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OPTIMISING THE EFFECT OF STIMULANTS ON CITRIC ACID PRODUCTION FROM COCOYAM STARCH USING *ASPERGILLUS NIGER*

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ABSTRACT
Additives such as low molecular weight alcohols, trace metals, phytate, lipids etc have been reported to stimulate citric acid production. Hence the objective of this study was to investigate the effect of stimulating the metabolic activity of Aspergillus niger for the purpose of improved citric acid production from cocoyam starch. A three-variable, three-level Box-Behnken design (BBD) was used to develop a statistical model to study the effects of Zinc (II) ion, Iron (III) ion and methanol on the production of citric acid. Response surface methodology (RSM) was used to optimise the effects of these stimulants. The results of analysis of variance (ANOVA) carried out on the model showed that the model was statistically significant ($p< 0.0001$) and did not show lack of fit ($R^2=0.997$). The results also showed that citric acid production increased when the levels of zinc and methanol were increased. Intermediate levels of iron were required to produce citric acid at optimum levels. Results obtained from RSM showed that the optimum levels of zinc, iron and methanol were 4.5 g/L, 6.87 g/L and 3.0 %v/v respectively. Under these conditions, the maximum citric acid concentration was obtained as 108 g/L. Validation of the model indicated no significant difference between predicted and experimental values.

Keywords: *Optimisation, Citric acid, Cocoyam, Fermentation, Methanol*

1. INTRODUCTION
Citric acid is a tri-carboxylic organic acid which finds a lot of uses in the food, beverage, pharmaceutical, chemical, cosmetic and other industries for applications such as acidulation, anti-oxidation, flavour enhancement, preservation, plasticizer and as a synergistic agent [1,2]. Due to the increasing demand of citric acid, it has been established that producing citric acid from synthetic or chemical methods cannot compete favourably with biotechnological means [3]. Hence, a large proportion of the world's demand for citric acid is satisfied from biotechnological means specifically submerged microbial fermentation of sucrose or molasses using the filamentous fungus, *Aspergillus niger* [4]. *Aspergillus niger* is the preferred fermenting organism for commercial scale production of citric acid because it has the capacity to ferment a wide range of cheap substrates and it can produce high yields of citric acid even at low pH values without the production of unwanted by products [5].

Nigeria is the world's largest producer of cocoyam, accounting for about 37% of total world output [6]. However, as a result of lack of proper facilities for the storage of the cocoyam tubers, large amounts in the order of millions of tons are reportedly destroyed through pest infestation, deterioration, physical damage to the tubers, pilfering etc [7]. Since the cocoyam tuber is rich in starch, the starch can be hydrolysed and fermented to produce value added products such as bioalcohols and citric acid, thereby recovering the losses resulting from wastage and expanding the usage range of these tubers [8].

The production of citric acid using *Aspergillus niger* has been reported to be influenced by the medium composition such as the concentration of carbon, nitrogen, phosphorous, potassium, trace elements (zinc, iron, manganese) and stimulants such as low molecular weight alcohol [9]. Several reports have shown the stimulatory effects of additives on citric acid production [10-12]. To improve citric acid production, stimulators such as trace metals, low

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molecular weight alcohols, phytate, lipids etc have been used [5]. Thus, citric acid productivity by *Aspergillus niger* could be further improved by optimising the medium composition as well as the effect of the stimulants. Experimental design method coupled with response surface methodology (RSM) has been reported to be very effective in achieving this and it has been successfully applied to the optimisation of many bioprocesses [13-16].

Hence the objective of this study was to optimise the effect of stimulants on citric acid production from cocoyam starch using *Aspergillus niger*. A three variable Box-Behnken design for response surface methodology was used to study the simultaneous effect of three independent variables (Fe^{3+}, $Zn^{2+}$and CH_3OH concentration) for optimum citric acid production.

