



OPTIMISATION OF SOLID STATE FERMENTATION OF BANANA PEELS FOR CITRIC ACID PRODUCTION

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ABSTRACT

*In this study, design of experiment (DOE) for response surface methodology (RSM) was used to analyse and optimise the simultaneous effect of solid substrate loading, initial pH and inoculum density on citric acid production from banana peels via solid state fermentation using *Aspergillus niger*. A three-variable, three-level Box-Behnken design was used to develop a statistical model to describe the relationship between citric acid concentration and the chosen independent variables and to optimise the production of citric acid using RSM. The model was statistically significant ($p < 0.0001$) with a low standard deviation (0.43) and did not show lack of fit ($R^2 = 0.999$). Citric acid production was significant at high levels of solid loading, low levels of pH and intermediate levels of inoculum density. Solid loading positively influenced citric acid production while the reverse was the case for initial pH. The optimum values of solid loading, pH and inoculum density were 20 g/L, 8 and 4.97×10^6 spores/mL respectively. Under these conditions, the citric acid concentration was obtained as 49.9 g/L. Validation of the statistical model showed an insignificant difference between experimental and model predicted results.*

Keywords: Optimisation, Citric acid, Banana peels, *Aspergillus niger*, Solid state fermentation

1. INTRODUCTION

Citric acid is a tricarboxylic acid that is utilised in a wide range of industries. It is an important microbial product used in the food and beverage industries as an acidifying, flavouring, preserving, chelating and buffering agent and also in other industries such as in the production of pharmaceuticals, cosmetics and textiles [1-3]. The use of citric acid in the food and beverage industry accounts for about 7% of the total citric acid produced annually while its use in the pharmaceutical and other industries account for 12% and 18% respectively [4, 5]. As a result of the high cost associated with chemical synthesis of citric acid, the biotechnological route via submerged fermentation using *Aspergillus niger* has been favoured [6].

Recently, a lot of research work has been done on solid state fermentation (SSF) with the view to adopting it as an alternative to submerged fermentation [2, 3, 6-10]. Solid state fermentation has some important advantages when compared with submerged fermentation. It has a higher citric acid yield, lower energy and water requirements, less risk

of bacterial contamination, less wastewater generation and less environmental concern regarding the disposal of solid waste [5]. By adopting SSF, solid waste materials such as abundantly available and cheap agricultural wastes such as sugarcane bagasse [7,8], apple pomace [9], banana peels [4], date palm [10], carob pod [11], grape pomace [12] can be used for the sustainable production of citric acid.

Banana peel is an example of a solid substrate that can be used for SSF. Nigeria is the fourth largest producer of banana in sub-Saharan Africa [13]. The processing of banana into products such as chips, puree/pulp, powder, jams, juice, bar, biscuits, wine etc results in the generation of solid banana wastes particularly the peels which account for about 40% of the total weight of the fresh banana fruit [14]. These wastes do not have any important use apart from being used as animal feed. Furthermore, they are typically disposed of inappropriately particularly in less developed countries thereby contributing to environmental problems [15]. As the waste material is rich in carbohydrate and other nutrients, it can serve as an

ideal substrate for citric acid production via solid state fermentation [16].

The production of citric acid has been reported to be influenced by fermentation conditions such as type and composition of fermentation medium, substrate type and concentration, agitation rate, aeration, temperature, pH etc [2, 17, 18]. The performance of the SSF process can be significantly improved if these conditions are optimised. Experimental design methods coupled with response surface methodology (RSM) have been reported to be very effective in achieving this and it has been successfully applied to the optimisation of many bioprocesses [19, 20]. Hence the objective of this study was to optimise the fermentation conditions for the production of citric acid from banana peels using *Aspergillus niger* in batch reactor. A three variable Box-Behnken design for response surface methodology was used to study the simultaneous effect of three independent variables (solids loading, pH and inoculum density) for optimum citric acid production.

2. MATERIALS AND METHODS

2.1 Feedstock Preparation

Banana peels were obtained from a local market in Benin City, Edo State, Nigeria and used as solid carbon substrate in this study. The chemical composition of banana peels has been previously reported in a study by Anhwange *et al.* [16] as shown in Table 1. The peels were washed with clean water to remove any adhering dirt after which they were dried in an oven at 60°C for 2 hours. The dried peels were then milled to obtain 2 mm particles.

