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Application of Box-Behnken Design for the Optimization of Citric Acid Production from Corn Starch Using *Aspergillus niger*

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Authors' contributions

This work was carried out in collaboration between all authors. Author NAA, in collaboration with author KIN, designed the study, performed the experiments, carried out the statistical analysis, and wrote the first draft of the manuscript. Authors NAA and KIN managed the literature searches. Authors FAA, SEO and COO provided analytical advice and manuscript correction. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Citric acid production from hydrolysed corn starch was optimized in this study. Response surface methodology (RSM) was employed for the analysis of the simultaneous effect of substrate concentration, broth pH and fermentation temperature on the concentration of citric acid produced during fermentation of hydrolysed corn starch. A three-variable, three-level Box-Behnken design (BBD) comprising 15 experimental runs was used to develop a second degree statistical model for the optimisation of the fermentation conditions. The optimal fermentation conditions that resulted in the maximum citric acid concentration were substrate concentration; 50 g/L, broth pH; 2.00 and fermentation temperature; 25ºC. Under these conditions, the concentration of citric acid was obtained to be 31.96 g/L. Validation of the model indicated no difference between predicted and observed values.

Keywords: Citric acid; Aspergillus niger; fermentation; box-behnken design; optimisation.

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1. INTRODUCTION

Citric acid is present in essentially all plants, many animal tissues and fluids. It is a constituent of wine, milk, cheese and it is abundant in most citrus fruits such as oranges, tangerines, lemon, berries, lime etc. It is also a metabolic product formed in the citric acid or Krebs cycle [1]. It finds application in the food and beverage, pharmaceutical, textile, crude oil and cosmetics industries as acidifying, flavouring, preserving, chelating and buffering agent [1,2].

Currently, submerged fermentation appears to be the most economical way of producing citric acid by *Aspergillus niger*. However, the growing demand for citric acid has increased the need for more economical methods to be developed [3–6].

Citric acid production during fermentation by *Aspergillus niger* is affected by factors such as carbon substrate source and concentration, temperature, pH, inoculum density, aeration, agitation, moisture content etc [7,8]. In order to get the best performance during fermentation, these factors need to be optimised. The classical method of optimization involves varying one factor at a time and keeping the others constant. This is often useful but does not elucidate the effect of interaction between the various factors under consideration. Response surface methodology is an empirical statistical technique employed for multiple regression analysis of quantitative data obtained from statistically designed experiments by solving the multivariate equations simultaneously [9,10]. By making use of design of experiment for response surface methodology, the input levels of each factor as well as the level of the selected response can be quantified. The central composite, Box-Behnken and Doehlert designs are among the common designs used for response surface methodology. In this work, the optimization of citric acid production by Aspergillus niger was studied. The objective of this study was to optimise the effect of carbon substrate level, fermentation temperature, and broth pH. Using the Box-Behnken design of experiments, a mathematical correlation between substrate level, fermentation temperature, and pH was developed to obtain maximum citric acid concentration.

2. MATERIALS AND METHODS

2.1 Microorganism

Aspergillus niger ATCC 9142 was obtained from the biotechnology division of the Federal Institute of Industrial Research Oshodi (FIIRO), Lagos, Nigeria. It was maintained on Potato Dextrose Agar (PDA) slants and stored in a refrigerator at 4^{o}C until it was needed.

2.2 Substrate and Pretreatment

Industrial grade corn starch was obtained from the Federal Institute of Industrial Research Oshodi (FIIRO), Lagos, Nigeria. Dilute acid hydrolysis of corn starch was carried out in an autoclave using 0.1M hydrochloric acid. The carbon substrate concentration range was 30-50 g/L, the broth pH range was 2-8 and the fermentation temperature range was 25–35ºC. The hydrolysis reaction was quenched by adding 1.0 M sodium hydroxide. After acid hydrolysis, the reaction mixture was filtered and the filtrate was collected for citric acid production.

