

Research Article

Optimization of Medium Constituents for Citric Acid Production from Groundnut Husk by *Aspergillus niger*

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ABSTRACT

Citric acid is one of the multipurpose organic acids used in food and pharmaceutical applications. It is extensively used as preservative, flavourant and acidulant. The effect of medium constituents affecting citric acid production using groundnut husk by *Aspergillus niger* was studied. The most significant variables (urea, MnSO₄ and FeSO₄) associated with citric acid production were optimized using Face Centred Central Composite Design (FCCCD). Maximum citric acid production of 5.34 mg/g was obtained when the concentrations of urea, MnSO₄, and FeSO₄ were set at 0.04 %, 0.024 %, and 0.125 %, respectively. The coefficient of determination (R²) and adjusted R² values of 0.9853 and 0.9720 were obtained respectively with a non-significant lack of fit value of 0.2304. The results obtained from this study demonstrated that groundnut husk-based medium can be utilized for citric acid production by *A. niger*.

Keywords: *Aspergillus niger*, Central Composite Design, Citric acid, Groundnut husk, Optimization

INTRODUCTION

Citric acid, a common metabolite of plants and animals is widely used in the beverage, food, pharmaceutical and chemical industries as flavouring, preserving and antifoaming agent (Iralapati and Kummari, 2014). Several microorganisms were found to accumulate citric acid during their growth and metabolism on various homogenous and heterogeneous substrates; these include *Aspergillus flavus*, *A. niger*, *A. phoenicus*, *A.saitoi*, *A. luchensis*, *A. lanosius*, *A. awamori*, *A. wenti*, *A. fonsecaeus*, *Absidia* sp., *Aschochyta* sp., *Botrytis* sp., *Acremonium* sp., *Eupenicillium* sp., *Mucor piriformis*, *Penicillium janthinellum*, *P. restrictum*, *Talaromyces* sp., *Trichoderma viride* and *Ustilina vulgaris*, (Mattey and Kristiansen, 1999; Magnuson and Lasure, 2004;

Li *et al.*, 2007; Rigo *et al.*, 2009; Silva *et al.*, 2011; Hanapi *et al.*, 2011; Shankar and Sivakumar, 2016). Some agricultural residues such as kiwi fruit peel, pineapple waste, banana peel, cassava peel, sugar beet, sugarcane molasses, corn steep liquor, wheat straw and oil palm empty fruit bunches, have been reported for production of citric acid (Hang and Woodams, 1995; Pintado *et al.*, 1998; Bari *et al.*, 2009; Luciana *et al.*, 2000; Vandenberghe *et al.*, 1999).

Groundnut husk is one of the lignocellulosic materials, whose average annual production in Nigeria was reported to be 551,129.8 metric tonnes, accounting for 20% of the total groundnut production of 2,755,649 metric tonnes (Benjamin, 2016). Thus, groundnut husk has been reported to be used for formation of various products of biological importance

(Salihi *et al.*, 2014). To produce any bio-products of high quality at a low cost, it is important to utilize a microbial system with good production efficiency and minimum by-products generation (Kusumoto, 2001).

Initial setup of an optimal medium for any production process includes selection of growth factors, nitrogen and carbon sources as well as inorganic salts (Ooijkaas *et al.*, 2000). Other factors to be considered include substrate composition, physical parameters (temperature, particle size, pH and moisture content), substrate permeability and inoculum concentration which are vital for improving production (Rigo *et al.*, 2009; Silva *et al.*, 2011). Generally, optimization processes could be achieved either by using statistical or empirical methods. Empirical approaches encompass the one-factor-at-a-time technique which has been linked with several limitations including its inability to determine the interaction among variables, time consumption and inefficiency (Chan *et al.*, 2009). Thus, one-factor-at-a-time method sometimes leads to erratic results and erroneous conclusions.

On the other hand, several biotechnological and biochemical procedures have been effectively demonstrated and optimized using Response surface methodology (RSM) (Liyana-Pathirana and Shahidi, 2005). The RSM estimates the effects of a number of factors and determines the interactions between the dependent and independent variables. It also aids in the design of experiments, developing various models, and analysing the ideal situations for suitable responses (Li *et al.*, 2007).

