Statistical Optimization of Citric Acid Production using Cladosporium sp Isolated from Sathuragiri Hills

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ABSTRACT
Response surface methodology (RSM) was used to optimize the cultivation conditions for citric acid production, 30 experimental runs with different combinations of four factors and five levels were carried out. The variables used for the factorial analysis were pH, temperature, glucose and ammonium chloride, named X1, X2, X3 and X4 this design, respectively. The effects of the four independent variables on citric acid production and the experimental response along with the predicted response obtained from the regression equation for each run are shown. It can be seen that, there was a considerable variation in the citric acid production depending on the four chosen variables. The maximum citric acid production (17.3 g/L) was achieved. This adequately indicated that choosing appropriate cultivation conditions could evidently enhance the yield of citric acid.

Keywords: Citric acid, RSM, CCD,Cladosporium sp, Optimization

INTRODUCTION
Citric acid (C6H8O7, 2−hydroxy−1,2,3−propane tricarboxylic acid), a natural constituent and common metabolite of plants and animals, is the most versatile and widely used organic acid in the field of food (60%) and pharmaceuticals (10%). It has got several other applications in various other fields. Citric acid is known as the most important organic acid produced in tonnage by fermentation and is the most exploited biochemical/biotechnological product [1]. It has an annual production of 1.6 million tons[2] with annual growth demand/consumption rate of 3.5-4.0%[3].

Citric acid is responsible for the tart taste of various fruits in which it occurs (i.e. lemons, limes, figs, oranges, pears and goose-berries). Hence, citric acid is used to impart a pleasant tart flavours to foods and beverages. It is used in the industries to achieve acidulation, antioxidation, emulsification, preservation, flavour enhancement, and as plasticizer and synergistic agent[4]. The wide applicability of citric acid in industries is attributed to its low/non-toxicity, high solubility; biodegradability and palatability[4].

Many microorganisms such as fungi, bacteria and yeast can produce citric acid. A large number of these microorganisms have been employed for citric acid production, but only a few of them can produce citric acid in industrial scale [5]. The supply of natural citric acid is very limited and the demand can only be satisfied by biotechnological processes[6].

RSM has already been successfully applied for the optimization of enzymatic hydrolysis of other bioprocesses. Response surface methodology (RSM) is a useful tool which integrates mathematical and statistical approaches to analyze the effects of defined independent variables on the response without the need for prior knowledge of a predetermined relationship between the response function and the variable. RSM is now considered as a standard statistical approach for designing experiments, building models, evaluating the effects of many factors and finding the optimal conditions for desirable responses and reducing the number of required experiments[7-11].

Response Surface Methodology (RSM) has been employed for the statistical optimization of the variables [12]. RSM explores the relationships between input and response variables. The optimization involves: Performing the statistically designed experiments, estimating the coefficients in a mathematical model and predicting the response and checking the adequacy of the model [13].

MATERIALS AND METHODS
STATISTICAL ANALYSIS FOR CITRIC ACID PRODUCTION - CCD AND RSM
Statistical optimization of nutrient supplements
From the optimized nutrient composition for Cladosporium sp growth rate, the effect of the pH, temperature, carbon source (Glucose) and nitrogen source (Ammonium chloride) level were studied using Central Composite Design (CCD). A Central Composite Design consists of:
1) A complete 2K factorial design, where the factor levels are coded to the usual -1, +1 value. This is called the factorial portion of the design.
2) No center points (no \( \alpha \geq 1 \))
3) Two axial points on the axis of the design variable at a distance of \( \pm a \) from the design center. This is called the axial portion of the design.

The total number of design points is thus equal to \( \alpha = \frac{2K}{4} \)

For this investigation, pH (X1), temperature (X2), glucose (X3) and ammonium chloride (X4) are the independent variables in a series of citric acid production experiment.

Thus \( K = 4 \alpha = 2 \times \frac{4}{4} \alpha = 2 \)

A CCD with six star points \( (a = 2) \) and six replicates at the center point (no 6) with a total number of experiments \( N = 30 \).

The experiments was conducted by five different level o were employed simultaneously covering the spectrum of variables for the production of citric acid in the Central Composite Design. Table 1 indicates the range and levels of the independent variables selected for the production of citric acid.

To understand the effects of the parameters pH, temperature, glucose, ammonium chloride, and their interactions on the production of citric acid process, statistically designed experiments were used.