2. MATERIALS AND METHODS

2.1 Cocoyam Starch Preparation

Cocoyam tubers were obtained from Benin City, Edo State, Nigeria.The cocoyam tuber is rich in carbohydrate containing abouit 77.9% starch making it an ideal substrate for producing citric acid [77]. The tubers were washed in clean water to remove the adhering dirt after which they were peeled manually and crushed using a mill. The crushed pulp was sieved with a sieve of Teflon cloth. The starch obtained was allowed to settle for about 12 h. It was decanted and the starch cake obtained was oven dried. The dried starch was then packed in a clean container for storage [7].

2.2 Two Step Enzymatic Hydrolysis of Cocoyam Starch

A solution of starch was prepared by weighing 20 g of starch into 80 mL of a solution containing 40 ppm Ca^{2+}. The pH was adjusted to 6.5 using citrate-phosphate buffer. The slurried starch was gelatinized by heating the mixture to 97 ^oC for 10 minutes after which 1% (v/v) of bacterial α-amylase was added for liquefaction to take place at a temperature 61 ^oC and a pH of 6.5 for 55 minutes. Enzyme activities were stopped by heating the mixture to boil. The final mixture was centrifuged at 10,000 rpm for 10 minutes and the supernatants were analyzed for reducing sugar. The liquefied starch was later subjected to saccharification using fungal glucoamylase at a temperature of 52 ^oC and a pH of 4.5 for 44 minutes and the mixture was treated as stated above [7].

2.3 Microorganism, Inoculum Preparation and Fermentation

Aspergillus niger ATCC 9142, obtained from the Biotechnology division of the Federal Institute of Industrial Research Oshodi (FIIRO), Lagos, Nigeria was used throughout the study as the fermenting organism. Conidia suspensions of fungal strains were obtained from cultures grown on potato dextrose agar slants at 30 ^oC for 5 to 7 days. The spores were washed with sterilised 0.8% Tween 80 solution by shaking vigorously for 1 minute. Spores were counted with a haemocytometer to obtain approximately 2 x 10^7 spores/mL. The composition (g/L) of the fermentation medium used for citric acid production was as described by Lotfy et al. [18]. $NaNO_3$, 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.The pH of the culture medium was adjusted to 5.5 by adding a sterile solution of sulphuric acid. 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 30 ^oC.

2.4 Analytical Methods

The concentration of citric acid produced during fermentation was determined using the pyridine-acetic anhydride method as reported by Marrier and Boulet [19]. This was accomplished by adding 1 mL of the filtered fermentation broth along with 1.30 mL of pyridine and 5.7 mL of acetic anhydride in a test tube. The test tube was then placed in a water bath at 32 ^oC for 30 min. The absorbance of the sample was measured at 405 nm using a UV-Vis spectrophotometer (PG Instruments model T70). The concentration of citric acid in the sample was determined from a citric acid calibration curve which was prepared from known concentrations of citric acid.

2.5 Design of Experiment

A three variable Box Behnken design (BBD) for response surface methodology was used to develop a statistical model for the fermentation process. The levels of variables optimised are shown in Table 1. The experimental design was developed using Design Expert® 7.0.0 (Stat-ease, Inc. Minneapolis, USA). The coded and actual values of the independent variables were calculated as follows.

$$X_i = \frac{X_i - X_o}{\Delta X_i} \quad (1)$$

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 centre point and ΔX_i is the step change in the actual value of the independent variable. The following generalised second order polynomial equation was used to estimate the response of the dependent variable.

$$Y_i = b_o + \sum b_i X_i + \sum b_j X_i X_j \sum b_{ii} X_i^2 + e_i \qquad (2)$$

Y_i is the dependent variable or predicted response, 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 error term.

