Response surface optimization of effective medium constituents for the production of alkaline protease from a newly isolated strain of *Pseudomonas aeruginosa*

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Optimization of the fermentation medium for maximum alkaline protease production was carried out with a new strain of *Pseudomonas aeruginosa* (B-2). Replacing the protein source/inducer (albumin in place of casein) brought about significant increase in yield after 48 hr of inoculation. The three most effective medium constituents identified by initial screening method of Plackett-Burman were albumin, \((\text{NH}_4)_2\text{SO}_4\) and glucose. Central Composite Design (CCD) and Response Surface Methodology (RSM) were used in the design of the experiment and in the analysis of the results. Optimum levels of the effective medium constituents were albumin (6.586%); \((\text{NH}_4)_2\text{SO}_4\), 0.164%; and glucose, 6.72%. The alkaline protease production increased from 533460 to 793492 UI\(^1\). The present study is a novel approach on formulating a suitable production medium using statistical optimization that can substantially increase the alkaline protease production for *Pseudomonas aeruginosa* (B-2).

**Keywords:** Central Composite Design, Response Surface Methodology, Proteases, Fermentation.

Proteases constitute one of the most important groups of industrial enzymes and have applications in different industries viz., detergent, food, pharmaceutical, leather and silk etc. Proteases account for 30% of the total worldwide enzyme production. *Bacillus*\(^1\)-\(^3\) and *Pseudomonas*\(^4\) are the potential producers of alkaline protease. Alkaline protease secreted by *Pseudomonas* is characterized by its high activity at alkaline pH, broad substrate specificity and organic solvent resistance\(^5\). These properties of alkaline proteases secreted by *Pseudomonas* make them suitable for use in the detergent industry.

Alkaline proteases are generally produced by submerged fermentation, normally optimization of medium composition is done so as to obtain maximum yield from minimum possible inputs, thus minimizing the amount of unutilized components at the end of fermentation. No defined medium has yet been established for optimum production of alkaline protease from *Pseudomonas aeruginosa*. The conventional method of optimization involves varying one parameter at a time and keeping the others at a fixed level is extremely time consuming and expensive when a large number of variables are evaluated at different levels. Response Surface Optimization of the three most effective constituents screened by Plackett-Burman design fairly reduces the total number of experiments required (only 20) and also manifests any possible interaction effect between the medium constituents. (N +1) No. of experiments in Plackett-Burman screening \{where N = no. of medium constituents + no. of dummy variables(D)\} followed by a \(2^3\) factorial central composite design of experiment\(^6\) and response surface optimization\(^7\)-\(^9\) was performed in the present investigation. All the experiments were carried out in triplicate and average values were reported.

The present study is a novel approach on formulating a suitable production medium using statistical optimization that can substantially increase the alkaline protease production for *Pseudomonas aeruginosa* (B-2).

**Materials and Methods**

Microorganism and inoculum preparation—A new strain of *Pseudomonas aeruginosa* B-2 isolated in our laboratory, was used as the producer of protease enzyme. Inoculum was prepared by transferring 5ml of suspension prepared from a fresh slant culture into Erlenmeyer flask (250 ml) containing 50 ml of sterile inoculum medium. The inoculum medium was nutrient broth (pH 7.2) consisting of beef extract, 10 g; peptone, 10 g and NaCl, 5 g and sterilized at 15 psi for 15 min. The flask was kept on a rotary shaker at 180 rpm at 37°C.

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Reference medium selection—Five ml of 24 hr old inoculum of *Pseudomonas aeruginosa* (B-2) was added to 50 ml of production medium in 250 ml Erlenmeyer flasks and incubated on a rotary shaker at 180 rpm 37°C for 48 hr. The compositions of two production medium tested were as follows:

1. **Casein medium:** Glucose, 5 g; casein, 2%; \((\text{NH}_4)_2\text{SO}_4, 1 \text{ g;} \quad \text{K}_2\text{HPO}_4, 3 \text{ g;} \quad \text{KH}_2\text{PO}_4, 1 \text{ g;} \quad \text{trace elements solution*}, 1 \text{ ml;} \quad \text{CaCl}_2, 100 \mu \text{g;} \quad \text{and Tween-80, 0.5%}. \quad \text{The pH was maintained at 7.}

