Formulation of medium constituents by multiresponse analysis of central composite design to enhance chitinase production in \textit{Pantoea dispersa}

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In the present study, a high chitinase producing strain \textit{Pantoea dispersa} was isolated from the sea dumps at Bhavnagar, India. Chitin, urea, CaCl\textsubscript{2} and MgSO\textsubscript{4}.7H\textsubscript{2}O were variables used in central composite design for chitinase production. Chitinase, biomass and pH were the responses used in different models to evaluate individually fit ones. Quadratic model was found to be fit for chitinase response whereas in the case of biomass and pH, linear model was found to be fit without the effect of others. Chitinase production was optimized with respect to other responses such as biomass and pH in multiresponse analysis of response surface design by using desirability approach. In multiresponse analysis, following medium formulation (g/l), chitin, 15; urea, 0.32; CaCl\textsubscript{2}, 0.10 and MgSO\textsubscript{4}.7H\textsubscript{2}O, 0.08 was found to predict optimum chitinase production of 482.77 units/ml with overall highest desirability of 0.854 as compared to other formulations. The selection of model was done on the basis of high Adjusted R-squared value and lowered p-value for each model in individual analysis of each response. In multiresponse experiment, it was found that for response chitinase quadratic model and for responses pH and biomass linear models were well fit. Through desirability analysis, it was found that in the chitinase production, pH was essential as compared to biomass in \textit{P. dispersa}. Endochitinase and chitobiase activities were also studied.

**Keywords:** R-Squared value, biomass, medium components, \textit{Pantoea dispersa}, pH, p-value, Response surface design, Chitinase production

Experimental design methods have found broad applications in many disciplines. In fact, we may view experimentation as part of the scientific process and as one of the ways of learning how the systems or processes work. Experimental design is a critically important tool in the engineering world for improving the performance of a manufacturing process. It also has extensive applications in the development of new media constituents\textsuperscript{1,2}. In a characterization experiment, we are usually interested in determining, which formulation of media variables affect the response. A logical step is to optimize the important factors that leads to the best possible response. There are number of designs available for optimization of process, from these central composite design is one of the most important experimental designs used in the process optimization studies\textsuperscript{3-4}. Response surface methodology (RSM) is a collection of mathematical and statistical techniques that are useful for the modeling and analysis of problems in which a response of interest is influenced by several variables and objective is to optimize this response\textsuperscript{5}.

In our present multiresponse experiment, we simultaneously model behavior of the response variables chitinase, biomass and pH in \textit{P. dispersa} as a function of input variables such as chitin, urea, CaCl\textsubscript{2} and MgSO\textsubscript{4}.7H\textsubscript{2}O within some region of interest. The model adequacy checking was also carried out, which is necessary to—(1) examine the fitted model to ensure that it provides an adequate approximation to the true system and (2) to verify that none of the response surface model assumptions are violated. Optimization of the multiresponse function using desirability function approach was done, which is an analytical technique based on the concept of utility or desirability associated with a given response function\textsuperscript{6,7}.

Present report was an attempt to formulate a suitable production medium using multiresponse analysis that can substantially increase the chitinase production by \textit{P. dispersa}.

**Materials and Methods**

Organism, culture conditions for the growth—The culture \textit{P. dispersa} was isolated in our laboratory from sea dumps, in Bhavnagar, India. The organism was...
cultivated on chitin agar medium consisting of (g/l) acid swollen chitin 0.5, yeast extract, 0.5; (NH₄)₂SO₄, 1.0; MgSO₄.7H₂O, 0.3 KH₂PO₄, 1.36 and agar 30. The pH of the medium was adjusted to 7.2. The medium was sterilized by autoclaving at 121°C for 15 min³.

Acid swollen chitin degrading activity—Chitinase was assayed as described by Vyas and Deshpande⁶. The assay system consisted of 10 mg of acid swollen chitin, 50 μmole of acetate buffer (pH 5) and suitable concentration of enzyme in a total system of 3 ml. The incubation was done at 50°C for 10 min. The product was estimated by Nelson method¹⁰. One unit of chitinase activity was defined as the amount of enzyme required to liberate 1 μmole of N-acetyl-D glucosamine equivalent at 50°C per hr.