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

Independent Variable	Symbols	Coded and Actual level		
		-1	0	1
CH_3OH (%v/v)	X_1	0	1.5	3
Fe^{3+} (g/L)	X_2	0	5	10
Zn^{2+} (g/L)	X_3	0	2.25	4.5

3. RESULTS AND DISCUSSION

3.1 Statistical Analysis

The Box Behnken design resulted in 17 experimental runs as shown in Table 2. The response variable was chosen as the citric acid concentration. Citric acid concentration was chosen as the response because it is the most convenient measure of the amount of citric acid produced during fermentation. Equation (3) is the quadratic statistical model in terms of actual variables that was obtained after applying multiple regression analysis to the experimental data presented in Table 2.

$$Y = 84.07 - 22.74X_1 + 0.582X_2 - 16.47X_3 - 0.22X_1X_2 + 5.48X_1X_3 + 1.16X_2X_3 + 5.52X_1^2 - 0.37X_2^2 + 1.23X_3^2 \qquad (3)$$

The values of citric acid concentration predicted by Equation (3) are also presented in Table 2. The results of analysis of variance (ANOVA) carried out to determine the fit of the statistical model are presented in Tables 3 and 4. The ANOVA results showed that the model was statistically significant with a very low p value ($p<0.0001$) as shown in Table 3. The model did not show lack of fit as seen from the "lack of fit" p value of 0.657. The first order main effects of methanol, iron and zinc concentration were significant as evident from their respective p values. This suggests that changes in the levels of these stimulants could affect the growth rate of the fungal cells as well as citric acid production [20]. The parity plot comparing experimental values of the response and those predicted by the statistical model showed that there was an acceptable level of correlation between the experimental and model predicted results as shown in Figure 1. This is evident from the fact that the data points all clustered around the 45° diagonal line showing that there was minimal deviation between experimental and predicted values thus indicating optimal fit of the model.

Statistical information for ANOVA showed that the model had a high coefficient of determination (R^2) as shown in Table 4. This shows that the model was able to adequately represent the relationship between the chosen factors (methanol, iron (III) and zinc (II) concentration) and the response (citric acid concentration).

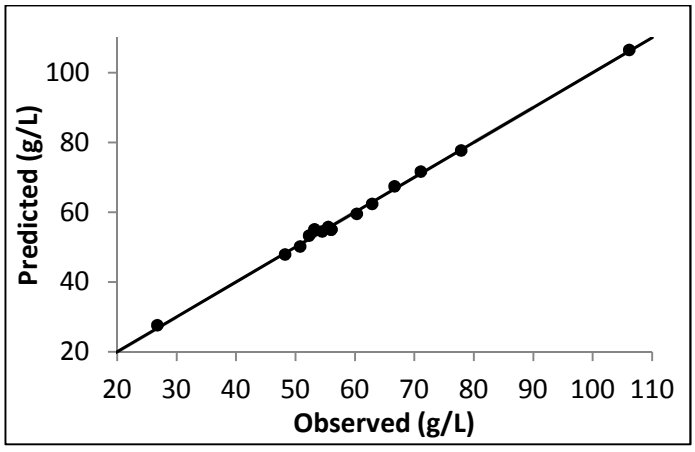

Figure 1: Comparison between predicted and observed values of citric acid concentration

An R^2 value of 0.997 means that the model was able to explain 99.7% of the variability observed in the response. The standard deviation was observed to be relatively small compared to the mean further validating the model. The coefficient of variation (CV) was obtained as 2.35. This value gives an indication of the degree of precision with which the treatments were carried out [21]. The relatively low value of CV obtained showed that the treatments were carried out with high precision and reliability [22]. The Adequate precision for the model was obtained as 73.95 showing that the model can be used to navigate the design space [23].

Table 2: Three variable Box-Behnken design for citric acid production

Runs	Factors						Responses
	Coded levels			Actual Values			Citric acid (g/l)
	X_1	X_2	X_3	X_1	X_2	X_3	Observed
1	1	1	0	3	10	2.25	52.34
2	0	1	-1	1.5	10	0	71.14
3	0	0	0	1.5	5	2.25	48.31
4	0	0	0	1.5	5	2.25	60.38
5	0	0	0	1.5	5	2.25	77.92
6	1	0	-1	3	5	0	55.58
7	-1	0	-1	0	5	0	54.57
8	0	0	0	1.5	5	2.25	106.19
9	-1	0	1	0	5	4.5	62.98
10	-1	-1	0	0	0	2.25	26.80
11	1	0	1	3	5	4.5	50.85
12	0	1	1	1.5	10	4.5	66.75
13	-1	1	0	0	10	2.25	56.06
14	1	-1	0	3	0	2.25	53.26
15	0	-1	-1	1.5	0	0	56.06
16	0	-1	1	1.5	0	4.5	53.26
17	0	0	0	1.5	5	2.25	56.06