Table 1: chemical composition of banana peels [16]

Component	Composition (wt %)
Carbohydrate	59.0
Protein	0.9
Crude fibre	21.7
Phytate	0.28
Crude lipid	1.7
Ash	8.5
Moisture	6.7

2.2 Microorganism and Inoculum Preparation

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 °C 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 the desired number of spores [2].

2.3 Solid State Fermentation

Solid state fermentation was carried out in 250 mL Erlenmeyer flasks with a working volume of 100 mL. The solid substrate was weighed into the flask and to this a supplemental salt solution was added to achieve the desired moisture level. The composition of the salt solution was as follows (g/L): MgSO₄·7H₂O:0.15; CaCl₂:0.015; ZnSO₄·7H₂O:0.002; MnSO₄·H₂O:0.006; FeCl₃·6H₂O:0.015. Yeast extract was taken as nitrogen source. The contents were thoroughly mixed, cotton plugged and autoclaved at 121°C (15 psi) for 20 min [3]. After cooling, the sterilised substrate including media was inoculated with 2 mL of inoculum and then incubated at varying conditions in a rotary shaking incubator for 5 days. The solid substrate loading, pH and the inoculum density were varied according to the experimental design. At the end of fermentation, the medium was diluted with distilled water (1:4 w/v) and then filtered and the filtrate was used for analysis [4].

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 [21]. 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 °C 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. The pH of the sample was determined using a Uican 9450 model pH meter [2].

2.5 Experimental Design

A three variable Box-Behnken design (BBD) for response surface methodology was used to develop a statistical model to describe the production of citric acid from banana peels. The range of the variables that were optimised is shown in Table 2. The Box-Behnken design is suitable for the exploration of quadratic

response surfaces and this design can be used to develop a second degree polynomial model which can be utilised for optimisation purposes [17]. The number of experimental run for this design is obtained from Equation (1).

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

Where k is the number of factors and c_p is the number of replications at the center point. 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 using Equation (2).

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

Where x_j and X_j 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_j is the step change of X_j .

Table 2: Experimental range and level of the independent variables

Variables	Symbol	Coded Levels		
		-1	0	+1
Solids loading (g/L)	X_1	5	12.5	20
pH	X_2	2	5	8
Inoculum density	X_3	0.5×10^6	3.25×10^6	6×10^6

The following generalised second order polynomial equation was used to estimate the response of the dependent variable [22].

$$Y_i = b_o + \sum b_i X_i + \sum b_{ij} X_i X_j + \sum b_{ii} X_i^2 + e_i \tag{3}$$

Where Y_i is the dependent variable or predicted response, X_i and X_j are the independent variables, b_o is offset term, b_i and b_{ij} are the single and interaction effect coefficients and e_i is the error term. The Design Expert software was used for regression and graphical analysis of the experimental data. The goodness of fit of the model for citric acid production was evaluated by the coefficient of determination (R^2) and analysis of variance (ANOVA). The optimum values of the variables tested were obtained by numerical optimisation based on the criterion of desirability [23].

3. RESULTS AND DISCUSSION

3.1 Statistical Analysis

Table 3 shows the result of the 17 experimental runs carried out according to the BBD. Multiple regression

analysis was applied to the experimental data to obtain Equation (4) which was used to estimate the response (citric acid concentration).

$$y = 90.70 - 4.71X_1 - 1.8.69X_2 - 0.38X_3 + 0.53X_1X_2 + 0.12X_1X_3 + 0.51X_2X_3 + 0.12X_1^2 + 0.89X_2^2 - 0.62X_3^2 \tag{4}$$

The values of citric acid concentration predicted by Equation (4) are also shown in Table 2 along with the experimental data. The significance of the fit of the citric acid model was assessed by performing analysis of variance (ANOVA).