2.3 Culture Medium, Inoculum and Fermentation

The constitution (g/L) of the fermentation medium used for citric acid production was as described by Lotfy et al, [11]. Glucose, (30-50); $NaNO_3$, 4.0; KH_2PO_3, 1.0; $MgSO_4.7H_2O$, 0.23; $FeCl_3$, 0.02; $ZnSO_4$, 0.0012; $MnCl_2.H_2O$, 0.0012. Conidia suspensions of fungal strains were obtained from cultures grown on potato dextrose agar slants at 30ºC for 5 to 7 days. The spores were washed with sterilized 0.8% Tween 80 solution by shaking vigorously for 1 minute. Spores were counted with a haemocytometer to obtain approximately 10^8 spores/ mL. Surface fermentation was carried out in 250 mL Erlenmeyer flasks. The flask containing the fermentation medium was inoculated with 0.5 mL of the inoculum and then incubated at 25-35ºC.

2.4 Analytical Methods

The citric acid content of the final hydrolysate was determined using the method of Marier & Boulet [12]. The pH of the sample was determined using a Unican 9450 model pH meter.

2.5 Design of Experiment

A three variable Box-Behnken design for response surface methodology was used to study the combined effect of substrate concentration, broth pH and fermentation temperature on citric acid concentration over three levels. The range and levels of the variables optimized are shown in Table 1. The Box-Behnken design is suitable for the exploration of quadratic response surfaces and generates a second degree polynomial model, which in turn is used in optimizing a process using a small number of experimental runs. This design requires an experimental number of runs according to:

$$N = k^2 + k + c_p \tag{1}$$

Where k is the factor number which is 3 in this case and c_p is the number of replications at the center point which is also 3 in this case. The design which was developed using Design Expert® 7.0.0 (Stat-ease, Inc. Minneapolis, USA), resulted in 15 experimental runs as shown in Table 2. The 15 experimental runs were randomized to maximize the effects of unexplained variability in the observed responses due to extraneous factors. The levels of the independent variables as shown in Table 1 were selected based on preliminary experiments. Since the pH conditions change in the course of fermentation, the pH range of 2- 8 had to be tested to optimize growth conditions. The choice of the pH range was also based on previous studies [13,14]. The relation between the coded and actual values is described as follows:

$$x_i = \frac{X_i - X_o}{\Delta X_i} \tag{2}$$

Where x_i and X_i are the coded and actual values of the independent variable respectively. X_o is the actual value of the independent variable at the center point, and ΔX_i is the step change of X_i. A second degree polynomial was fitted to the experimental data using the statistical package Design Expert® 7.0.0 to estimate the response of the dependent variable and predict the optimal point. The second degree polynomial was expressed as follows:

$$Y = b_o + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_{12} X_1 X_2 + b_{13} X_1 X_3 + b_{23} X_2 X_3 + b_{11} X_1^2 + b_{22} X_2^2 + b_{33} X_3^2 \quad (3)$$

Where Y is predicted response, X_1, X_2 and X_3 are independent variables, b_0 is offset term, b_1, b_2, b_3 are linear effects, b_{11}, b_{22}, b_{13} are interaction terms.

Table 1. Coded and actual levels of the factors for three factor Box-Behnken design

Independent variables	Symbols	Coded and actual levels		
		-1	0	+1
Substrate concentration (g/L)	X_1	30	40	50
pH (-)	X_2	2	5	8
Temperature (ºC)	X_3	25	30	35

3. RESULTS AND DISCUSSION

3.1 Statistical Analysis

The results obtained from the 15 experimental runs carried out according to the Box-Behnken design are summarised in Table 2. The proposed second degree polynomial was fitted to the data presented in Table 2 using multiple linear regressions to determine the optimum fermentation conditions that resulted in the maximum concentration of citric acid. The effects of substrate concentration, broth pH and fermentation temperature were quantitatively evaluated using response surface curves. By applying multiple regression analysis on the experimental data, the following second degree polynomial was found to represent the relationship between the concentration of citric acid and substrate concentration, broth pH and fermentation temperature adequately.

$$\begin{aligned} Y = {} & 52.241 - 2.621X_1 - 0.577X_2 + 0.842X_3 + 0.072X_1X_2 - 0.000722X_1X_3 \\ & - 0.068X_2X_3 - 1.895X_1^2 - 2.653X_2^2 - 0.00066X_3^2 \end{aligned} \quad (4)$$

The predicted levels of citric acid concentration using Equation (4) are given in Table 2 along with experimental data. The significance of the fit of the second-order polynomial for the concentration of citric acid was assessed by carrying out analysis of variance (ANOVA) with results shown in Tables 3 and 4.

