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Citric Acid Production from Sugarcane Bagasse: A Case of Optimization using Response Surface Methodology

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ABSTRACT

This study investigated the use of response surface methodology (RSM) for optimizing the effect of trace metals and chelating agents on citric acid production from sugarcane bagasse. A three level Box-Behnken design was used to plan the experiments while RSM was used to optimise citric acid production as well as determining the effect of ammonium oxalate, copper and EDTA on citric acid production. The statistical model developed using RSM was statistically significant ($p<0.05$) and showed a good fit with the experimental data ($R^2= 0.954$). The optimal levels of the independent variables were determined to be 0.05 g/l of copper, 10 g/l of ammonium oxalate and 0.29 g/l of EDTA, after numerical optimization by RSM was carried out. The maximum concentration of citric acid produced was obtained to be 29 g/l.

1. INTRODUCTION

Citric acid is a weak organic acid that can be found practically in all plants and in many animal tissues or fluids. There are vast uses of citric acid, with it being used in the food and beverage industry as an additive and a natural food preservative. It finds application in the canning industry, pharmaceutical industry and the detergent industry (Kuforiji et al., 2010).

It has been reported that over 90% of citric acid produced worldwide is by fermentation. The production of citric acid by fermentation can be carried out in three ways which are; submerged fermentation, surface fermentation and solid state fermentation. However, most industrial scale production of citric acid is achieved by solid state fermentation using *Aspergillus niger* because this option has lower energy and water requirements, less risk of bacterial contamination, less wastewater generation and less environmental concern as a result of the use of solid waste for the production of value-added products like citric acid (Amenaghawon et al., 2014).

A large number of microorganisms can be used for fermentation, though few can produce citric acid on an industrial scale, hence the most preferred microorganism for commercial production is the *Aspergillus niger* (Hayder, 2012).

Citric acid production by microbial fermentation is affected by various factors which include; the nutritional composition of the fermentation media, environmental conditions, deficiency of trace metals and dissolved oxygen. Other factors that also affect the production of citric acid are the types and concentration of sugars, chelating effect on metal ions, ammonium nitrate and aeration (Hauka et al., 2005). The optimization of these factors is imperative to obtain the maximum yield of citric acid. Traditionally, citric acid production is optimized by varying one factor at a time while keeping the others constant. Although this method is useful, it involves the use of large experimental runs to find the optimal conditions. It also does not explain the effect of interaction between the factors under consideration (Montgomery, 2005).

In the last decades response surface methodology (RSM) which is an empirical statistical technique employed for multiple regression analysis of quantitative data, has been used for the analysis and optimization of experimental results (Fang et al., 2010). This is due to the fact that RSM is able to clarify and explicate the interaction of independent variables and their effect on responses. This method is very effective and has been successfully applied to the optimisation of many bioprocesses (Montgomery, 2005; Anupama et al., 2010; Fang et al., 2010; Tian et al., 2011; Amenaghawon et al., 2015; Velu et al., 2016).

This study focused on modelling and optimisation of the effect of trace metals and chelating agents on citric acid produced from sugarcane bagasse using *Aspergillus niger*. A mathematical model was developed to predict the production of citric acid and the suitability of RSM as an optimisation tool was then assessed.

2. MATERIALS AND METHODS

2.1. Feedstock Collection, Preparation and Pretreatment

Fresh sugarcane bagasse was obtained from a local sugar processing factory located in Lagos State, Nigeria. The bagasse was subsequently sun dried in order to reduce its moisture content to prevent biodeterioration by microbial action. The dried bagasse was then milled to a particle size of 2 mm using a laboratory mill. It was then pretreated in an autoclave using dilute NaOH solution.