2. **Albumin medium:** Glucose, 5 g; albumin, 2%; \((\text{NH}_4)_2\text{SO}_4, 1 \text{ g;} \quad \text{K}_2\text{HPO}_4, 3 \text{ g;} \quad \text{KH}_2\text{PO}_4, 1 \text{ g;} \quad \text{trace elements solution*}, 1 \text{ ml;} \quad \text{CaCl}_2, 100 \mu \text{g;} \quad \text{and Tween-80, 0.5%}. \quad \text{The pH was maintained at 7.}

Effect of glucose level on yield in two different production media (casein and albumin) at 24-120 hr of age was also studied (Table 1). Repression of protease yield due to higher glucose concentration \((\geq 1\%)) did not occur after 48 hr of fermentation in albumin medium, hence that was selected as the reference/starting medium for optimization studies.

**Plackett-Burman screening of effective medium constituents**—Simultaneous optimization of all the medium constituents requires a large number of experiments hence, three most effective medium constituents were screened and selected for optimization by Response Surface Methodology (RSM), this was done by Plackett-Burman screening method, the design of the experiment (runs 1-12) and the effect of the medium constituents has been shown in Table 2. Test of significance (Student’s *t* test, *P*-value and confidence level) of the effect determined the effectiveness of the medium constituents. A confidence level of more than 95% was considered to

### Table 1—Effect of culture age and varying concentration of glucose (0.1 – 3.0%) on yield in production medium containing casein or albumin

<table>
<thead>
<tr>
<th>Casein media*</th>
<th>Albumin media**</th>
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<tbody>
<tr>
<td>Age (hr)</td>
<td>0.10% 0.50% 1.00% 2.00% 3.00% 0.10% 0.50% 1.00% 2.00% 3.00%</td>
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<tr>
<td>0</td>
<td>0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00</td>
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<tr>
<td>24</td>
<td>243.38 386.74 200.04 133.36 126.69 300.06 420.08 400.08 333.40 326.73</td>
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<tr>
<td>48</td>
<td>366.74 453.42 286.72 253.38 173.37 413.42 433.44 473.45 526.79 533.46</td>
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<tr>
<td>72</td>
<td>386.74 446.76 406.75 320.06 246.72 446.76 433.44 513.46 533.46 526.77</td>
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<tr>
<td>96</td>
<td>433.42 453.42 453.42 413.42 293.39 433.44 446.76 513.46 533.46 533.46</td>
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<tr>
<td>120</td>
<td>440.09 446.76 453.42 453.42 386.74 413.42 433.44 513.46 513.46 513.46</td>
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</table>

*Media contained casein, 2%; and \((\text{NH}_4)_2\text{SO}_4, 0.1\%.

**Media contained albumin, 2%; and \((\text{NH}_4)_2\text{SO}_4, 0.1\%.

### Table 2—Design of experiments and significance of effects of constituents in Plackett-Burman Screening

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Albumin</th>
<th>((\text{NH}_4)_2\text{SO}_4)</th>
<th>Tween –80</th>
<th>Trace El.</th>
<th>CaCl_2</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
<th>D4</th>
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<th>Yield Uml(^{-1})</th>
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Effect -145.58 354.52 45.565 -27.78 7.7793 12.225
*t* value 7.9169 19.279 2.4778 -1.5110 0.4230 0.6648
*P* value 0.0000 0.000 0.0307 0.0159 0.06804 0.5199
Confidence level 100 100 96.9300 84.1000 31.9600 48.01

D=Dummy constituents/variable. Effect = significant for confidence level > 95. H=high; L=low
be significant. The high and low levels of medium constituents involved in Plackett-Burman Design have been shown in Table 3.

Varying concentration of albumin, (NH₄)₂SO₄ and glucose showed a significant effect on proteases yield (Table 2) while variation of rest of the constituents (Tween-80, trace elements and CaCl₂) showed insignificant effect on the yield. Effect \( E(x_i) \) of the medium constituent was calculated as—

\[
E(x_i) = 2(\sum H_i - \sum L_i)/N \quad \ldots (1)
\]

where, \( N \) = number of trials (12), \( \sum H_i \) = Sum of the yields of the runs where the level of \( i^{th} \) constituent is high, \( \sum L_i \) = Sum of the yields where level of \( i^{th} \) constituent is low.