The coefficient of determination (R^2) of the model was 0.976 (Table 3), which indicated that the model adequately represented the real relationship between the variables under consideration. An R^2 value of 0.976 means that 97.6% of the variability was explained by the model and only 2.40% was as a result of chance. The coefficient of variation (C.V.) obtained was 6.72%. The Coefficient of Variation (C.V) indicates the degree of precision with which the treatments were carried out. A low value of C.V suggest a high reliability of the experiment [10,15]. Adequate precision value (13.921) measures the signal to- noise ratio, and a ratio greater than 4 is generally desirable [16].

Table 2. Three factor Box-Behnken design with experimental as well as predicted responses of dependent variable (citric acid concentration, g/L)

Runs	Factors						Response	
	Coded values			Actual values			Citric acid concentration (g/L)	
	X_1	X_2	X_3	X_1	X_2	X_3	Observed	Predicted
1	+1	+1	0	50	8	30	20.47	21.11
2	+1	-1	0	50	2	30	24.48	23.34
3	-1	-1	0	30	2	30	15.69	14.05
4	-1	0	-1	30	5	25	15.87	15.96
5	0	-1	-1	40	2	25	23.45	23.01
6	0	0	0	40	5	30	15.26	15.51
7	0	0	0	40	5	30	14.99	15.51
8	0	+1	+1	40	8	35	36.42	35.87
9	-1	0	+1	30	5	35	13.68	14.10
10	-1	+1	0	30	8	30	15.37	16.52
11	+1	0	-1	50	5	25	30.72	29.30
12	+1	0	+1	50	5	35	14.73	14.64
13	0	+1	-1	40	8	25	12.05	13.82
14	0	0	0	40	5	30	16.27	16.51
15	0	-1	+1	40	2	35	12.20	11.43

Table 3. Statistical information for ANOVA

Source	Response value
R-squared	0.976
Adjusted R-squared	0.945
Standard deviation	1.530
C.V %	6.720
Adeq. precision	13.921

Results obtained after carrying out ANOVA is presented in Table 4. Values of ‘‘Prob. > F’’ less than 0.05 indicate the model terms are significant. Values greater than 0.10 indicate the model terms are not significant. A model F-value of 14.30 and a very low probability value [(Prob > F) less than 0.0001] imply significant model fit. From the regression model of citric acid concentration, the model terms X_1, X_2, X_3, X_1^2 and X_2^2 were significant with a probability of 95%. The terms X_1X_2 and X_2X_3 were also significant indicating that there was interaction between substrate concentration and broth pH as well as broth pH and fermentation temperature. The interaction between the terms X_1 and X_3 however had no significant effect on the concentration of citric acid produced during fermentation. The "Lack of Fit" F-value of 2.15 implies that there is insignificant lack of fit. The "Lack of Fit" (Prob > F) value of 0.27 implies that there is only 27 % chance that the “Lack of Fit" F-value could occur due to noise.

Table 4. Analysis of variance (ANOVA) for quadratic model of citric acid concentration

Sources	Sum of squares	df	Mean squares	F value	p- value [Prob >F]
Model	413.21	9	56.42	14.30	<0.0001
X_1–Substrate concentration	17.61	1	17.61	6.48	0.03
X_2 – pH	0.45	1	0.45	17.11	0.0061
X_3 - Temperature	298.81	1	298.81	129.26	<0.0001
X_1X_2	1.89E-04	1	1.89E-04	5.31E-05	0.01
X_1X_3	0.20	1	0.20	7.28	0.87*
X_2X_3	2.32	1	2.32	0.50	0.005
X_1^2	5.34	1	5.34	5.53	0.01
X_2^2	8.20	1	8.20	3.01	0.02
X_3^2	99.41	1	99.41	51.04	0.99*
Residual	20.93	8	2.69		
Lack of Fit	14.28	4	2.57	2.15	0.27
Pure Error	6.65	4	2.06		
Cor Total	536.13	16			

**not significant*

3.2 Optimization of Citric Acid Fermentation

In order to optimise the variables that influence the production of citric acid from corn starch, response surface plots were generated from the regression model. The three-dimensional (3D) plots were generated by keeping one variable constant at the centre point and varying the others within the experimental range. The resulting response surfaces showed the effect of substrate concentration, broth pH and fermentation temperature on citric acid concentration.