Ethylene glycol chitin degrading activity [endochitinase]—Endochitinase activity was studied only in unoptimized⁸ and optimized medium (result of this paper). The assay system consisted of 10 mg of ethylene glycol chitin, 50 μmole of acetate buffer (pH 5) and suitable concentration of enzyme in a total system of 3 ml. The incubation was done at 50°C for 10 min. The product was estimated by Nelson method¹⁰. One unit of chitinase activity was defined as the amount of enzyme required to liberate 1 μmole of N-acetyl-D glucosamine equivalent at 50°C per hr.

pNP-NAG degrading activity [chitobiase]—Similarly, chitobiase activity was studied for both unoptimized⁸ and optimized medium. Chitobiase activity was determined by measuring the release of p-nitrophenyl from pNP-NAG (p-nitrophenyl N-acetyl-D glucosamine). The assay system consisted of 0.5 mg of pNP-NAG, 50 μmole of acetate buffer (pH 5) and suitable concentration of enzyme in a total system of 1 ml. The incubation was done at 50°C for 10 min. The reaction was terminated with 0.5 N of NaOH (2 ml). The amount of p-nitrophenol was estimated by taking absorbance at 410 nm. One unit of chitobiase activity was defined as the amount of enzyme required to liberate 1 μmole of p-nitrophenol at 50°C per hr.

Method for the determination of the growth—Growth was estimated in terms of total protein content. The cells were hydrolyzed with 1 N of NaOH (1ml) in boiling water bath for 20 min. The suitable concentrations of hydrolyzed cells were taken in a total system of 1ml and protein was assayed by method of Lowry et al.¹¹.

Optimization procedure—The optimization of medium constituents for production of chitinase by P. dispersa was carried out in three stages—screening, optimization and verification experiments.

Screening experiment—To determine which few process variables, out of nineteen (chitin, glucose, peptone, yeast extract, urea, (NH₄)₂SO₄, NH₄NO₃, FeCl₃·H₂O, NaCl, MgCl₂·6H₂O, Na₂SO₄, CaCl₂, KCl, Na₂CO₃, KBr, H₂BO₃, MgSO₄·7H₂O, KNO₃ and KH₂PO₄) have an important affect on chitinase activity, Plackett-Burman design was used (data not shown). The screened components were chitin, urea, CaCl₂ and MgSO₄·7H₂O with P-value < 0.05.

Optimization experiment—The multiple response analysis of response surface design using desirability approach was used to optimize the screened components for optimization of chitinase production with respect to pH and biomass. Multi-response analysis involves first building an appropriate response surface model for each response and then trying to find a set of operating conditions that was in some sense optimized all responses or at least keeps them in desired ranges. The design used for the optimization experiment is given in Table 1 with coded values of variables at various levels. This design is central composite design; with twelve replicates at the centre point with total number of 36 trials. To explain the behavior of the whole process first we modeled each individual response; chitinase activity, pH and biomass. Different models are available in Central composite design of statistical software package Design-Expert. They are namely 1) Linear, 2) 2F1, and 3) Quadratic, which are used in present study. The model we selected for chitinase activity (Y₁) as a function of chitin (X₁), urea (X₂), CaCl₂ (X₃) and MgSO₄·7H₂O (X₄) is a second order (quadratic) polynomial model. It is of the form

\[ Y₁ = β₀ + ΣβₓᵢXᵢ + Σβₓᵢ²Xᵢ² + ΣΣβₓₓᵢXₓᵢ + ε; i = 1,2,3,4 \]  

... (1)

Where xᵢ represents the coded values of Xi, the squared terms (xᵢ²) represent the curvature in the response surface and the multiplicative terms (xᵢxⱼ) represents the interaction term. Also we assumed that ε ~ N(0, σ²) and xᵢ’s are independent.

The model we selected for response pH (Y₂) and response biomass (Y₃) as a function of chitin (X₁), urea (X₂), CaCl₂ (X₃) and MgSO₄·7H₂O (X₄) is a linear model. They are of the form
\[
Y_2 = \beta_0 + \sum \beta_i x_i + \varepsilon; i = 1,2,3,4 \quad \ldots \quad (2)
\]
\[
Y_3 = \beta_0 + \sum \beta_i x_i + \varepsilon; i = 1,2,3,4 \quad \ldots \quad (3)
\]

Where the terms in the models have usual meaning as described earlier with same assumptions.