Table 3: Box-Behnken design matrix for citric acid production

Run no	Factors						Response
	Coded values			Actual values			Citric acid conc. (g/L)
	X_1	X_2	X_3	X_1	X_2	X_3	Observed
1	-1	-1	0	5	2	3.25	39.01
2	1	-1	0	20	2	3.25	33.71
3	-1	1	0	5	8	3.25	5.86
4	1	1	0	20	8	3.25	48.12
5	-1	0	-1	5	5	0.5	13.49
6	1	0	-1	20	5	0.5	26.30
7	-1	0	1	5	5	6	6.69
8	1	0	1	20	5	6	29.52
9	0	-1	-1	12.5	2	0.5	30.04
10	0	1	-1	12.5	8	0.5	12.72
11	0	-1	1	12.5	2	6	19.93
12	0	1	1	12.5	8	6	19.43
13	0	0	0	12.5	5	3.25	16.81
14	0	0	0	12.5	5	3.25	17.72
15	0	0	0	12.5	5	3.25	16.81
16	0	0	0	12.5	5	3.25	17.72
17	0	0	0	12.5	5	3.25	16.81

The model was statistically significant as seen from the very low p value ($p < 0.0001$) as shown in Table 4. The lack of fit p value of 0.7346 indicates that the model did not show lack of fit. Results presented in Table 4 also show that all the model terms were significant as seen from their very low p values. Figure 1 is a parity plot showing a comparison between experimental values of the response and those predicted by the statistical model. The Figure shows that there was an acceptable level of correlation between the experimental and model predicted results. 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 [3].

Table 5 shows the statistical information for ANOVA for the citric acid model. The model had a high coefficient of determination (R^2) which showed that it was able to adequately represent the relationship between the independent variables and the response (citric acid concentration). An R^2 value of 0.999 means that 99.9% of the variability in the response could be explained by the model. A low standard deviation of 0.43 means that there was very little deviation of the individual values of the response from the mean. The coefficient of variation (CV) was obtained as 1.98. This value gives an indication of the degree of precision with which the treatments were carried out [24]. The relatively low value of CV obtained showed that the treatments were carried out with high precision and reliability [25]. The Adequate precision for the model was obtained as 126.52 showing that the model can be used to navigate the design space [26].

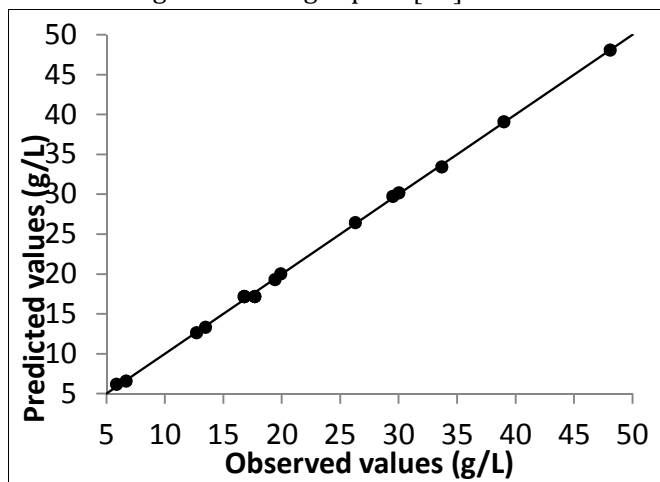


Figure 1: Parity plot showing comparison between experimental and predicted values

Table 4: ANOVA results for citric acid production model

Source	Sum of Squares	df	Mean Square	F value	p value
Model	2027.87	9	225.32	1206.96	< 0.0001
X ₁	658.78	1	658.78	3528.89	< 0.0001
X ₂	167.04	1	167.04	894.77	< 0.0001
X ₃	6.09	1	6.09	32.62	0.0007
X ₁ X ₂	565.36	1	565.36	3028.44	< 0.0001
X ₁ X ₃	25.05	1	25.05	134.21	< 0.0001
X ₂ X ₃	70.70	1	70.70	378.69	< 0.0001
X ₁ ²	177.13	1	177.13	948.82	< 0.0001
X ₂ ²	270.50	1	270.50	1448.99	< 0.0001
X ₃ ²	91.38	1	91.38	489.48	< 0.0001
Residual	1.31	7	0.19		
Lack of Fit	0.33	3	0.11	0.44	0.7346
Pure Error	0.98	4	0.25		
Cor Total	2029.18	16			

Table 5: Statistical information for ANOVA of citric acid model

Parameter	Response
R-Squared	0.999
Adjusted R-Squared	0.998
Mean	21.80
Standard Deviation	0.43
C.V %	1.98
Adeq. Precision	126.52