The application of RSM for optimization of production processes is aimed at reducing the cost of expensive analytical methods. Therefore, RSM is faster and gives better information than the classical one-factor-at-a-time method (Li *et al.*, 2007; Ozdemir *et al.*, 2008). The present work utilizes statistical approach using RSM to optimize the fermentation parameters for improved citric acid production using groundnut husk as the basal substrate by *Aspergillus niger*. The three medium components (FeSO₄, MnSO₄ and Urea) used in this study were based on preliminary screening experiments carried out by Kudu *et al.* (2018).

MATERIALS AND METHODS

Chemicals and equipment

All reagents and materials used in this study were of analytical grade. Some of the equipment used include water bath (Grant OLS 200), shaker (IKA-Labortechnik. KS 501), haemocytometer, incubator (Gen lab.Limited), weighing balance (Sartorius Entris 124-1S) and autoclave (Omega prestige medical model 220220).

Sample collection

The main sample used in this study was groundnut husk obtained from Institute for Agricultural Research (IAR) Ahmadu Bello University, Zaria. Authentication of the sample (groundnut husk) was done at the Department of Crop Protection A.B.U., Zaria, where a voucher No. of Smcot 0011 was assigned. Also, *Aspergillus niger* was gotten from culture collection of Mycology laboratory, Department of Crop Protection, Ahmadu Bello University Zaria.

Inoculum preparation (*Aspergillus niger*)

The fungi *Aspergillus niger* was cultivated on potato dextrose agar plates at 30°C for 7 days. The technique described by Alam, *et al.* (2004) was used to prepare the inoculum. Sterile distilled water (25 ml) with the aid of bent glass rod was used to wash the spores from each culture plate; the suspension was filtered using a filter paper and a hemocytometer was used to estimate the quantity of spores present in the filtrate per millilitre.

Solid state fermentation

The total fermentation media was fixed at 20g, consisting of 40% substrate (groundnut husk), and 60% moisture content in the form of distilled water and inoculum. The fermentation set up was prepared in 250ml Erlenmeyer flasks, initial pH was adjusted to 5.0±0.1 and autoclaved at 121°C and 15 psi, for 20 min. All flasks were cooled to room temperature; thereafter 5% (v/w) *Aspergillus niger* (1×10⁷ spore/ml) was inoculated into each flask and incubated for 7 days at 30°C according to the design matrix.

Extraction of citric acid

After seven days of incubation, recovery of citric acid was carried out according to Khattab *et al.* (2017) and Ali *et al.* (2002). A spatula was used to mix thoroughly the fermented substrate after adding one hundred millilitres (100ml) of distilled water into each flask. A rotatory shaker fixed at 150 rpm was used to homogenize the content of the flask for 60 minutes at room temperature (Tran *et al.*, 1998). The supernatant obtained after centrifuging for 15 minutes at 5,000 rpm was used for citric acid estimation.

Estimation of citric acid concentration

Citric acid was estimated according to the method reported by Marrier and Boulet (1958) using pyridine-acetic anhydride. One millilitre of the culture supernatant along with 1.30 ml of pyridine was added in test tubes and swirled gently. Then 5.70 ml of acetic anhydride was added into each test tube and incubated in a water bath at 30°C for 30 min. The absorbance was measured at 405 nm and citric acid

contents of the sample were estimated with reference to the standard.

Response Surface Methodology: Face-Centred Central Composite Design (FCCCD)

Following Plackett-Burman design and One-factor-at-a-time experiments, three best factors were chosen for optimization; the selection criteria were based on the media components contributing significantly to the overall yield as described by Kudu *et al.* (2018). The FCCCD experimental combination is based on 2^{3+2k+n} ; where n is the number of experimental repetitions at the centre point and k is the number of independent variables. This design was based on six replications at the centre point, three levels and three factors making the total experimental runs to be 20 using Design Expert Software (version 10.0.3, stat Ease corporation, USA). Ranges selected for each factor were 0.10-0.25, 0.015-0.025 and 0.02-0.04 for FeSO₄, MnSO₄ and urea respectively. Overall model significance, coefficient of determination (R²), adjusted R² and lack of fit were determined. Surface and contour plots were utilized in demonstrating the association between the response (citric acid concentration) and the independent variables.

Experimental model validation

The statistical model developed using FCCCD was validated with respect to all the three variables within the design space. Three combinations of the selected media constituents (FeSO₄, MnSO₄ and urea) were utilized in estimating the citric acid production and the results were compared with the predicted responses.