2.2. Microorganism and Inoculum Preparation

The fermenting organism *Aspergillus niger* ATCC 9167 was obtained from the Department of Microbiology, University of Benin. Conidia suspensions of fungal strains were obtained from cultures grown on potato dextrose agar slants at 30°C for 5 to 7 days. The spores were washed with sterilized 0.8% Tween 80 solution by shaking vigorously for one minute. Spores were counted with a haemocytometer to obtain approximately 2×10^7 spores/ml (Amenaghawon et al., 2014).

2.3. Solid State Fermentation

Erlenmeyer flasks (250ml) were used to carry out the solid state fermentation for the production of citric acid using sugarcane bagasse and *Aspergillus niger*. The composition of the un-optimized fermentation medium was 0.015g/l of $FeCl_3$, 0.0012g/l of $ZnSO_4$, 0.015g/l of $CaCl_2$. The concentration of ammonium oxalate, copper and EDTA were determined according to the experimental design. The solid substrate (sugarcane bagasse) was added in the flask and wetted with the nutrient medium to the desired moisture content. The contents were then thoroughly mixed, cotton plugged and autoclaved at 121ºC, 15 psi for 15 mins. After cooling, the substrate plus media was then inoculated with 2 ml of inoculum (2×10^7 spores/ml) and then incubated at 30 ºC for 5 days. After 5 days, the medium was diluted with 100 ml of distilled water, filtered and the subsequent filtrate was analyzed.

2.4. Analytical Method

The concentration of citric acid produced during fermentation was determined using the pyridine-acetic anhydride method (Marrier and Boulet, 1958).

2.5. Design of Experiment

A three variable Box-Behnken design was used to plan the experiments for analysing the effect of the factors on citric acid production. The range of the variables that were optimised is shown in Table 1. Equation 1 is a quadratic response model which was used to fit the experimental data and this was achieved by using multiple regression analysis to estimate the values of the coefficients of the model. Analysis of variance (ANOVA) was then used to assess the quality and significance of the model.

$$Y_i = b_o + \sum b_i X_i + \sum b_{ij} X_i X_j + \sum b_{ii} X_i^2 + e_i \tag{1}$$

Y_i is the predicted response or dependent variable, X_i and X_j are the independent variables, b_o is the offset term, b_i and b_{ij} are the single and interaction effect coefficients and e_i is the experimental error term. The low, middle, and high levels of each variable were coded as -1, 0, and +1, respectively. The factors were coded according to Equation 2.

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

Where x_i and X_i are the coded and actual values of the factors respectively. X_o is the actual value of the factors at the centre point and ΔX_i is the step change in the actual value of the factors. Design Expert® 7.0.0 (Stat-ease, Inc. Minneapolis, USA), a statistical software used for the experimental design, regression analysis and analysis of variance (ANOVA).

Table 1: Experimental range and level of the independent variables

Variables	Unit	Symbol	Coded levels		
			-1	0	+1
Copper	g/l	X_1	0.00	0.025	0.05
Ammonium oxalate	g/l	X_2	0.00	5.00	10.00
EDTA	g/l	X_3	0.00	0.15	0.30

3. RESULTS AND DISCUSSION

3.1. Statistical Analysis

The coded and actual values of the independent variables X_1 (copper), X_2 (ammonium oxalate), and X_3 (EDTA) as obtained from the Design Expert software with the corresponding values of citric acid concentration obtained from experiment and those predicted by the statistical model (Equation 3) are shown in Table 2.

$$\begin{aligned} Y = {} & 12.81 - 122.74X_1 + 0.53X_2 + 31X_3 + 0.89X_1X_2 + 97.73X_1X_3 \\ & - 1.56X_2X_3 + 2882.72X_1^2 + 0.074X_2^2 - 34.52X_3^2 \end{aligned} \tag{3}$$

The ANOVA results presented in Table 3 reveals that the statistical model was statistically significant, since the F value is large (16.15) and p value is very small (p=0.0007). The model terms representing the concentrations of ammonium oxalate and EDTA i.e. X_2, and X_3 respectively were statistically significant indicating that changes in the concentration of these substances could significantly affect citric acid production.