Table 6: Citric acid model coefficient estimate and confidence intervals

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	17.17	1	0.19	16.72	17.63	
X ₁	9.07	1	0.15	8.71	9.44	1
X ₂	-4.57	1	0.15	-4.93	-4.21	1
X ₃	-0.87	1	0.15	-1.23	-0.51	1
X ₁ X ₂	11.89	1	0.22	11.38	12.40	1
X ₁ X ₃	2.50	1	0.22	1.99	3.01	1
X ₂ X ₃	4.20	1	0.22	3.69	4.71	1
X ₁ ²	6.49	1	0.21	5.99	6.98	1.01
X ₂ ²	8.02	1	0.21	7.52	8.51	1.01
X ₃ ²	-4.66	1	0.21	-5.16	-4.16	1.01

3.2 Optimisation of Citric Acid Production

Three dimensional (3D) response surface plots were generated to determine the optimum levels of the variables that were investigated in this study. The plots were generated by keeping one variable constant at the center point and varying the others within the experimental range. The resulting response surfaces showed the effect of solids loading, pH and inoculum density on citric acid production.

Figure 2 shows the effect of solid loading and pH on citric acid production. The trend observed indicates that citric acid production was favoured at high solids loading. This is evident from the increase in citric acid concentration when the solid loading was increased. The amount of solids used for solid state fermentation determines the amount of fermentable sugars that can be liberated for the purpose of producing citric acid. Since citric acid production is enhanced at increased sugar concentration, it will be expected that increasing the solid loading will also have a similar effect. The increase in citric acid concentration in the course of fermentation could be attributed to the consumption of sugar substrate by the *Aspergillus niger* cells to produce citric acid [7, 9]. These results are in agreement with those reported by previous

researchers. Kim [27] reported a similar increase in citric acid concentration with increase in sugar substrate loading for the production of citric acid from peat moss. Al-Sheri and Mostafa [28] also reported a similar trend for the production of citric acid from date palm syrup using immobilized *Aspergillus niger* cells. The initial pH of the fermentation medium displayed a significant overall negative effect on citric acid concentration as shown in Figure 2. This is evident from the inverse relationship between the pH and the production of citric acid. This result is also corroborated by the negative value of the coefficient estimate of the model term representing pH (i.e. X_2) as shown in Table 6. At low solid loading, citric acid production was observed to increase with a decrease in pH. This observation could be attributed to the fact that a low pH is required to begin citric acid production. The low pH value also provides a sterile environment which reduces the risk of contamination as well as inhibiting the production of unwanted by products such as oxalic and gluconic acids which reduces citric acid yield [29]. With increasing solid loading, citric acid concentration was observed to increase with pH. Maximum citric acid concentration was recorded at a pH of 8 and a solid loading of 20 g/L. Similar results have also been reported in the literature. Barrington and Kim [18] reported an optimum pH of 7.1 for the production of citric acid from peat moss. In another study, Ali *et al.* [30] reported an optimum pH of 6 for the production of citric acid from cane molasses in a stirred fermenter using *Aspergillus niger*.

The interactive effect of inoculum density and initial pH on the fermentation process is shown in Figure 3. Inoculum density is an important fermentation variable that has been reported to affect citric acid production [31]. Citric acid production displayed a quadratic relationship with the inoculum density showing that the citric acid concentration increased with inoculum density up to a certain level beyond which any further increase in inoculum density did not positively affect citric acid production as shown in Figures 3 and 4. This observation has also been reported by previous researchers. According to Nampoothiri *et al.* [32], metabolite formation during fermentation generally increases with increase in inoculum density up to a particular limit. At low values of inoculum density, there is the risk of contamination as a result of insufficient microbial cell population and these results in a decline in the production of metabolic products [18]. A high inoculum density results in population over crowding which consequently results in increased competition for nutrients as well as rapid consumption of the nutrients [33]. Reports in the literature indicate that an inoculum density between 1×10^4 to 1×10^9 spores/mL is typically favourable for citric acid production by *Aspergillus niger* [34]. The optimum inoculum density obtained in this study (i.e. 4.97×10^6 spores/mL) falls within this range thus corroborating the results obtained by previous researchers. The optimisation of the statistical model resulted in the selection of the optimum fermentation conditions and their respective levels. The maximum citric acid concentration predicted by the model was 49.9 g/L.