RESULTS AND DISCUSSION

Table 1 shows the design matrix containing twenty (20) experimental runs for the three factors (FeSO₄, MnSO₄ and urea) varied at three levels with six replications at centre points where citric acid production was taken as the response. From the results obtained in Table 1, citric acid production varied from 3.93 to 5.30 mg/g indicating the dependence of citric acid concentrations on the selected media constituents. Run 11 gave the highest citric acid yield of 5.30 mg/g and the predicted yield of 5.26 mg/g. Lowest production of 3.93 mg/g was obtained in run 20 where all the media constituents were set at their lowest concentrations. Higher citric acid yields were also obtained in runs 2 and 17 with values of 5.11 mg/g and 5.22 mg/g respectively when the media constituents were set at their highest concentrations (Table1). The values obtained for the six centre points (runs 4, 8, 9, 10, 15 and 16) ranged from 4.85 mg/g to 5.09 mg/g.

Table 1: Face Centered Central Composite Design Matrix for Citric acid production using groundnut husk based medium by *Aspergillus niger*.

Run	FeSO ₄ (%)	MnSO ₄ (%)	Urea (%)	Citric acid (Experimental) (mg/g)	Citric acid (Predicted) (mg/g)
1	0.250	0.015	0.02	4.59	4.56
2	0.250	0.020	0.03	5.11	4.99
3	0.100	0.015	0.04	4.27	4.27
4	0.175	0.020	0.03	4.96	5.07
5	0.250	0.020	0.04	4.89	4.91
6	0.175	0.015	0.03	4.31	4.33
7	0.100	0.025	0.02	4.76	4.70
8	0.175	0.020	0.03	5.02	5.07
9	0.175	0.020	0.03	5.09	5.07
10	0.175	0.020	0.03	5.01	5.07
11	0.250	0.025	0.04	5.30	5.26
12	0.175	0.020	0.02	4.75	4.77
13	0.175	0.025	0.04	4.89	4.90
14	0.250	0.025	0.03	4.99	5.09
15	0.175	0.020	0.03	5.07	5.07
16	0.175	0.020	0.03	4.85	5.07
17	0.250	0.025	0.02	5.22	5.25
18	0.100	0.020	0.03	4.45	4.51
19	0.250	0.015	0.04	4.61	4.64
20	0.100	0.015	0.02	3.93	3.94

Analysis of variance (ANOVA) was used to define the model competence by means of the Fisher's statistical analysis and Table 2 shows the results obtained, the two linear terms (A and B) had p-values of < 0.0001, while a p value of 0.0019 was recorded for the linear term C. Similarly, only one quadratic term (B²) and one interaction term (AC) were significant with p-values of 0.0043 and 0.0348 respectively. From the developed model, coefficient of determination (R²) of 0.9853 was obtained and the values for adjusted R² and predicted R² were found to be 0.9720 and 0.8566 respectively. The predicted and experimental responses were closer to each other as evident from the insignificant lack of fit (p-value of 0.2304).

Table 2: Analysis of Variance for Citric Acid Production by *Aspergillus niger* using groundnut husk as substrate

Source	Sum of Squares	F-value	p-value	Remark
Model	1.92	74.39	< 0.0001	Significant
A-FeSO ₄	0.59	205.56	< 0.0001	Significant
B-MnSO ₄	1.19	414.34	< 0.0001	Significant
C-Urea	0.050	17.55	0.0019	Significant
AB	2.112 × 10 ⁻³	0.74	0.4112	Not significant
AC	0.017	5.96	0.0348	Significant
BC	2.813 × 10 ⁻³	0.98	0.3458	Not significant
A ²	4.105 × 10 ⁻³	1.43	0.2595	Not significant
B ²	0.039	13.47	0.0043	Significant
C ²	7.255 × 10 ⁻³	2.53	0.1431	Not significant
Lack of Fit	0.019	2.01	0.2304	Not significant

The coefficient of determination $R^2 = 0.9853$, Adjusted $R^2 = 0.9720$ and Predicted $R^2 = 0.8566$.