After building the appropriate models, model adequacy checking was performed which checks whether all the assumptions of fitted models are satisfied.

**Model diagnostics**—These diagnostics include—

(a) Normal probability plot of residuals,
(b) Plot of studentized residuals versus predicted values,
(c) Plot of Outliers versus Run number,
(d) Box-Cox plot for power transformation.

(a) Normal probability plot indicates whether the residuals follow normal distribution and in that case the points will follow a straight line.

(b) The plot of studentized residuals versus predicted values indicates whether the assumption of constant variance for residuals is satisfied and in that case points will show random pattern.

(c) Plot of Outliers versus Run number checks whether a run is consistent with the other runs, assuming the chosen model holds. Prediction of the response at each run was made. The residual was evaluated using the \( t \)-test. If a value is greater than \( \pm 3.5 \) means the corresponding point indicates an outlying observation.

(d) The Box-Cox plot is a tool, which helps in determining the most appropriate power transformation to apply to response data. Most data transformation can be described by the power function, \( \sigma = f(\mu \alpha) \), where sigma (\( \sigma \)) is the standard deviation and mu (\( \mu \)) is the mean and the \( \alpha \) is the power. If the standard deviation associated with an observation is proportional to the mean raised to the \( \alpha \) power, then the transforming the observation by \( 1-\alpha \) (or \( \lambda \)) power gives scale satisfying the equal variance requirements of the statistical model. Commonly used transformations are \( \lambda = -1 \) inverse, \( \lambda = 0 \) natural log, \( \lambda = 0.5 \) square root, \( \lambda = 1 \) no transformation. The lowest point in the Box-Cox represents the value of \( \lambda \), which results in the minimum residual sum of squares in the transformed model. The plot shows the minimum \( \lambda \) values as well as lambda as the 95% confidence range. If the 95% confidence interval around this \( \lambda \) includes one, no transformation is required.

**Desirability analysis**—After fitting the models and residual analysis of all three responses, for optimization of these multiresponses simultaneously an optimization techniques popularized by Derringer and Suich\(^{12} \) was used. Their procedure makes use of desirability functions. The general approach is to first convert each response \( y_i \) into an individual desirability function \( d_i \) that varies for the range \( 0 \leq d_i \leq 1 \), where as if the response \( y_i \) is at its goal or target then, \( d_i = 1 \), and if the response is out side acceptable region \( d_i = 0 \). Then the design variables are chosen to maximize the overall desirability,

\[
D = (d_1^* d_2^* \ldots \ldots \ldots \ldots \ldots \ldots d_n^*)^{1/n}
\]

Where, \( n \) is number of responses.

Statistical software package Design-Expert (Version 6.0.10, State-Ease, Minneapolis, MN, USA) was used to design and analyze experiment.

**Verification experiment**—Verification of resulted formulation was checked in terms of chitinase production with respect to pH and biomass as described previously.

All the experiments were done in triplicate and the values presented were the means of three independent determinations.

**Results and Discussion**

**Multiresponse analysis**

Individual response in each model of central composite design—In screening experiment by Plackett-Burman design, the variables such as chitin, urea, CaCl\(_2\) and MgSO\(_4\).7H\(_2\)O showed significant behavior with \( p \)-value \(<0.05 \) (data not shown), which were further used in the production of chitinase by \( P.\) dispersa with respect to biomass and pH in shake flask condition at 30±2°C. The amount has been taken in g/l of each variable in each trial as shown in Table 1. The chitinase activity, biomass and pH value of each trial are also given in Table 1. The centre point in the design was repeated for twelve times for estimation of error. The initial pH of the medium of each trial was adjusted to 7.2. By using Design Expert software different models were fit on (a) chitinase; (b) biomass; and (c) pH.