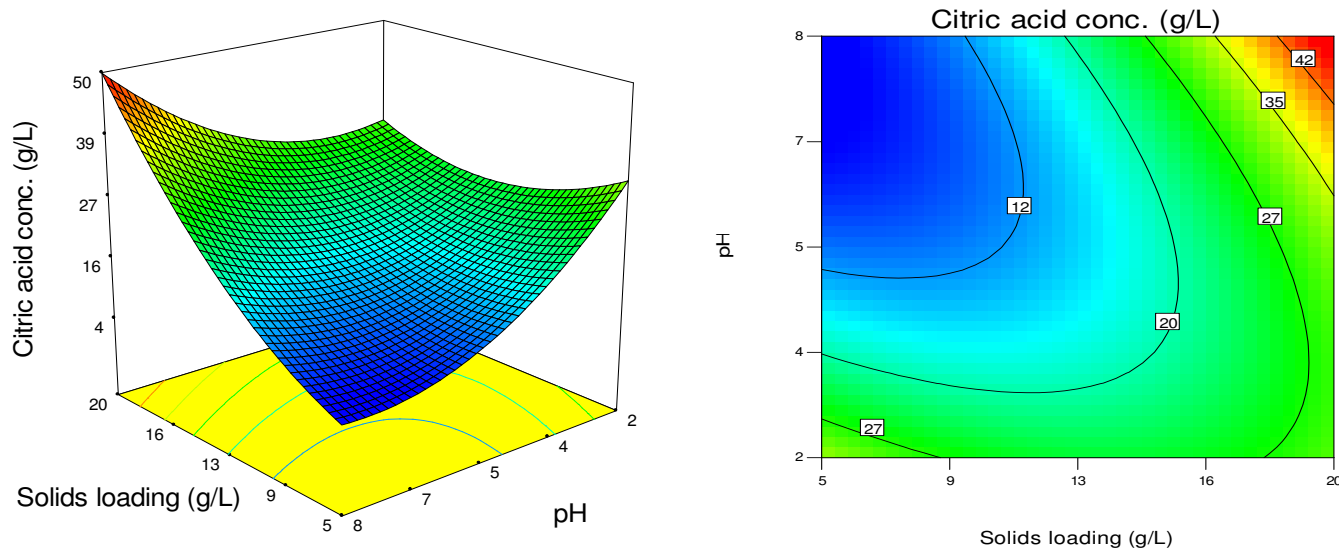


Figure 2: Response surface and corresponding contour plot showing the effects of solids loading and pH on citric acid concentration