Table 3: ANOVA for quadratic model for citric acid production

Source	Sum of squares	df	Mean square	F value	p-value
Model	4180.6	9	464.51	239.95	< 0.0001
X_1	452.46	1	452.46	233.72	< 0.0001
X_2	153.72	1	153.72	79.402	< 0.0001
X_3	379.27	1	379.27	195.91	< 0.0001
X_1X_2	11.303	1	11.303	5.8384	0.0463
X_1X_3	1367.7	1	1367.7	706.51	< 0.0001
X_2X_3	678.52	1	678.52	350.49	< 0.0001
X_1^2	648.61	1	648.61	335.04	< 0.0001
X_2^2	364.82	1	364.82	188.45	< 0.0001
X_3^2	162.57	1	162.57	83.973	< 0.0001
Residual	13.55	7	1.9359		
Lack of Fit	4.123	3	1.3744	0.5831	0.657
Pure Error	9.428	4	2.357		
Cor Total	4194.	16			

Table 4: Statistical information for Box-Behnken design

Variable	Value
Standard deviation	1.39
Mean	59.32
C.V.%	2.35
R^2	0.997
Predicted R^2	0.98
Adeq Precision	73.95

3.2 Optimisation of Citric Acid Production

Figures 2 to 4 represent the response surfaces describing the interactive effects of the tested variables on the response. Citric acid production was positively influenced by Zn^{2+}as shown in Figure 2. Citric acid concentration was observed to increase with increase in the concentration of Zn^{2+}. This trend could be attributed to the stimulating effect of Zn^{2+}on the fungal cells. Citric acid production was inhibited by Fe^{3+} as shown by the decrease in citric acid concentration when the concentration of Fe^{3+} was increased. This trend was observed at low levels of Zn^{2+}. However, at high levels of Zn^{2+}, the inhibitory effect of Fe^{3+} was suppressed such that optimum levels of citric acid production was recorded at intermediate levels of Fe^{3+}. A similar trend was reported by Majolli and Aguirre [24]. A search of the literature revealed that there is no generally established trend with respect to the effects of these traces metals. Some studies have reported stimulatory effects while others have reported inhibitory effects [25]. Nevertheless, it is well established that trace metal ions particularly divalent metal ions such as zinc, iron, manganese etc can significantly affect citric acid production by *Aspergillus niger* [26]. Hence, Soccol *et al*. [27] advised that it is necessary to take the interdependence of the fermentation medium constituents into account and that citric acid production could be improved only if a strict control of the trace metal availability is achieved.

Low molecular weight alcohols such as methanol and ethanol have been known to positively influence citric acid production [5]. Figure 3 shows the effect of the interaction between methanol concentration and Fe^{3+} concentration on citric acid production. Citric acid production increased generally in the presence of methanol. A similar trend has been reported by previous researchers. Alben and Erkmen [28] reported that the addition of methanol at a concentration of 1 to 4% resulted in a significant increase in the amount of citric acid produced by *Aspergillus niger* in spent grain liquor in brewery wastes. El-Holi and Al-Delaimy [29] attributed these observations to the reduction of the inhibitory effect of metal ions. They also suggested that methanol could also have served as carbon substrate for the microbial cells. It has also been suggested that the addition of methanol under optimised conditions increases the permeability of the cell membrane of *Aspergillus niger* which subsequently increases the transfer of nutrients, which in turn increases the secretion of

citric acid across the cell membrane [30]. Figure 3 also shows that the inhibitory effect of Fe^{3+} was suppressed in the presence of methanol.

The results presented in Figure 4 corroborated those presented in Figures 2 and 3 with respect to the trends observed for Zn^{2+} and methanol respectively. The optimisation of the statistical model resulted in the selection of the optimum fermentation conditions and their respective levels.

