Process optimization for citric acid production from raw glycerol using response surface methodology

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Statistical experimental design was applied for the optimization of medium constituents for citric acid production by Yarrowia lipolytica NCIM 3589 in submerged fermentation using raw glycerol as the carbon source. Response surface methodology (RSM) involving central composite design (CCD) was adopted to evaluate the amount of citric acid produced by most important factors, such as carbon concentration, nitrogen concentration, and salt solution concentration. A second order polynomial regression model was fitted and was found adequate with $R^2$ of 0.9485. The optimum conditions were found to be carbon concentration 38.77 g/L, nitrogen concentration 0.401 g/L, and salt solution concentration 12.3 % (v/v). Citric acid production at these optimum conditions was 13.41 g/L.

Keywords: Citric acid, central composite design, raw glycerol, response surface methodology, Yarrowia lipolytica

Introduction

Citric acid is a commercially valuable microbial product, mainly produced by submerged fermentation of medium of sucrose or molasses using Aspergillus niger1. Yarrowia lipolytica, besides A. niger, is also considered as a potential producer of citric acid2-6. The growth of these microorganisms on sugars resulted in a significant citric acid accumulation in the culture medium. While other strains grown on an industrial derivative of animal fat resulted in higher cellular lipid accumulation with negligible amount of organic acids, Y. lipolytica LGM S(7)1 grown on raw glycerol in a nitrogen limited media led to secretion of citric acid instead of production of reserve lipid7. In the study, growth and citric acid production parameters were similar to those obtained on glucose (35 g L$^{-1}$). Raw glycerol is an industrial feedstock appearing in increasing quantities as the main by-product of bio-diesel production facilities. However, instead of raw glycerol, pure glycerol was used as the carbon substrate in microbial fermentations, because the salts present in the raw glycerol exert important inhibitory effects on many microorganisms. A few strains of Clostridium butyricum have also been reported to grow on raw glycerol and produced 1,3-propanediol with concomitant production of acetic acid and butyric acid8.

The traditional ‘one-factor at a time’ technique used for optimizing a multivariable system is not only time consuming but often misses easily the alternative effects between components. Also, this method requires carrying out a number of experiments to determine the optimum levels, which are false. These drawbacks of single factor optimization process can be eliminated by optimizing all the affecting parameters collectively by central composite design (CCD) using response surface methodology (RSM). Recently, many statistical experimental design methods have been employed in bioprocess optimization. Among them, RSM is the one suitable for identifying the effect of individual variables and for seeking the optimum conditions for a multivariable system efficiently. This method has been successfully applied to optimize fermentation media9-11. A detailed account of this technique has been outlined12. Basically, this optimization process involves three major steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model and predicating the response and checking the adequacy of the model. Hence, the authors report the application of the RSM using the Box-Wilson design13 of experiments to develop a

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mathematical correlation between the carbon concentration, nitrogen concentration and salt solution content of fermentation and concentration of citric acid.

Materials and Methods

Substrate
Raw glycerol is a light brown semi solid obtained as the byproduct in biodiesel production and is taken from Energy Engineering Laboratory, Department of Chemical Engineering, Andhra University, Visakhapatnam, India.

Microorganism
Y. lipolytica NCIM 3589 obtained from National Chemical Laboratory, Pune, India was used throughout the study.

Growth Conditions
The culture was maintained on MGYP slants having the composition (%): malt extract 0.3, glucose 1.0, yeast extract 0.3, peptone 0.5 and agar agar 2.0. The pH of the medium was adjusted to 6.4–6.8 and culture was incubated at 30°C for 48 h. Sub-culturing was carried out once in every 2 wk and the culture was stored at 4°C.

Inoculum Preparation
The yeast strain was cultivated in a medium containing (per L of distilled water): 5 g peptone, 3 g yeast extract and 3 g sodium chloride, at 30°C on a shaker at 200 rpm for 24 h.