The developed quadratic equation for citric acid production by *A. niger* using groundnut husk as a substrate is represented by the equation:

$$\text{Citric acid (mg/g)} = + 4.80 + 0.24A + 0.34B + 0.071C - 0.016AB - 0.046AC - 0.019BC - 0.039A^2 - 0.12 B^2 + 0.051C^2$$

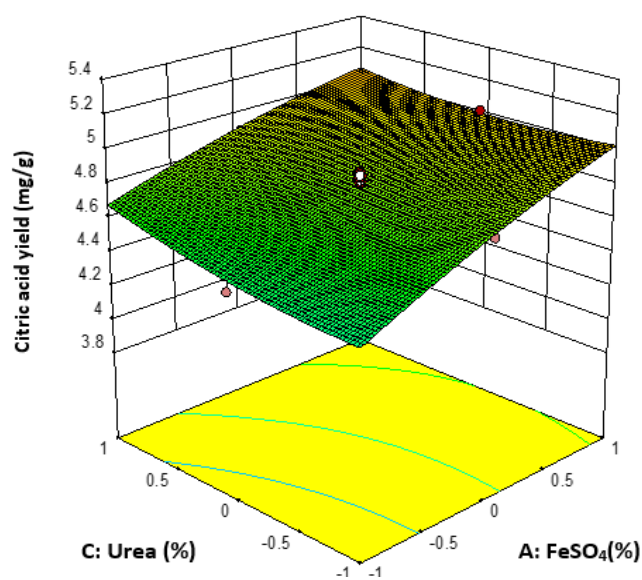
where A, B and C stand for FeSO₄, MnSO₄ and urea respectively.

The suitability of the model was confirmed by conducting a set of five experiments as indicated in Table 3. The results obtained for the validation experiments showed that maximum citric acid yield of 5.34 mg/g were attained in run 1 when urea, FeSO₄ and MnSO₄ was at 0.125%, 0.024% and 0.040% respectively. Similarly, run 5 gave a good citric acid yield of 5.26 mg/g at 0.124% urea, 0.025% FeSO₄ and 0.040% MnSO₄.

Table 3: Validation of Experimental Model for Production of Citric acid by *Aspergillus niger*

Run	Factors			Response	
	FeSO ₄ (%)	MnSO ₄ (%)	Urea (%)	Predicted Value of Citric acid (mg/g)	Experimental Value of Citric acid (mg/g)
1	0.125	0.024	0.040	5.33	5.34±0.16
2	0.125	0.024	0.040	5.27	5.02±0.03
3	0.123	0.024	0.040	5.26	4.92±0.24
4	0.125	0.025	0.040	5.07	4.99±0.10
5	0.124	0.025	0.040	5.26	5.23±0.02

The 3D surface plots shown in Figures 1-3 are graphical depictions of the regression equation, fixing one variable constant while changing the two variables within the ranges considered. The interaction indicated in Figure 1 between FeSO₄ and urea showed the interaction between the variables on citric acid production with p-value of 0.0348 (Table 2). On the other hand, the interactions shown in Figures 2 and 3 between FeSO₄ and MnSO₄, and that of MnSO₄ and urea were insignificant as evident from the spherical nature of the response surface plots which agree with their p-values of 0.4112 and 0.3458 respectively (as obtained in Table 2).

**Figure 1:** 3D Response surface plot depicting the effect of Urea and FeSO₄ at fixed concentration of MnSO₄ on Citric acid produced from groundnut husk

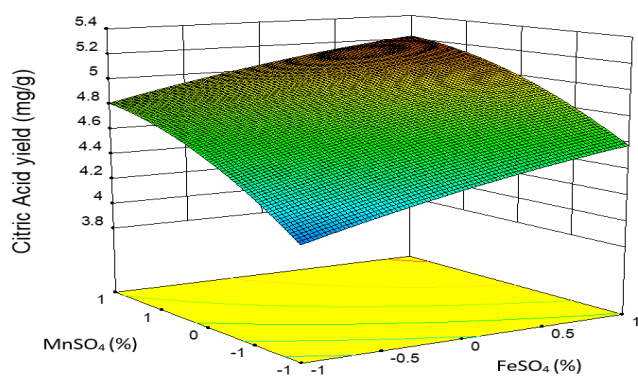


Figure 2: 3D Response surface plot depicting the effect of MnSO_4 and FeSO_4 fixed concentration of Urea on Citric acid produced from groundnut husk

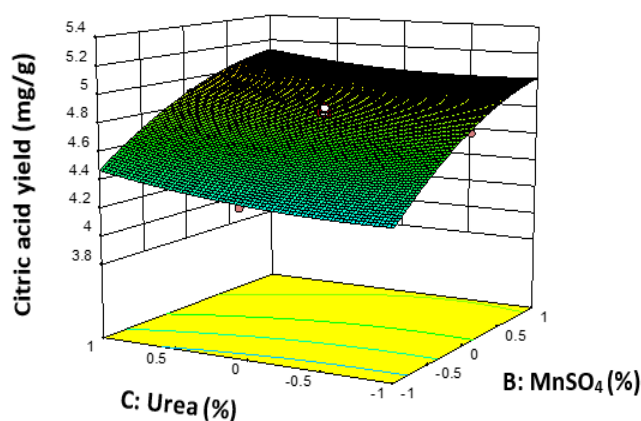


Figure 3: 3D Response surface plot depicting the effect of MnSO_4 and Urea at fixed concentration of FeSO_4 on Citric acid produced from groundnut husk