Table 2: Box-Behnken design matrix for citric acid production

Run no	Factors (g/l)			Citric acid concentration (g/L)	
	X1	X2	X3	Experiment	Predicted
1	1	0.025	0	0.30	19.52
2	2	0.000	10	0.15	27.73
3	3	0.000	0	0.15	16.32
4	4	0.025	5	0.15	20.52
5	5	0.000	5	0.00	17.78
6	6	0.050	5	0.00	18.91
7	7	0.050	0	0.15	17.28
8	8	0.050	10	0.15	29.14
9	9	0.050	5	0.30	22.94
10	10	0.025	5	0.15	19.58
11	11	0.000	5	0.30	20.34
12	12	0.025	5	0.15	19.77
13	13	0.025	5	0.15	18.97
14	14	0.025	10	0.00	23.15
15	15	0.025	10	0.30	26.68
16	16	0.025	0	0.00	11.31
17	17	0.025	5	0.15	16.62

Table 3: Analysis of variance for statistical model of citric acid concentration

Source	Sum of squares	df	Mean square	F Value	P value
Model	305.17	9	33.91	16.15	0.0007
X_1	4.65	1	4.65	2.22	0.1802
X_2	223.30	1	223.30	106.39	< 0.0001
X_3	42.04	1	42.04	20.03	0.0029
X_1X_2	0.049	1	0.049	0.023	0.8825
X_1X_3	0.54	1	0.54	0.26	0.6284
X_2X_3	5.48	1	5.48	2.61	0.1502
X_1^2	11.84	1	11.84	5.64	0.0493
X_2^2	14.40	1	14.40	6.86	0.0345
X_3^2	2.54	1	2.54	1.21	0.3076
Residual	14.69	7	2.10		
Lack of fit	5.84	3	1.95	0.88	0.5226
Pure error	8.85	4	2.21		
Cor total	319.86	16			

The R^2 value was obtained as 0.954 meaning that 95.4% of the variability in the response could be explained by the model as shown in Table 4. It is generally desirable for the predicted R-squared and the adjusted R-squared values to be within 0.20 of each other, otherwise there may be a problem with either the data or the model. For this study, the values of 0.954 and 0.895 obtained for the predicted R-squared and the adjusted R-squared values respectively satisfied this requirement indicating that the model was adequate to represent the experimental data. A low standard deviation of 1.45 means that there was very little deviation of the individual values of the response from the mean. The coefficient of variation (CV) is the standard deviation expressed as a percentage of the mean and experimental data is considered reproducible if the CV is not greater than 10%. In this study, the CV obtained was 7.11%. Adequate precision value measures signal to noise ratio. A ratio greater than 4 is desirable. A ratio of 15.62 was obtained in this study which indicates an adequate signal.

Table 4: Statistical information of Box-Behnken design

Parameter	Value
Standard deviation	1.450
Mean	20.390
C.V. %	0.665
R-Squared	0.954
Adj R-Squared	0.895
Adeq Precision	15.620

3.2. Analysis of response surface plots

Three-dimensional (3D) response surface plots were generated to evaluate the effect of the independent variables on citric acid production as shown in Figures 1 to 3. The plots were obtained by keeping two variables constant at the center points and varying the third within the experimental range. Figure 1 shows the effect of copper and ammonium oxalate on citric acid production. The trend observed indicates that citric acid production was enhanced by the addition of ammonium oxalate. This trend was observed both at low and at high concentrations of copper.