(a) **Chitinase response**—For modeling of chitinase as a function of chitin, urea, CaCl\(_2\) and MgSO\(_4\).7H\(_2\)O,
The estimated quadratic model in terms of coded factors is

\[ \text{Chitinase} = +465.50+73.37^*x_1+3.88^*x_2+17.76^*x_3 +2.71^*x_4+52.28^*x_1^2-62.43^*x_2^2-6.27^*x_3^2-7.74^*x_4^2 -6.00^*x_1^2-18.13^*x_1^3+28.81^*x_1^4+8.64^*x_2^3-7.16^*x_2^4+20.06^*x_3^4 \]  

and in terms of actual factors is

\[ 12383.33333^*(\text{MgSO}_4\cdot7\text{H}_2\text{O})^2 - 36^*\text{P. dispersa} \]

and Adjusted R-squared 80.16%. The contribution of factors was found to be significant (p-value <0.0001 and Adjusted R-squared 80.16%). The estimated quadratic model for chitinase production was found to be influencing individually as well as in combination. Chitinase activity was found to be significant in explaining the behavior of chitinase at 5% level of significance. The significance of \( x_1^2 \); (chitin), \( x_2^2 \); (urea) and \( x_1^3 \); (chitin* MgSO\(_4\).7H\(_2\)O) were found to be significant. In the ANOVA (Analysis of variance) through quadratic model for chitinase production the contribution of factors \( x_1 \); chitin, \( x_2 \); (chitin), \( x_2^2 \); (urea) and \( x_1^3 \); (chitin* MgSO\(_4\).7H\(_2\)O) were found to be significant in explaining the behavior of chitinase at 5% level of significance. The significance of \( x_1^2 \); (chitin), \( x_2^2 \); (urea) indicated that there was a curvature in the response surface of chitinase. Chitin was found to be influencing individually as well as in combination.

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<th>( x_2 ); Urea (g/l)</th>
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interaction with MgSO₄·7H₂O in chitinase production. Also a high value of adjusted R-squared of 80.16% was observed, which indicated a good agreement between experimental and predicted values of chitinase production as explained by quadratic model. But the lack of fit test was found to be significant ($P < 0.05$) and there was a large gap between Adjusted and Predicted R-squared (33.96%), which indicates the presence of block effect in the experiment.

(b) pH response—In the case of the pH, both linear and quadratic models were significant ($P<0.001$). But the gap between Adjusted (69.00%) and Predicted R-squared (61.38%) was less in case of linear model as compared to quadratic model (Adjusted R-squared = 87.66%, Predicted R-squared = 57.87%); hence linear model was selected for modeling the behavior of pH response. The fitted linear model in terms of coded factors is

$$pH = 48.36 + 0.25x_1 - 0.020x_2 - 0.053x_3 + 0.065x_4 \ldots (6)$$

and in terms of actual factors is

$$pH = +7.86972 + 0.050667 \cdot \text{chitin} - 0.13333 \cdot \text{urea} - 1.06667 \cdot \text{CaCl}_2 + 2.60000 \cdot \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \ldots (7)$$

In the ANOVA (Analysis of variance) through linear model for pH response, the contribution of factors $x_1$: chitin and $x_4$: MgSO₄·7H₂O were found to be significant in explaining the behavior of pH at 5% level of significance. The Adjusted R-squared was 69.00% and the gap between Adjusted and Predicted R-squared was less, indicating a good agreement between experimental and predicted values of pH response as explained by linear model. But the significance of lack of fit test ($P < 0.05$) indicated the presence of block effect in the experiment.

(c) Biomass response—Similarly, in the case of the biomass, linear model was found to be significant ($P<0.0001$). The estimated linear model in terms of coded factors is

$$\text{Biomass} = +0.69 + 0.20x_1 + 0.045x_2 + 1.667E-0.03x_3 + 0.020x_4 \ldots (8)$$

In terms of actual factors is

$$\text{Biomass} = +0.12639 + 0.039333 \cdot \text{chitin} + 0.30000 \cdot \text{urea} + 0.033333 \cdot \text{CaCl}_2 + 0.80000 \cdot \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \ldots (9)$$

In the ANOVA (Analysis of variance) though linear model for biomass response, only the effect of $x_1$: chitin was found to be significant at 5% level of significance. Here the Adjusted R-squared was also 62.36% and the gap between Adjusted and Predicted R-squared (50.73%) was less, indicating a good agreement between experimental and predicted values of biomass as a response explained by linear model. But the significance of lack of fit test ($p$-value) again indicated the presence of block effects in the experiment.

Model diagnostics—Model adequacy checking was also performed, which is an important part of data analysis procedure. These diagnostics include—(a) Normal probability plot of residuals, (b) Plot of studentized residuals versus predicted values, (c) Plot of Outliers versus Run number and (d) Box-Cox plot for power transformation.