**Statistical Analysis Software**

Experimental designs and the polynomial coefficients were calculated and analyzed using a trial version of Design-Expert software (version 7.1.6., Stat-Ease Inc., Minneapolis, USA). Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA).

**Table 1. Range and levels of the independent variables selected for the production of citric acid**

| Variables       | -\( \alpha \) | Low value | Coded variable | High value | +\( \alpha \) |
|-----------------|--------------|-----------|---------------|------------|------------|
| pH              | 5            | 5.5       | 6             | 6.5        | 7          |
| Temperature     | 20           | 25        | 30            | 35         | 40         |
| Glucose         | 0            | 0.5       | 1             | 1.5        | 2          |
| Ammonium chloride | 0     | 0.25      | 0.50          | 0.75       | 1          |

**Table 2. Central composite design for citric acid production by Cladosporium sp.**

| Std | Run | Factor:1 A:pH | Factor:2 B:Temperature | Factor:3 C:Glucose | Factor:4 D:Ammonium Chloride | Citric acid g/L |
|-----|-----|---------------|------------------------|-------------------|-------------------------------|----------------|
| 11  | 1   | 5.50          | 35.00                  | 0.50              | 0.75                          | 12.3           |
| 25  | 2   | 6.00          | 30.00                  | 1.00              | 0.50                          | 17.3           |
| 17  | 3   | 5.00          | 30.00                  | 1.00              | 0.50                          | 13.6           |
| 10  | 4   | 6.50          | 25.00                  | 0.05              | 0.75                          | 14.1           |
| 19  | 5   | 6.00          | 20.00                  | 1.00              | 0.50                          | 13.2           |
| 14  | 6   | 6.50          | 25.00                  | 1.50              | 0.75                          | 12.7           |
| 12  | 7   | 6.50          | 35.00                  | 0.50              | 0.75                          | 14.3           |
| 18  | 8   | 7.00          | 30.00                  | 1.00              | 0.50                          | 13.6           |
| 29  | 9   | 6.00          | 30.00                  | 1.00              | 0.50                          | 17.3           |
| 16  | 10  | 6.50          | 35.00                  | 1.50              | 0.75                          | 13.9           |
| 5   | 11  | 5.50          | 25.00                  | 1.50              | 0.25                          | 14.1           |
| 22  | 12  | 6.00          | 30.00                  | 2.00              | 0.50                          | 15.3           |
| 15  | 13  | 5.50          | 35.00                  | 1.50              | 0.75                          | 13.2           |
| 30  | 14  | 6.00          | 30.00                  | 1.00              | 0.50                          | 17.3           |
| 6   | 15  | 6.50          | 25.00                  | 1.50              | 0.25                          | 13.1           |
| 27  | 16  | 6.00          | 30.00                  | 1.00              | 0.50                          | 17.3           |
| 3   | 17  | 5.50          | 35.00                  | 0.50              | 0.25                          | 12.6           |
| 9   | 18  | 5.50          | 25.00                  | 0.50              | 0.75                          | 13.2           |
| 13  | 19  | 5.50          | 25.00                  | 1.50              | 0.75                          | 14.4           |
| 4   | 20  | 6.00          | 35.00                  | 0.50              | 0.25                          | 15.2           |
| 24  | 21  | 6.00          | 30.00                  | 1.00              | 1.00                          | 15.3           |
| 26  | 22  | 6.00          | 30.00                  | 1.00              | 0.50                          | 17.3           |
| 1   | 23  | 5.50          | 25.00                  | 0.50              | 0.25                          | 13.3           |
| 23  | 24  | 6.00          | 30.00                  | 1.00              | 0.00                          | 15.2           |
| 28  | 25  | 6.00          | 30.00                  | 1.00              | 0.50                          | 17.3           |
| 7   | 26  | 5.50          | 35.00                  | 1.50              | 0.25                          | 15.0           |
| 8   | 27  | 6.50          | 35.00                  | 1.50              | 0.25                          | 14.6           |
| 2   | 28  | 6.50          | 25.00                  | 0.50              | 0.25                          | 14.7           |
| 20  | 29  | 6.00          | 40.00                  | 1.00              | 0.50                          | 14.3           |
| 21  | 30  | 6.00          | 30.00                  | 0.00              | 0.50                          | 13.6           |
**RESULT**

**Central composite design**
Response surface methodology was used to optimize the levels of the significant variables identified by the 2-level fractional factorial design. A CCD matrix was developed depending on the number of factors considered for optimization. Based on the identification of variables by the 2-level fractional factorial, a central composite design was developed for variables significantly affecting citric acid production. All the non-significant factors were maintained at central points (‘0’ coded level) of the levels used in the 2-level fractional factorial design.