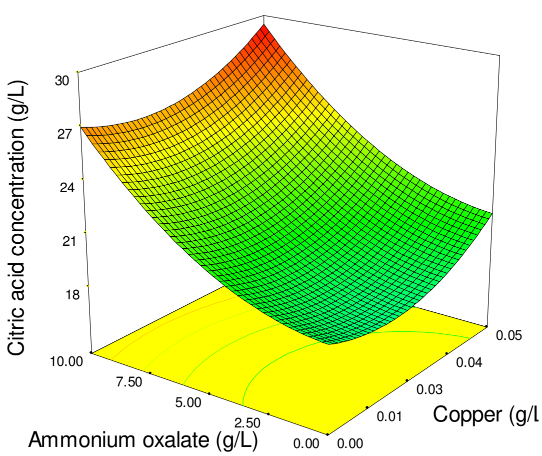

Figure 1: Effect of ammonium oxalate and copper on citric acid production

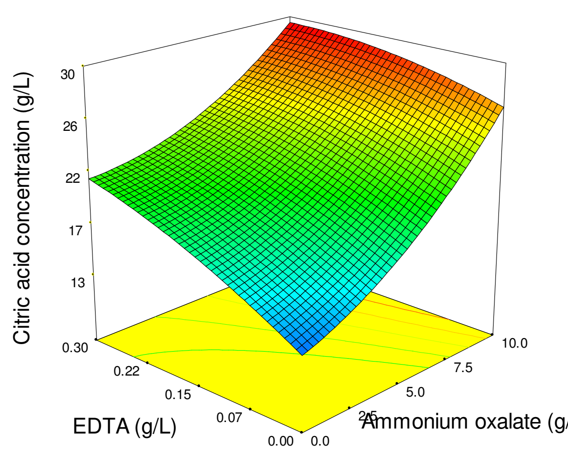

Figure 2: Effect of EDTA and ammonium oxalate on citric acid production

Chelating agents such as ammonium oxalate serve to mop up residual inhibitory metals present in the substrate. This was achieved by the ammonium oxalate decreasing the absorption of metals like calcium by binding with the metal and decreasing its availability in solution thereby reducing its inhibitory effects (Hauka et al., 2005; Amenaghawon et al., 2017). Copper also exhibited a stimulatory effect on citric acid production as seen in the upward trend of citric acid concentration when the concentration of copper was increased (Figure 1). This observation could be attributed to the fact that in small amounts, copper is essential for the action of a variety of enzymes in the microbial cell and is required for both growth and citric acid production (Amenaghawon et al., 2017). Similar observations have been reported by previous investigators (Mashhoor et al., 1987; Rasmy, 1999).

EDTA also enhanced citric acid production as seen in Figure 2. This observation is also attributed to the chelating properties possessed by EDTA as it forms complexes with metal ions present in the medium to promote microbial growth and citric acid production by minimizing the availability of these metal ions which tend to inhibit citric acid production (Hauka et al., 2005).

3.3 Numerical optimisation

Results obtained from numerical optimisation carried out using the Design Expert software revealed that the optimal citric acid concentration was 29 g/l. This was obtained at copper, ammonium oxalate and EDTA concentrations of 0.05 g/l, 10 g/l and 0.29 g/l respectively.

3.4 Validation of Statistical Model

To confirm the validity of the statistical model for citric acid production, triplicate confirmation experiments were performed at the specified optimum conditions. The results showed that the citric acid concentration obtained was close to the predicted value. The good correlation between predicted and experimental values after optimization justified the validity of the response model and the existence of an optimum point.

CONCLUSION

This study investigated the use of RSM for optimizing the effect of trace metals and chelating agents on citric acid production from sugarcane bagasse and the following conclusions drawn.

- The Box-Behnken design of experiment coupled with RSM can be used in the optimization of citric acid production.
- The production of citric acid is favoured by high levels of copper, ammonium oxalate and EDTA.
- The optimal levels of the independent variables were determined to be 0.05g/l of copper, 10 g/l of ammonium oxalate and 0.29 g/l of EDTA, after numerical optimization by RSM was carried out. The maximum concentration of citric acid produced was also obtained to be 29.129g/l.

5. CONFLICT OF INTEREST

There is no conflict of interest associated with this work.

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