure Error | 358.0333 | 5 | 71.606667 | | | |
| Cor Total | 25099.43 | 19 | | | | |

**Table:2b.** Analysis of variance table (ANOVA for Response Surface Quadratic Model CCD)

| **Std. Dev.** | 9.599124 | | **R-Squared** | 0.963288741 |
|---|---|---|---|---|
| **Mean** | 151.42 | | **Adj R-Squared** | 0.930248608 |
| **C.V. %** | 6.339403 | | **Pred R-Squared** | 0.808648351 |
| **PRESS** | 4802.818 | | **Adeq Precision** | 20.17159153 |

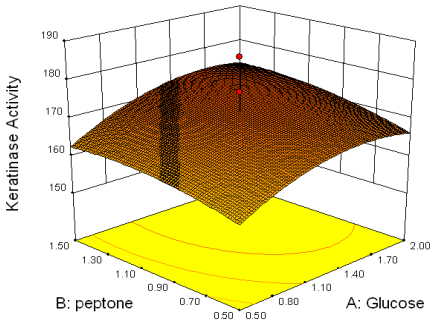
**Table: 2c.** Regression values by CCD

| **Run** | **A:Glucose** | **B:peptone** | **C:time** | **Actual** | **predicted** | **residual** |
|---|---|---|---|---|---|---|
| 1 | 0.5 | 1.5 | 72 | 1690 | 1730.8913 | -4.89125 |
| 2 | -0.01 | 1 | 48 | 1510 | 1500.833 | 0.167012 |
| 3 | 1.25 | 0.15 | 48 | 1620 | 1600.242 | 1.757988 |
| 4 | 1.25 | 1.84 | 48 | 500 | 570.24228 | -7.24228 |
| 5 | 1.25 | 1 | 48 | 1860 | 1820.5452 | 3.454849 |
| 6 | 1.25 | 1 | 48 | 1960 | 1820.5452 | 13.45485 |

**Table: 3.** Validation of the model

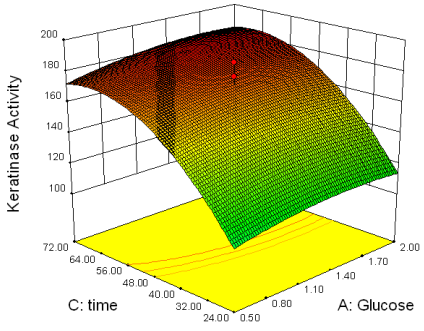
**Figure 1**. Contour plots of enzyme activity as a function of the interactions of two variables by keeping the other at centre level: (a) interactions of peptone and glucose with time, (b) interactions of time and Glucose with peptone at 1%, and interactions of peptone and (c) time with glucose at 1.25%.

(a)

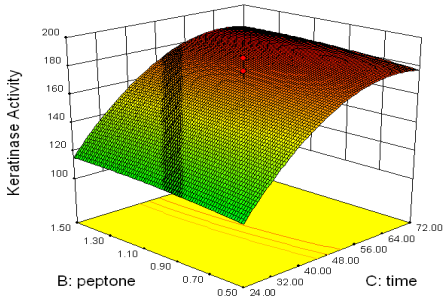



Actual Factor C Time 48 hrs

(b)




Actual Factor B Peptone 1.00




(c) Actual Factor A Glucose 1.2

which is a pair wise combinations of the three factors by keeping the one at its optimum level [15,16].

### 3.4 Adequacy of the model

Adequacy of the model was confirmed by experimental rechecking using a random set of six production combinations to test for keratinase production [15,17]. Final optimized conditions revealed the predicted response for keratinase production was 1820U/ml, and the observed validated experimental value was 1960U/ml shown in table 3. Thus the validity of the model became confirmed, and the experimental values were found to be fairly close to the predicted values.

## [IV] CONCLUSION

The present study reveals that newly isolated *Bacillus subtilis* RSE163 is a potential keratinase producer. The response surface technology used in the study provides information on the growth requirement of this organism and also helps for choosing the optimized conditions for keratinase production. Statistical optimization (CCD) of media increases the keratinase production up to 4 folds (1960U/mL) using1.25% glucose, 1% peptone with 48

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