Table 1 shows the five levels of variables chosen for trials in CCD. Response surface methodology (RSM) was used to optimize cultivation conditions for citric acid production, 30 experimental runs with different combinations of four factors and five levels were carried out (Table 2). The variables used for the factorial analysis were pH, temperature, glucose and ammonium chloride, named X1, X2, X3 and X4 this design, respectively. The effects of the four independent variables on citric acid production and the experimental response along with the predicted response obtained from the regression equation for each run are shown in Table 3. It can be seen from Table 3, there was a considerable variation in the citric acid production depending on the four chosen variables. The maximum citric acid production (17.3 g/L) was achieved. This adequately indicated that choosing appropriate cultivation conditions could evidently enhance the yield of citric acid. By applying multiple regression analysis on the experimental data, the following second order polynomial equation was found to explain the regression analysis on the experimental data, the following second order polynomial equation was found to explain the mutual interactions between the best variables. The parameter estimates and the corresponding P-values with the parameter estimate, are given in Table 4. The P-values are used as a tool to check the significance of each of the coefficients which, in turn, are necessary to understand the pattern of the mutual interactions between the best variables. The parameter estimates and the corresponding P-values showed that among the independent variables, X1 (pH), X2 (Temperature), X3 (glucose) and X4 (ammonium chloride) had a significant effect on citric acid production. So, compared with the traditional ‘one- variable at- a-time’ approach which is unable to detect the frequent interactions occurring between two or more factors although they often do occur, RSM has immeasurable effects and tremendous advantages.

The independent variables were fitted to the second order model equation and examined for the goodness of fit. Several indicators were used to evaluate the adequacy of the fitted model and the results are shown in Table 8. The determination coefficient R2 value, correlation coefficient R value, coefficients of variation (CV) and model significance (F-value) were used to judge the adequacy of the model. R2, or coefficient of determination, is the proportion of variation in the response attributed to the model rather than to random error. Suggested for a good fit of a model, R2 should be at least 80%. The determination coefficient (R2) implies that the sample variation of 95.94% for citric acid production using corn ears as substrate is attributed to the independent variables, and only about 4.06% of the total variation cannot be explained by the model. The closer value of R (correlation coefficient) to 1, the better is the correlation between the experimental and predicted values. Here the value of R (0.9594) for Eq. (3.2) being close to 1 indicated a close agreement between the experimental results and the theoretical values predicted by the model equation. The coefficient of variation (CV) is the ratio of the standard error of estimate to the mean value of the observed response, expressed as a percentage. A model can be considered reasonably reproducible if the CV is not greater than 10%. Usually, the higher value of CV, the lower is the reliability of experiment. Here, a lower value of CV (2.92) indicated a greater reliability of the experiments performed. The model significance (F-value) indicates the level of confidence that the selected model cannot be due to experimental error. Linear and quadratic terms were significant at the 1% level. Therefore, the quadratic model was selected in this optimization study. The Student T distribution and the corresponding P value, along with the parameter estimate, are given in Table 4. The P-values are used as a tool to check the significance of each of the coefficients which, in turn, are necessary to understand the pattern of the mutual interactions between the best variables. The parameter estimates and the corresponding P-values showed that among the independent variables, X1 (pH), X2 (Temperature), X3 (glucose) and X4 (ammonium chloride) had a significant effect on citric acid production. So, compared with the traditional ‘one- variable at- a-time’ approach which is unable to detect the frequent interactions occurring between two or more factors although they often do occur, RSM has immeasurable effects and tremendous advantages.