Production Medium
The composition of basal medium for citric acid production was (in g/L): KH₂PO₄, 7; Na₂HPO₄, 7; MgSO₄.7H₂O, 1.5; CaCl₂, 0.15; ZnSO₄.7H₂O, 0.02; MnSO₄.4H₂O, 0.06; and FeCl₃.6H₂O, 0.15. Raw glycerol was used as carbon source. Ammonium sulphate and yeast extract (1:1) were taken as nitrogen source. The basal medium with carbon and nitrogen sources was sterilized at 121°C (15 psi) for 20 min. A cell suspension of inoculum was added to the basal medium. The flasks were incubated in an orbital shaker at 30°C on 150 rpm for 72 h.

Analytical Technique
Citric acid was estimated by the acetic anhydride and pyridine method of Marrier and Boulet.

Experimental Design
Once the variables having the statistically significant influence on the responses were identified, a CCD was used to optimize the levels of these variables. The full CCD, based on three basic principles of an ideal experimental design, primarily consists of (1) a complete 2ⁿ factorial design, where n is the number of test variables, (2) n₀ center points (n₀ ≥ 1) and (3) two axial points on the axis of each design variable at a distance of 2ⁿ/3 from the design center. Hence, the total number of design points \( N = 2^n + 2n + n_0 \). For statistical calculations the variables \( X_i \) are coded as \( x_i \) according to Eq (1):

\[
x_i = \frac{X_i - x_i}{\Delta x_j}, \quad (i = 1, 2, 3, \ldots, k)
\]

where \( x_i \) is dimensionless value of an independent variable, \( X_i \) is the real value of an independent variable, \( x_j \) is the real value of the independent variable at the center point and \( \Delta x_j \) is the step change.

The second degree polynomials [Eq (2)] are calculated with the statistical package STATISTICA 6.0 (Stat-Ease Inc., Tulsa, OK, USA) to estimate the response of the dependent variable.

\[
Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3
\]

where \( Y \) is predicted response, \( X_1, X_2, X_3 \) are independent variables, \( b_0 \) is offset term, \( b_1, b_2, b_3 \) are linear effects, \( b_{11}, b_{22}, b_{33} \) are squared effects and \( b_{12}, b_{23}, b_{13} \) are interaction terms.

Results and Discussion
Based on the results of the preliminary experimental runs, carbon concentration (\( X_1 \), g/L), nitrogen concentration (\( X_2 \), g/L) and salt solution concentration (\( X_3 \), % v/v) were chosen as the independent input variables and amount of citric acid produced (\( Y \), g/L) was used as the dependent output variable. A CCD was employed to analyze the interactive effect of these parameters and to arrive at an optimum. A summary of the independent variables and their variation levels is given in Table 1. A 2³–factorial central composite experimental design, with six axial points and six replications at the center points (n₀=6) leading to a total number of 20 experiments (Table 2) was employed for the optimization of the parameters.

The calculated regression equation for the optimization of medium constituents showed that
citric acid production (\(Y\)) is a function of the concentration of carbon concentration (\(X_1\)), nitrogen concentration (\(X_2\)) and salt solution concentration (\(X_3\)). By applying multiple regression analysis to the experimental data, the following second order polynomial equation was found to represent the citric acid production adequately.

\[
Y = -72.925 + 1.688X_1 + 175.967X_2 + 2.949X_3 - 0.018X_1^2 - 194.342X_2^2 - 0.086X_3^2 - 0.26X_1X_2 - 0.013X_1X_3 - 0.812X_2X_3.
\] (3)

The coefficients of the regression model [Eq (3)] calculated are listed in Table 3, in which they contain three linear, three quadratic and three interaction terms and one block term. The significance of each coefficient was determined by student’s \(t\)-test and \(p\)-values, which are listed in Table 3. The larger the magnitude of the \(t\)-value and smaller the \(p\)-value, the more significant is the corresponding coefficient\(^{15,16}\). This implies that the first order and second order main effects of carbon concentration, nitrogen concentration and salt solution concentration are highly significant as is evident from their respective \(p\)-values. This indicates that they can act as limiting nutrients and small variations in their concentration will alter either growth rate or product formation rate or both to a considerable extent. The interaction terms were found to be insignificant (\(p>0.05\)) which are also presented in Table 3. The parity plot (Fig. 1) showed a satisfactory correlation between the experimental and predicted values of citric acid production, wherein the points cluster around the diagonal line indicated the good fit of the model, since the deviation between the experimental and predicted values was minimal.