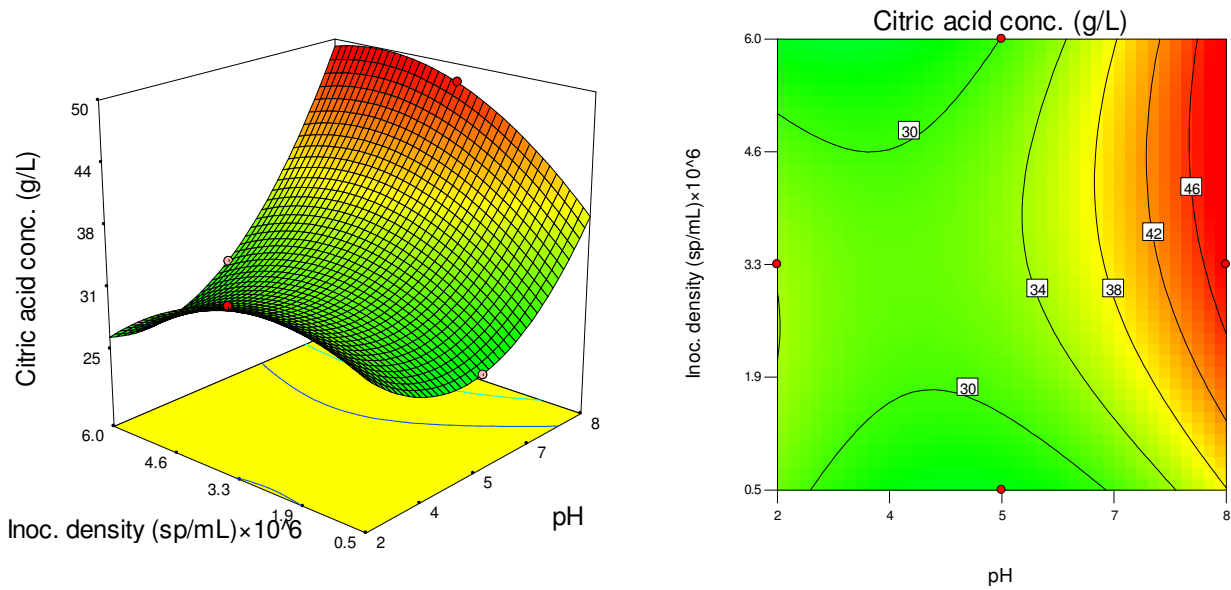


Figure 3: Response surface and corresponding contour plot showing the effects of inoculum density and pH on citric acid concentration

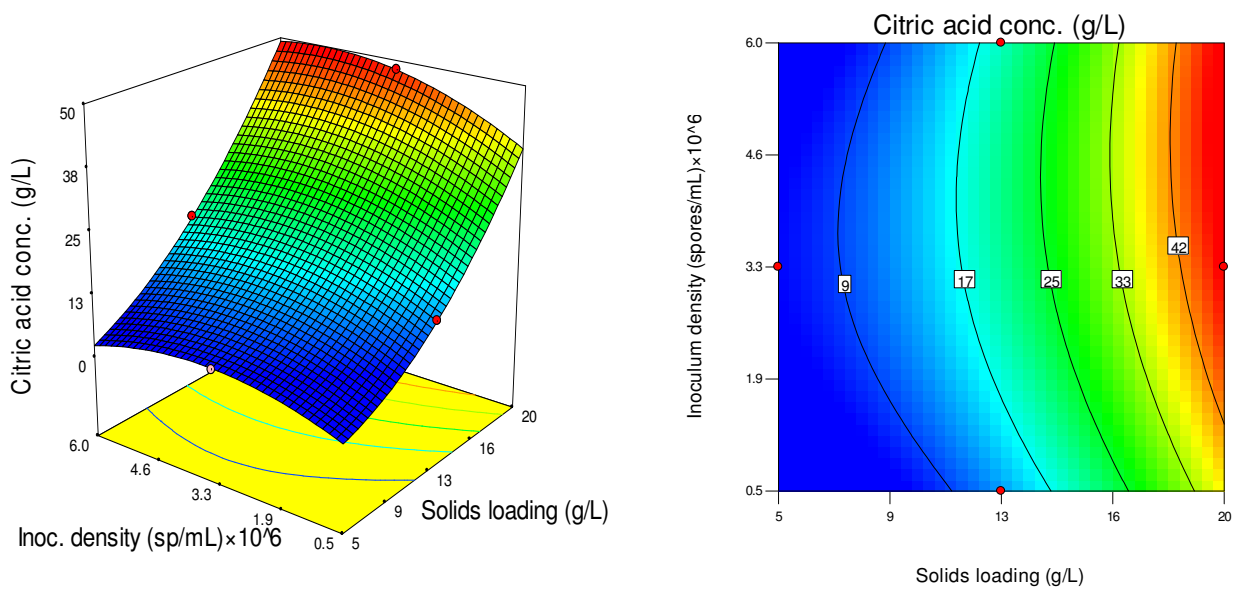


Figure 4: Response surface and corresponding contour plot showing the effects of inoculum density and solids loading on citric acid concentration

The final optimised conditions for the production citric acid as obtained from RSM were a solid loading of 20 g/L, a pH of 8 and an inoculum density of 4.97×10^6 spores/mL. 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 banana peels via solid state 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 solid loading, initial pH and inoculum density. 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

($R^2=0.999$) with a low standard deviation. High level of solid loading, low levels of pH and intermediate levels of inoculum density were favourable for citric acid production. Citric acid production was positively influenced by solid loading while the reverse was the case for initial pH. The optimum values of solid loading, pH and inoculum density were 20 g/L, 8 and 4.97×10^6 spores/mL respectively. Under these conditions, the citric acid concentration was obtained as 49.9 g/L.

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