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**Table 3. Observed and predicted responses for the experiments performed using CCD design for citric acid production.**

| Std | Run | Factor:1 A: pH | Factor:2 B: Temperature | Factor:3 C: Glucose | Factor:4 D: Ammonium Chloride | Actual Citric acid g/L | Predicted Citric acid g/L |
|-----|-----|----------------|-------------------------|---------------------|-------------------------------|------------------------|---------------------------|
| 11  | 1   | 5.50           | 35.00                   | 0.50                | 0.75                          | 12.3                   | 12.6                      |
| 25  | 2   | 6.00           | 30.00                   | 1.00                | 0.50                          | 17.3                   | 17.3                      |
| 17  | 3   | 5.00           | 30.00                   | 1.00                | 0.75                          | 13.6                   | 13.1                      |
| 10  | 4   | 6.50           | 25.00                   | 0.05                | 0.75                          | 14.1                   | 14.0                      |
| 19  | 5   | 6.00           | 20.00                   | 1.00                | 0.50                          | 13.2                   | 13.1                      |
| 14  | 6   | 6.50           | 25.00                   | 1.50                | 0.75                          | 12.7                   | 13.0                      |
Table 4. Analysis of variance (ANOVA) for quadratic model for citric acid production

| Sources                  | Sum of Squares | Df | Mean Square | F Value | p-value Prob > F |
|--------------------------|----------------|----|-------------|---------|------------------|
| Model                    | 64.85          | 14 | 4.63        | 25.35   | < 0.0001         |
| A-Ph                     | 0.45           | 1  | 0.45        | 2.48    | 0.1359           |
| B-Temperature            | 1.00           | 1  | 1.00        | 5.48    | 0.0335\( ^c \)  |
| C-Glucose                | 0.51           | 1  | 0.51        | 2.79    | 0.1154           |
| D-Ammonium chloride      | 1.45           | 1  | 1.45        | 7.94    | 0.0130\( ^c \)  |
| AB                       | 1.05           | 1  | 1.05        | 5.75    | 0.0299\( ^c \)  |
| AC                       | 4.10           | 1  | 4.10        | 22.44   | 0.0003\( ^c \)  |
| AD                       | 0.031          | 1  | 0.031       | 0.17    | 0.6880           |
| BC                       | 0.28           | 1  | 0.28        | 1.51    | 0.2383           |
| BD                       | 0.53           | 1  | 0.53        | 2.88    | 0.1105           |
| CD                       | 0.031          | 1  | 0.031       | 0.17    | 0.6880           |
| A\(^2\)                  | 26.02          | 1  | 26.02       | 142.39  | < 0.0001\( ^c \) |
| B\(^2\)                  | 24.05          | 1  | 24.05       | 131.64  | < 0.0001\( ^c \) |
| C\(^2\)                  | 15.90          | 1  | 15.90       | 87.04   | < 0.0001\( ^c \) |
| D\(^2\)                  | 12.00          | 1  | 12.00       | 65.68   | < 0.0001\( ^c \) |
| Residual                 | 2.74           | 15 | 0.18        |         |                  |
| Lack of Fit              | 2.74           | 10 | 0.27        |         |                  |
| Pure Error               | 0.000          | 5  | 0.000       |         |                  |
| Cor Total                | 67.59          | 29 |             |         |                  |

R\(^2\)=0.9594; AdjR\(^2\)=0.9216; C.V.\%=2.92; Cmodel terms are significant.
The significant factors identified by manual optimization design were considered for the next stage in the medium optimization using response surface optimization technique for the future study. The analysis of variance (ANOVA) was employed (shown in Table 4) for the determination of significant parameters. ANOVA consists of classifying and cross-classifying statistical results and testing whether the means of a specified classification differ significantly. The F-value is the ratio of the mean square due to regression to the mean square due to error and indicates the influence (significance) of each controlled factor on the tested model.

Where Y1, was the citric acid production, X1 the pH, X2 the temperature, X3 the glucose and X4 the ammonium chloride. The Model F-value of 25.35 implies the model is significant. There is only 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case B, D, AB, AC, A², B², C², D² are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The "Pred R-Squared" of 0.7664 is in reasonable agreement with the "Adj R-Squared" of 0.9216. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. This ratio of 15.370 indicates an adequate signal. This model can be used to navigate the design space.

The above model can be used to predict the citric acid production within the limits of the experimental factors. Figure 1 shows that the actual response values agree well with the predicted response values.

The interaction effects of variables on citric acid production were studied by plotting 3D surface curves against any two independent variables, while keeping another variable at its central (0) level. The 3D curves of the calculated response (citric acid production) and contour plots from the interactions between the variables are shown in Figs. 2a-2e.

The predicted values from the regression equation closely agreed with that obtained from experimental values. Validation of the experimental model was tested by carrying out the batch experiment under optimal operation conditions. Three repeated experiments were performed and the results are compared. The citric acid obtained from experiments was very close to the actual response predicted by the regression model, which proved the validity of the model. At these optimized conditions the maximum citric acid production was found to be 17.3 g/L. The results show a close concordance between the expected and obtained activity level.