Hence glucose, albumin and (NH₄)₂SO₄ were the test variables selected for optimization, while rest of the constituents (Tween-80, 0.5%; trace elements, 1ml; CaCl₂, 100 μg; K₂HPO₄, 3 g; and KH₂PO₄, 1 g) were kept at constant level in each flask in Central Composite Design (CCD).

**Central Composite Design (CCD) and Response Surface Optimization**—Table 4 demonstrates the experiments performed according to the CCD proposed by Box and Wilson, each row of Table 4 corresponds to a single experiment. The central values (zero level) chosen for CCD were albumin, 4.25%; (NH₄)₂SO₄, 0.155%; and glucose, 4.25%. In developing the regression equation, the test variables were coded according to the equation—

\[
X_i = (x_i - x_{ci})/\Delta x_i \quad \ldots (2)
\]

where \( X_i \) is the coded value of the \( i^{th} \) independent variable, \( x_i \) is the natural value, \( x_{ci} \) is the natural value at the center point and \( \Delta x_i \) is the interval.

Total 20 experiments that included eight cube points (runs 1-8), six star points (runs 9-14) and six replicas of the central points (runs 15-20) were required to fit the second order polynomial model. The concentrations of test variables (albumin, (NH₄)₂SO₄ and glucose in coded and uncoded units) in the production medium were varied according to CCD (Table 4). Samples (5 ml) were withdrawn from each flask after 48 hr of fermentation at 37°C and centrifuged at 1000 rpm for 10 min. The clear
supernatant broth was used to determine the enzyme yield. Protease activity was determined by a modified procedure based on the method of Tsuchida et al.\textsuperscript{10}. One protease unit is defined as amount of enzyme liberating 1 µmole tyrosine/min under defined conditions.

Statistical software ‘Statistica’ was used to perform regression and graphical analysis of the results obtained from CCD. A second order polynomial response equation (of the form given below) comprising equation linear, quadratic and interaction terms was obtained.

\[
Y = b_0 + \sum b_i X_i + \sum b_i^2 X_i^2 + \sum b_{ij} X_i X_j \quad \ldots (3)
\]

where \(Y\) is enzyme yield in Uml\(^{-1}\), \(b_0\) is the intercept, \(b_i\) is the coefficient for linear direct effect, \(b_i^2\) is the coefficient for quadratic effect and is responsible for curvatures in the model, \(b_{ij}\) is the coefficient for interaction effect a positive or negative significant value implies possible interaction between the medium constituents.

### Results and Discussion

**Analysis of Response Surface Model (RSM)—**The result of the second order response surface model (same for coded and uncoded test variables) fitting in the form of analysis of variable (ANOVA) is shown in Table 5.

The fisher F-test (regression) with a very low probability value (\(P\) Value \(~ 0\)) demonstrated a very high significance for the regression model. The goodness of fit of the model was checked by the determination coefficient (\(R^2\)). In this case, the value of the determination coefficient (\(R^2 = 0.959\)) indicated that the model did not explain only 4.1% of the total variations. The value of the adjusted determination coefficient (Adj. \(R^2 = 0.923\)) was also very high, which indicated a high significance of the model. There was no evidence of ‘lack-of-fit’ (\(P = 0.153 \gg 0.05\)). A high value of the correlation coefficient (\(R = 0.979\)) signified an excellent correlation between the independent variables. In Table 3 each of the observed values for Yield was compared with the predicted values. Variance and standard deviation of residuals (\(Var = 728.091; SD = 27\)) indicated that none of the individual residual values exceeded twice the square root of the variance of the residuals. All of the above considerations indicated an excellent adequacy of the regression model.