Figs. 1 to 3 represent the response surface and contour plots for the optimization of citric acid production. Fig. 1 shows the response surface and corresponding contour plots for citric acid concentration as a function of substrate concentration and broth pH. An increase in the substrate concentration with an accompanying decrease in broth pH resulted in an increase in citric acid concentration from about 9g/L to a maximum value of 31.96 g/L at 50 g/L substrate concentration and 2.00 broth pH.

The effect of fermentation temperature and broth pH on the concentration of citric acid is presented in Fig. 2. The trend observed indicate that high values of citric acid concentration were predicted for different combinations of low temperature and low broth pH as well as high temperature and high broth pH. The best citric acid concentration of about 30 g/L was obtained at a temperature of 25^{o}C and a broth pH of 2.00.

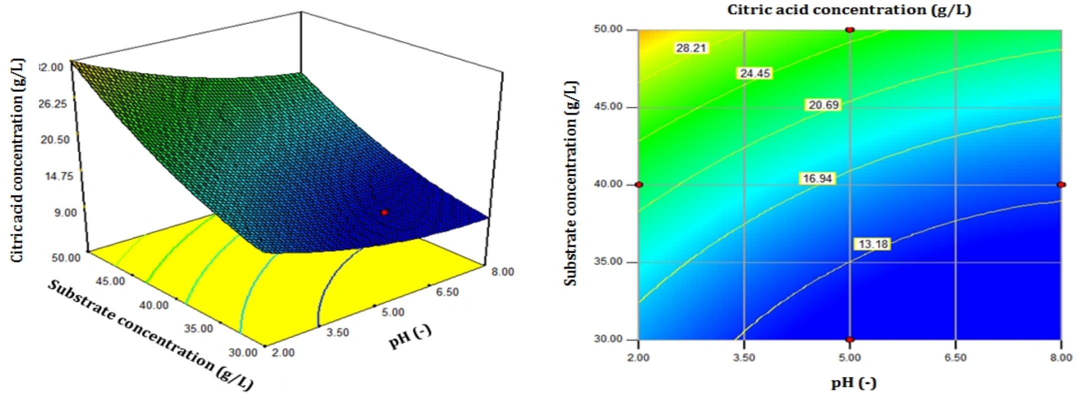

Fig. 1. Response surface plot and the corresponding contour plot showing the effects of substrate concentration and broth pH on citric acid concentration

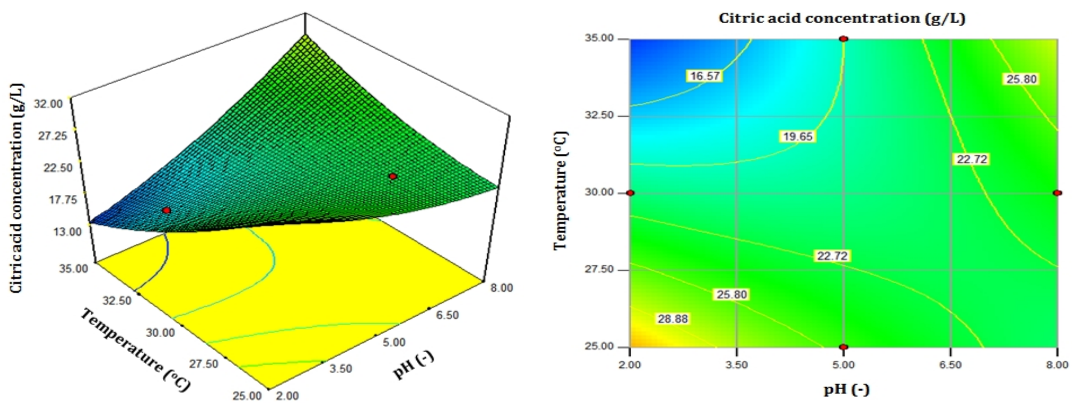

Fig. 2. Response surface plot and the corresponding contour plot showing the effects of temperature and broth pH on citric acid concentration