(a) Normal probability plot of residuals—In case of chitinase response for quadratic model and biomass response for linear model, normality assumption for residuals was satisfied since the residuals are plotted around the straight line as shown in Fig. 1a and Fig. 1b, respectively. Whereas in case of the pH response, the normality assumption for residuals was loosely satisfied in linear model since the pattern of the residuals creating a little bit S-shaped curve (Fig. 1c).

(b) Plot of studentized residual versus Predicted values—The pattern of the plot showed the random distribution of studentized residuals in all the responses indicating that the assumption of constant variance was true (Figs 2a, 2b and 2c).

(c) Outlier T—Here, for all the three responses, in the graph of outlier T versus run number where all the points were lying within ± 3.5 indicated that there was no outlying observation throughout and consistency was observed in all the runs (Figs 3a, 3b and 3c).

(d) Box-Cox plot—The Box-Cox plot of chitinase, biomass and pH responses was suggested lowest value of $\lambda$, which was one and also included in the 95% confidence interval as shown in Figs 4a, 4b and 4c, respectively. Thus, as per the above discussion, no power transformation was required for three responses.

Desirability analysis—The Design expert software package was used to optimize the chitinase production with respect to minimum biomass and maximum pH,
Normal Plot of Residuals

Fig. 1—Normal probability plot of residuals of (a) chitinase response; (b) biomass response; and (c) pH response

Plot of studentized versus predicted values of (a) chitinase response; (b) biomass response; and (c) pH response
Fig. 3—Outlier T for (a) chitinase response; (b) biomass response; and (c) pH response

Fig. 4—Box-Cox plot for power transformation in (a) chitinase response; (b) biomass response; and (c) pH response
keeping all the four independent variables within the range, which were found by experimental analysis (Table 1). We set weight for this individual desirability equal to unity. It was observed that in this analysis the medium consisting of (g/l) chitin, 15; urea, 0.32; CaCl\(_2\), 0.10 and MgSO\(_4\).7H\(_2\)O, 0.08 was found to predict optimum chitinase of 482.77 units/ml with overall highest desirability of 0.854 as compared to other formulations. It was also found that in the chitinase production, pH was essential as compared to biomass in *P. dispersa*. The effects of chitin and urea on the chitinase activity are shown in the contour plot (Fig. 5) of chitin versus urea with fixed CaCl\(_2\), 0.10(g/l) and MgSO\(_4\).7H\(_2\)O, 0.08(g/l). Hence, the above medium composition was suggested for enhanced chitinase production.

Verification of the experiment—optimized medium composition was used for verification of chitinase production with respect to pH and biomass. It was found that 484.87±0.97 units/ml chitinase was produced with biomass and pH as 0.69±0.05 g/l and 8.70±0.1, respectively.

Activity of endochitinase and chitobiase—Endochitinase and chitobiase activity was studied in both optimized and unoptimized medium using different substrates such as acid swollen chitin, glycol chitin and pNP-NAG as described previously.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Activity in unoptimized medium (units/ml)</th>
<th>Activity in optimized medium (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid swollen chitin</td>
<td>111.82 ± 2.25</td>
<td>484.87 ± 0.97</td>
</tr>
<tr>
<td>Ethylene glycol chitin</td>
<td>55.44 ± 0.81</td>
<td>235.68 ± 0.88</td>
</tr>
<tr>
<td>pNP-NAG</td>
<td>38.43 ± 0.71</td>
<td>108.02 ± 0.57</td>
</tr>
</tbody>
</table>

It was found that 4.33-fold chitinase production was increased through statistical optimized method as compared to unoptimized method when acid swollen chitin was used as a substrate in chitinase assay system. The endochitinase and chitobiase activity was also increased 4.25 and 2.28 fold, respectively by statistical optimized method (Table 2). By using statistical optimization method 35% increase in riboflavin production was reported in U.V. mutant of *Eremothecium ashbyii*\(^{13}\), 35% higher recombinant hirudin production in *Saccharomyces cerevisiae*\(^{14}\), 141% increase in chitinase production in *Alcaligenes xylosoxydans*\(^{15}\).

In summary, these results suggest that the statistical optimized medium shows a much high level of chitinase production as compared to unoptimized or basal medium.

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References

GOHEL et al.: FORMULATION OF MEDIUM CONSTITUENTS BY MULTIRESPONSE ANALYSIS