**Model coefficients and their significance**—The significance of each coefficient (coded and uncoded) was determined by Student’s \(t\) test and \(P\) values, which have been listed in Table 6. The second order or quadratic main effects of albumin, (NH\(_4\))\(_2\)SO\(_4\) and...
glucose are quite significant, as is evident from their respective $P$ values ($P_{x_1^2} = 0.000035$, $P_{x_2^2} = 0.000267$ and $P_{x_3^2} = 0.007758 < 0.01$), the negative values (coded units) of all the quadratic main effects suggested that all the constituents had a negative effect at higher concentration which was overcome by higher positive first order main effects ($P_{x_1} = 0.00$ and $P_{x_3} = 0.007235$). The fact that all the quadratic terms were significant suggested considerable curvature in the model. There was a significant ($P = 0.008981 < 0.01$) positive interaction between albumin and glucose, however interaction of glucose and $(\text{NH}_4)_2\text{SO}_4$ ($P = 0.95079 >> 0.01$) and interaction of albumin and $(\text{NH}_4)_2\text{SO}_4$ ($P = 0.66711 >> 0.01$) were not significant, first order main/linear effect of $(\text{NH}_4)_2\text{SO}_4$ which was insignificant, in the case of coded units ($P_{x_2} = 0.77537 >> 0.01$) was quite significant when the test variables are represented in uncoded units ($P_{x_2} = 0.001493 < 0.01$), this was due to scaling effect. For uncoded test variables the coefficient for $(\text{NH}_4)_2\text{SO}_4$ (3598.7) was larger than that for albumin and glucose. This was a scaling effect as the range for $(\text{NH}_4)_2\text{SO}_4$ was smaller than for albumin and glucose i.e., scaling effect masked the 'Importance' effect.

Response equations and optimum values—Application of RSM yielded the following regression equation, which was an empirical
relationship between the enzyme yield and test variables in coded units—

\[ Y = 706.2256 + 115.6045x_1 + 19.7963x_2 + 33.8224x_3 - 69.061x_1^2 - 53.7373x_2^2 - 32.5197x_3^2 - 5.8345x_1x_2 + 42.5085x_1x_3 + 0.8335x_2x_3. \] 

... (4)

where \( Y \) is the response, that is, the enzyme concentration in \( \text{Uml}^{-1} \), and \( x_1, x_2 \) and \( x_3 \) are the coded values of the test variables (albumin, \((\text{NH}_4)_2\text{SO}_4\) and glucose, respectively).

Similarly, response equation represented with test variables in uncoded units—

\[ Y = -248.2 + 183.9x_1 + 3598.7x_2 + 44.3x_3 - 19.6x_1^2 - 10223.5x_2^2 - 9.3x_3^2 - 42.9x_1x_2 + 12.1x_1x_3 + 6.1x_2x_3. \] 

... (5)

where \( x_1, x_2 \) and \( x_3 \) are the uncoded values (percentage) of the test variables.

Response surface plots (Figs 1-3) as a function of two variables at a time, maintaining the third variable at a fixed levels (zero, coded units), were helpful in understanding both the main and the interaction effects of these two variables.

Optimum concentrations of albumin, \((\text{NH}_4)_2\text{SO}_4\) and glucose was determined by performing the canonical analysis on the model coefficients of the response equation in uncoded units using MathCAD software. The optimal values of the test variables in uncoded units (percentages) was albumin, 6.586%; \((\text{NH}_4)_2\text{SO}_4\), 0.164%; and glucose, 6.72% with the corresponding predicted Yield = 801822 \( \text{UUm}^{-1} \), using the optimized conditions in incubation experiments resulted in the enzyme yield of 793492 \( \text{UUm}^{-1} \), this verified the fitness of the model. The alkaline protease production increased from 533460 to 793492 \( \text{UUm}^{-1} \), hence the response surface optimization of the quantities for effective medium constituents (albumin, \((\text{NH}_4)_2\text{SO}_4\) and glucose) led to an overall increase of 260032 \( \text{UUm}^{-1} \).

Enhanced production of chitinase and xylanase enzymes has been achieved with the help of Plackett-Burmann screening of media components, statistically designed experiments such as CCD and subsequent optimization analysis by RSM have also been very successful for the yield optimization. Concentration of \( \text{NH}_4\text{Cl}, \text{xylan} \) and salinity have been statistically optimized and resulted in a substantial increase of xylanase production, similarly increased production of chitinase has been achieved with firstly, the screening of medium components viz., tween-20, yeast extract and chitin followed by statistical optimization methods (CCD and RSM) \(^7\), \(^11\). In our studies also production of alkaline protease has been increased significantly using statistical optimization techniques.

References