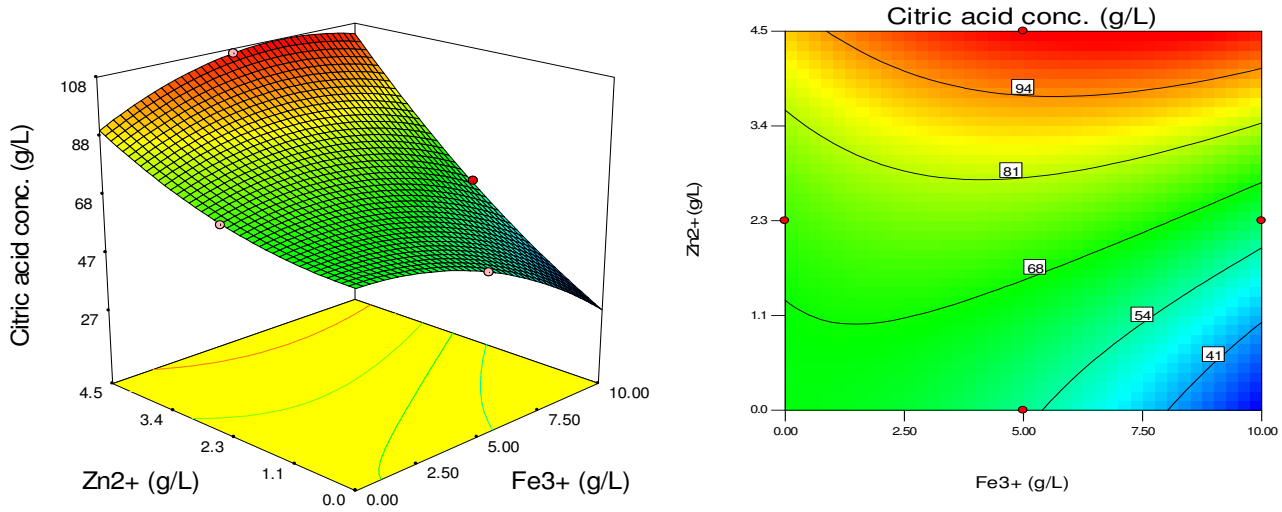

Figure 2: Response surface and corresponding contour plot showing the effect of Zinc (II) and iron III

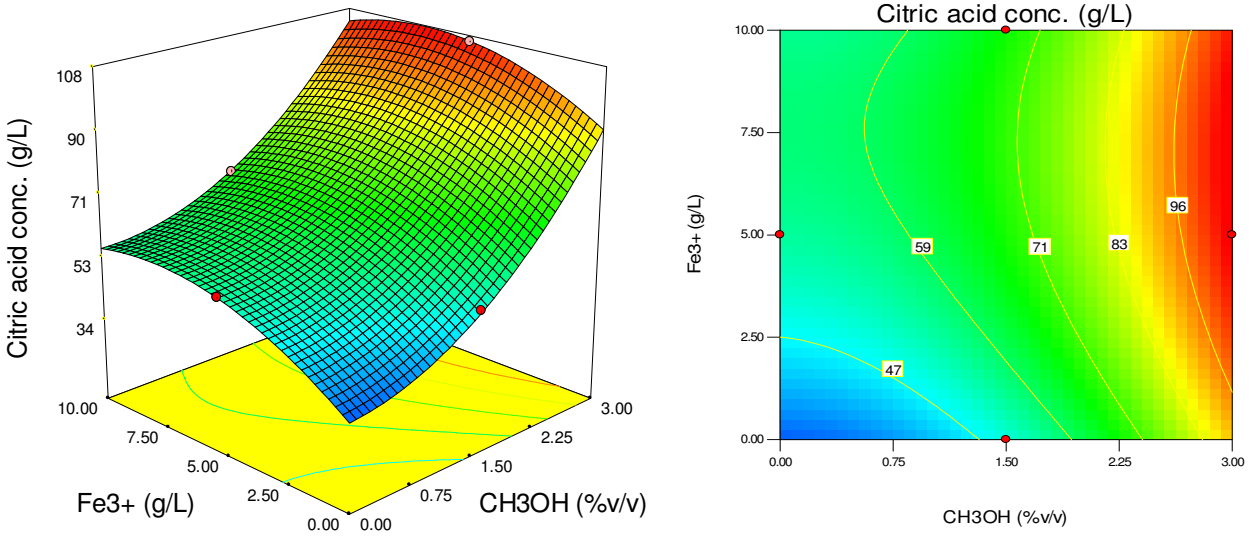

Figure 3: Response surface and corresponding contour plot showing the effect of iron (III) and methanol