Fig. 2a. 3D plot showing the effect of pH and temperature on citric acid production.
Fig. 2b. 3D plot showing the effect of pH and glucose on citric acid production.

Fig. 2c. 3D plot showing the effect of pH and ammonium chloride on citric acid production.

Fig. 2d. 3D plot showing the effect of temperature and glucose on citric acid production.
Fig. 2e. 3D plot showing the effect of temperature and ammonium chloride on citric acid production.

Fig. 2f. 3D plot showing the effect of glucose and ammonium chloride on citric acid production.

The shapes of the contour plots, circular or elliptical, indicate whether the mutual interactions between the variables are significant or not. A circular contour plot of response surfaces indicates that the interaction between the corresponding variables can be ignored, while an elliptical or saddle nature of the contour plot suggests that the interaction between the corresponding variables is significant (Fig. 3a-3f).

Fig. 3a. Contour plot showing the effect of pH and temperature on citric acid production.
Fig. 3b. Contour plot showing the effect of pH and glucose on citric acid production.

Fig. 3c. Contour plot showing the effect of pH and ammonium chloride on citric acid production.

Fig. 3d. Contour plot showing the effect of temperature and glucose on citric acid production.
The conventional method (i.e., change-one-factor-at-a-time) traditionally used for optimization of multifactor experimental design had limitations because (i) it generates large quantities of data which are often difficult to interpret (ii) it is time consuming and expensive (iii) ignores the effect of interactions among factors which have a great bearing on the response. To overcome these problems, a central composite design (CCD) and RSM were applied to determine the optimal levels of process variables on citric acid production. Only 30 experiments were necessary and the obtained model was adequate (P < 0.001). By solving the regression equation, the optimum process conditions were determined; substrate concentration 5g/L of corn ears, initial pH 6, fermentation temperature 30°C and ammonium chloride 0.5%. A maximum citric acid yield of 17.3 g/L was obtained at the optimized process conditions. The research results indicated that RSM not only helps to locate the optimum conditions of the process variables in order to enhance the maximum citric acid production, but also proves to be well suited to evaluating the main and interaction effects of the process variables on citric acid production from waste agricultural residues.

**DISCUSSION**

An investigation was carried for the production of citric acid from solid state fermentation of treated sugarcane bagasse using *Aspergillus niger*. Response surface methodology (RSM) was employed for the optimization of fermentation conditions namely broth pH, fermentation time and substrate loading. A three-variable, three-level Box-Behnken design (BBD) comprising 15 experimental runs was used to develop a statistical model for the optimization of fermentation conditions. The optimal fermentation conditions that resulted in the maximum citric acid concentration were broth pH, 2.0; fermentation time, 6 days and substrate loading, 80 g/L. Under these conditions, the concentration of citric acid produced was 18.63 g/L. Validation of the model indicated no difference between predicted and observed values as seen in the high correlation between model predicted results and experiment [14].
By using the response surface methodology has been optimized the production of citric acid. These variables were further optimized using a $2^4$ full factorial CCD (Central Composite Design) and a second order polynomial model equation was obtained. In the present study the value of the regression coefficient $R^2 = 0.6238$ which indicates that 62.38% of the variability in the response could be explained by the model. The adjusted $R^2$ value is 0.6238 which is also very high to advocate the significance of the model. Maximum yield was obtained from mutant type of A. niger MTCC 662 than wild strains were about 12.0g/100ml and 8.2g/100ml. It could be concluded that the A. niger MTCC662 mutant strains have produced high yield than wild strain of A.niger MTCC662 cultures [15].

Whereas, a work reports on the statistical design of experiments using Central composite design (CCD) for submerged fermentation of Citric acid and optimization of incubation time, fermentation temperature and O2 flow rate by Response surface methodology (RSM). For the three variables, CCD had designed fifteen different combinations of experiments. From the experiments, the concentrations of citric acid produced were estimated and from the results a second order polynomial equation was developed with theyield of citric acid as a function of Incubation time, Fermentation temperature and O2 flow rate. Different levels of the variables were found to have a significant positive effect on citric acid production. A maximum citric acid concentration of 52.49 g/l was obtained at Initial sucrose concentration of 155g/l, Initial medium pH of 6.0 and stirrer speed of 240 rpm with the optimized variables of incubation time: 5 days, fermentation temperature; 30º C and O2 flow rate: 1.0 ppm[16].

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