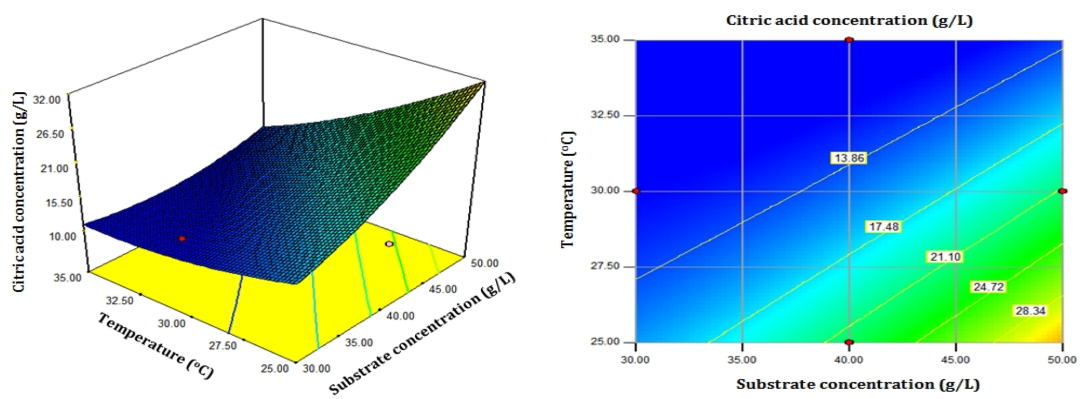

Fig. 3. Response surface plot and the corresponding contour plot showing the effects of temperature and substrate concentration on citric acid concentration

Fig. 3 shows the effect of the interaction between fermentation temperature and substrate concentration on citric acid concentration. An increase in substrate concentration with corresponding decrease in temperature resulted in an increase in citric acid concentration from about 12 g/L to a maximum value of 31.96 g/L at 50 g/L substrate concentration and 25ºC temperature.

In order to select the optimum conditions and their respective levels, the model was analysed. The maximum response predicted from the model was a citric acid concentration of 31.96 g/L. The final optimised fermentation conditions obtained with RSM were 50 g/L (substrate concentration), 2.00 (broth pH) and 25ºC (fermentation temperature). The high levels of citric acid recorded at pH 5 or 8 were not the optimised values as those particular experimental runs did not account for the optimised simultaneous effect of all three independent variables investigated. The optimal pH value of 2 obtained from response surface methodology is the pH value at which the simultaneous effect of all tested variables was manifested on the citric acid production.

The broth pH is important in two respects. Firstly, spore germination which is required for fermentation requires a pH of 5 and above to occur. Secondly, protons are released when nitrogen containing nutrients are absorbed by germinating spores during fermentation. This causes a decrease in the pH of the medium. Hence, a decrease in broth pH as fermentation progresses is an indication of citric acid production. The low pH has the effect of improving citric acid production, inhibits the production of unwanted organic acids which makes recovery difficult and provides a sterile environment which reduces the risk of contamination [7,8]. The fermentation temperature is important in that when cells are grown under non-ideal temperature conditions, they exhibit signs of adverse growth and metabolic production [17]. Nampoothiri et al. [18] reported that citric acid production could be affected by a slow germination of the fungi, slow metabolic activity, enzyme denaturation and reduced cell viability when *Aspergillus niger* cells are incubated under extremely low or high temperatures. Optimum temperatures are therefore favoured. The optimum temperature obtained in this study is in agreement with the fact that filamentous fungi such as *Aspergillus niger* are mesophilic thus requiring optimal temperatures around 25ºC for growth [19,20].

The validity of the results predicted by the regression model, was confirmed by carrying out repeated experiments under optimal fermentation conditions (i.e. substrate concentration; 50 g/L, broth pH; 2.00 and fermentation temperature; 25ºC). The results obtained from three replications demonstrated that the average of the maximum citric acid concentration (31.24 g/L) obtained was close to the predicted value (31.96 g/L). The excellent correlation between the predicted and measured values from these experiments indicates validity of response model.

4. CONCLUSION

In this work, the variables that could influence citric acid production from hydrolysed corn starch were optimised. They include substrate concentration, broth pH and fermentation temperature. The following conclusions can be drawn from the study.

- The use of response surface methodology to determine the optimum conditions for the production of citric acid from hydrolysed corn starch has been demonstrated.
- Citric acid fermentation is influenced by the concentration of substrate, broth pH and fermentation temperature.

- The concentration of citric acid produced during fermentation is related to substrate concentration, broth pH and fermentation temperature by a validated quadratic regression model.
- The quadratic regression model was able to predict to a high level of confidence, the concentration of citric acid produced during fermentation by *Aspergillus niger*.
- The combination of optimum fermentation conditions were a substrate concentration of 50 g/L, broth of pH 2.00, and fermentation temperature of 25ºC. Under these conditions, the maximum concentration of citric acid was obtained to be 31.96 g/L.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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