Discussion

In order to determine the concentrations of medium constituents to be utilized for optimization using RSM, three factors (FeSO_4 , MnSO_4 and urea) that contributed positively to citric acid production were taken into account based on the previous findings reported by Kudu *et al.* (2018) involving Plackett-Burman design and one-factor-at-a-time (OFAT) experiments. Response surface methodology is made up of a group of empirical techniques principally aimed at creating any link that exists among the experimental factors and the measured responses, with the target of creating a dependable and a realistic model that can describe the process (Salihu *et al.*, 2014). Therefore, RSM using FCCCD has been utilized as an essential tool to determine the optimum level of the selected medium constituents along with their interactions for citric acid production by *A. niger*.

Analysis of variance showed that the developed quadratic model was significant and based on the p-value there is only a 0.01% probability that the obtained F-value (74.39) could occur due to noise. The suitability of the model was tested using coefficient of determination (R^2) and a value of 0.9853 was obtained which shows that 98.53% of the total variations could be accounted for by the model equation and only 1.47 % of the total variations were not accountable by the model. Therefore, the closer the value of R^2 is to 1, the stronger the association between the experimental and theoretical values. Generally, the insignificant lack of fit was desirable and signifies that the entire data in this study were satisfactory and at the same time serve as an excellent predictor of the model (Rashid *et al.*, 2009). The p-value shows the pattern of interactions among the variables and determines the importance of each of the model terms (Salihu *et al.*, 2014).

The findings in this study showed that the selected factors can serve as limiting nutrients and slight changes in their concentrations will significantly alter either the rate of product formation and/or microbial growth rate (Imandi *et al.*, 2008). Similarly, the validation experiment was accompanied with the results obtained from the design matrix to ascertain that the data generated from FCCCD were based on experimentation and also to confirm the reproducibility of the results. Three dimensional plots involve an infinite combination of the two independent variables fixing the other variable constant. Thus, an elliptical contour is achieved when there is interaction between the independent variables (Muralidhar *et al.*, 2001). The plots further provide vivid representation of the pattern of interactions among the two variables and help in identifying their optimal levels (Rashid *et al.*, 2009).

Thus, findings from this study agree with what has been reported in the literature using *A. niger* on different substrates according to RSM based experiments. A wild *A. niger* strain was utilized for optimization of citric acid by Sekoai *et al.* (2018) using corn steep liquor and apple pomace as substrates. The coefficient of determination (R^2) of 0.920 was obtained with maximum citric acid yields of 62.0g/L and 68.26g/L for apple pomace and corn steep liquor respectively. Prasad *et al.* (2014) carried out statistical optimization of citric acid production using RSM by *A. niger* using sorghum malt media. This resulted in maximum citric acid yield of 0.12g/ml and a low R^2 value of 0.6238 was obtained. Also, Ayeni *et al.* (2019) reported maximum citric acid yield of 5.5g/L and an R^2 value of 0.895.

Following the optimization of medium components considered in this study, the selected factors considerably improved the citric acid production with maximum yield of

5.34 mg/g. Thus, *A. niger* under appropriate conditions is capable of utilizing agricultural residues (groundnut husk) during its metabolism by secreting citric acid as one of the primary metabolites during the growth phase (Rashid *et al.*, 2009). Results from this study contributed in developing a fermentation system for improved citric acid production where an inexpensive and available residue (groundnut husk), could be used as a substitute to more commonly used medium (glucose) for citric acid production.

CONCLUSION

This study employed the use of statistical experimental design using face centred central composite design (FCCCD) to determine the optimal concentrations and synergistic effects of the selected medium constituents (FeSO₄, MnSO₄ and urea) for improved citric acid production by *Aspergillus niger* using groundnut husk as the main substrate.

AUTHORS' CONTRIBUTIONS

All authors contributed to the study conception, design and the interpretation of data. The first draft of the manuscript was written by Fatima Abdullahi Kudu and all authors commented and revised the manuscript.

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None.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare during the experimentation, analysis and preparation of the manuscript.

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