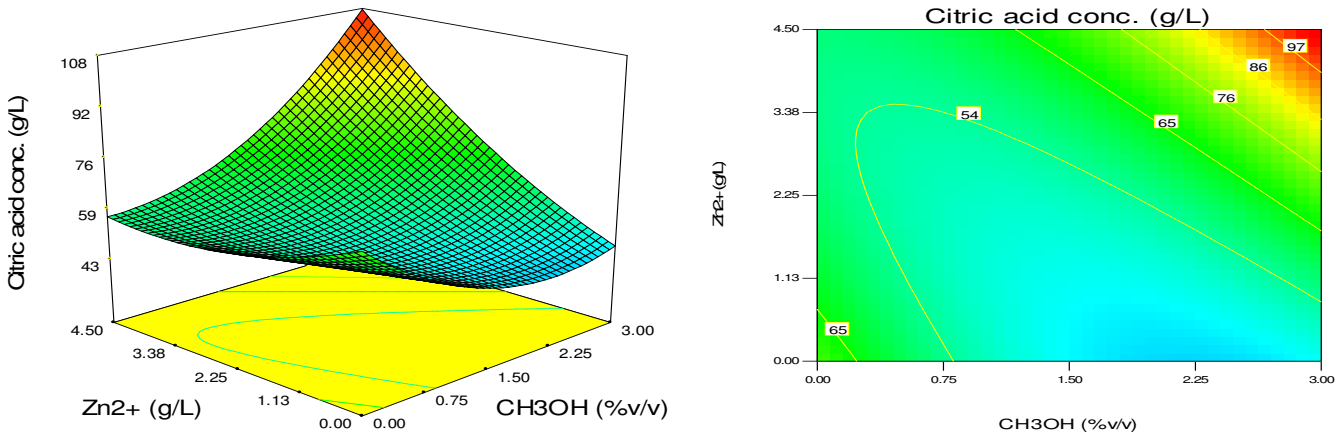

Figure 4: Response surface and corresponding contour plot showing the effect of Zinc (II) and methanol

The maximum citric acid concentration predicted by the model was 108 g/L. The final optimised conditions for the production citric acid as obtained from RSM were Zn^{2+} concentration of 4.5 g/L, Fe^{3+} concentration of 6.87 g/L and CH_3OH concentration of 3% v/v. The statistical model was validated by comparing model predicted results with those of repeated experiments carried out at the optimised conditions. The mean of the results obtained from three replications was close to that predicted by the model thus showing validity.

4. CONCLUSION

Citric acid production from cocoyam starch via submerged fermentation using *Aspergillus niger* was investigated in this study. A three-variable, three-level Box-Behnken design was used to study the simultaneous effect of stimulants such as Zn^{2+}, Fe^{3+}, CH_3OH on citric acid production. A statistically significant model ($p<0.0001$) was developed to describe the relationship between citric acid concentration and the chosen independent variables. The fermentation conditions were optimised using RSM. The statistical model showed a good fit with the experimental data ($R^2=0.997$) with a low standard deviation. High concentration of Zn^{2+}, high concentration of CH_3OH and intermediate concentration of Fe^{3+} were favourable for citric acid production. Citric acid production was positively influenced by Zn^{2+} and CH_3OH while the reverse was the case for Fe^{3+}. The optimum values of Zn^{2+}, Fe^{2+} and CH_3OH were 4.5 g/L, 6.87 g/L and 3% v/v respectively. Under these conditions, the citric acid concentration was obtained as 108 g/L.

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