The results of the second order response surface model fitting in the form of ANOVA are given in Table 4. It is required to test the significance and adequacy of the model. The Fisher variance ratio, the \(F\)-value (= \(S^2_p/S^2_e\)), is a statistically valid measure of how well the factors describe the variation in the data about its mean. The greater the \(F\)-value from unity, the more certain it is that the factors explain adequately the variation in the data about its mean, and the estimated factor effects are real. The analysis of variance of the regression model demonstrates that the model is highly significant, as is evident from the
Fisher’s $F$-test ($F_{model} = 20.48047$) and a very low probability value ($P_{model} = F_{0.000026}$). Moreover, the computed $F$-value ($F_{0.01(9,10)} = S^2_r/S^2_e = 20.48047 > F_{0.01(9,10)}^{tabular} = 4.94$) is greater than the tabular $F$-value ($F_{0.01(9,10)}^{tabular} = 4.94$) at the 1% level, indicating that the treatment differences are significant.

The goodness of the fit of the model was checked by the determination coefficient ($R^2$). The $R^2$ value provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. The $R^2$ value is always between 0 and 1. The closer the $R^2$ value to 1, the stronger the model is and the better it predicts the response\textsuperscript{13}. In this case, the value of the determination coefficient ($R^2 = 0.9485$) indicates that 94.85% of the variability in the response could be explained by the model. In addition, the value of the adjusted determination coefficient (Adj $R^2 = 0.9022$) is also very high to advocate for a high significance of the model. Also a higher value of the correlation coefficient ($R = 0.9739$) justifies an excellent correlation between the independent variables\textsuperscript{17}. The predicted optimum levels of carbon concentration, nitrogen concentration and salt solution concentration were obtained by applying the regression analysis to the Eq (3).

Figs 2-4 represent the response surface and contour plots for the optimization of medium constituents of citric acid production. The effects of the carbon and nitrogen concentrations on the citric acid production are shown in Fig. 2. An increase in the carbon with nitrogen concentration up to the optimum point increased the citric acid production to a maximum level and a further increase in the carbon with nitrogen concentration the trend is reversed. The interaction effect of the nitrogen concentration and salt solution concentration on the citric acid production in Fig. 3 clearly indicates a proper combination for production of citric acid. An increase in the salt solution concentration with nitrogen concentration increased the citric acid production gradually but at a higher salt solution concentration and nitrogen concentration the trend is reversed. The optimum for maximum citric acid production lies near the center point of the salt solution concentration and nitrogen concentration. A similar effect was observed for the carbon concentration at any level of the salt solution concentration. An increase in the carbon concentration with salt solution concentration up to the optimum point increased the citric acid production to maximum level and a further increase in the carbon concentration with salt solution concentration decreased the citric acid production as shown in Fig. 4. Therefore, an optimum was observed near the central value of carbon concentration, nitrogen concentration and salt solution concentration. The optimum values of the medium constituents for maximum citric acid production can be attained at the concentrations of 38.77 g/L carbon source, 0.401 g/L nitrogen source, and 12.3% (v/v) of salt solution. At these optimum medium concentrations, maximum citric acid production of 13.41 g/L was obtained.

Conclusion

The present study using the RSM with CCD enables to determine the optimal medium constituents of the fermentation medium for the production of citric acid. The validity of the model was proved by fitting the values of the variables in the second order polynomial equation and by actually carrying out the experiment at those predicted values for the three independent variables, i.e., carbon concentration, nitrogen concentration and salt solution concentration. Among the three variables tested for the correlation
between their concentrations and the production of citric acid, all the three variables showed significant influence on the production. The significant interactions between the three variables were also observed from the contour plots. The maximum amount of citric acid produced from raw glycerol was predicted to be 13.41 g/L when the optimized medium constituents of the fermentation medium were set as follows: carbon concentration 38.77 g/L, nitrogen concentration 0.401 g/L, and salt solution concentration 12.3% (v/v). The methodology as a whole proved to be adequate for the design and optimization of the bioprocess.

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References


