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Fermentation parameter optimization of microbial oxalic acid production from cashew apple juice

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Abstract

The potential of cashew apple juice (CAJ) as a carbon source for oxalic acid (OA) production via fermentation process was investigated in this study. The effects and interactions of CAJ concentration, time, pH, $NaNO_3$ concentration and methanol concentration on OA production were determined in a central composite design (CCD) and the process was modelled and optimized using response surface methodology (RSM). The results showed that OA fermentation can be described significantly ($p < 0.05$) by a quadratic model giving regression coefficient (R^2) of 0.9964. $NaNO_3$ concentration was the most significant positive variable while methanol was not a significant variable. A maximum OA concentration of 122.68 g/l could be obtained using the optimum levels of CAJ of 150.0 g/l, pH of 5.4, time of 7.31 days, $NaNO_3$ of 2 g/l and methanol of 1% volume. The production of OA was found to increase from 106.75 to 122.68 g/l using the statistically design optimization. This study revealed that CAJ could serve as an inexpensive and abundant feedstock for fermentative OA production, the resulting model could be used in the design of a typical pilot plant for a scale up production.

Keywords: Biochemical engineering, Applied microbiology, Food science, Mathematical modelling, Optimization

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1. Introduction

Oxalic acid (OA) is a strong organic acid, which belongs to the family of dicarboxylic acids. It sometimes occurs as a free acid but more commonly as a calcium salt. OA has very wide applications in pharmaceuticals, waste water treatment, food industry and hydrometallurgy. It serves as food preservatives, for example, as antibrowning agents for apple [1], in postharvest browning of litchi fruit [2] and in postharvest ripening of banana fruit [3]. It is also used for the removal of iron present in kaolin due to its high reduction power [4, 5].

Currently, the majority of OA is produced via chemical methods [6]. These methods include oxidation of olefins and glycols, decomposition of formates followed by H_2SO_4 treatment, oxidation of carbohydrates with HNO_3, fusion of sawdust with caustic soda and radiation processing of carbonate solutions and molasses [7]. It is clear that these methods will impact negatively on the environment and may not be commercially attractive, especially with the recent concern for development of environmental friendly processes such as the microbial fermentation for OA production [8].

OA is commonly found in nature in plants such as spinach, rhubarb and beet root [9]. Its biosynthesis has long been known to occur in a variety of organisms such as bacteria, fungi, plants, and animals [9, 10]. Nevertheless, filamentous fungus, *Aspergillus niger*, remains the microorganism of choice for OA production due to easy handling, ability to ferment a variety of cheap raw materials, and high yields [8]. A variety of substrates have been investigated for OA fermentation using *A. niger*, which include lactose permeate [11], milk whey [12], molasses [11, 13], post-refining fatty acids [14], lipids [15], glucose [7, 16], biodiesel-derived waste glycerol [5, 17] and sweet potato starch hydrolysate [18].

Cashew plant (*Anacardium occidentale* L) is an important tropical crop grown mainly in Nigeria, India, Brazil, Vietnam and Indonesia. It is highly drought-resistant, grows well on poor soils and has high productivity [19]. Cashew apple is the peduncle or pseudofruit of the cashew fruit, which is attached to the cashew nut, the real fruit [20]. It has high fructose, glucose and sucrose [19], minerals, vitamins, and some amino acids. Even though cashew apples processed can be consumed as juice, and other foodstuffs, *A. occidentale* cultivation is largely directed towards production of its nuts [21]. Like most countries producing cashew, bulk of the Nigerian cashew apple production is lost to spoilage. In order to put the cashew apple to effective use, many studies have been carried out to assess the potential of its juice as carbon source in fermentation processes such as in production of single cell protein [19, 22], wine [22], mannitol [21, 23], biosurfactant [20], lactic acid [21].

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Microbial synthesis of OA by *A. niger* is affected by factors which include pH, nitrogen source, carbon source and fermentation time. It has been reported that pH of the fermentation medium plays a critical role in OA production [15], in *A. niger*, medium pH of 4 causes induction of the enzyme oxaloacetate hydrolase responsible for OA production [24]. While pH of 6 has been reported in separate studies to be vital for OA accumulation in *A. niger* [4, 7, 12], medium pH of 4 led to the observed decrease in a another study [7].

Nitrogen is one of the most essential constituents of nutrient medium for fungal fermentation. Accumulation of OA is strongly influenced by the presence of nitrogen source such as $NaNO_3$ in the fermentation medium. Ruijter et al. [16] showed that an increase in $NaNO_3$ concentration from 6 to 60 mM resulted in an increase of molar yield of OA from 0.5 to 0.54, with a concomitant increase in dry weight from 3.2 to 4.2 g/l. High content of nitrogen in green syrup and molasses led to higher growth in *A. niger* than when lactose was used [11]. Addition of methanol ($1 - 6\%$volume) to the fermentation systems in *A. niger* has been demonstrated to improve the production of some organic acids such as citric acid [25, 26]. Addition of methanol to the fermentation medium for OA production using *A. niger* led to a 1.4 fold increase in the acid yield [14].

In our previous report, OA was produced using *A. niger* grown on CAJ under surface fermentation and the fermentation process involved was modelled and optimized using both artificial neural network and response surface methodology (RSM) [8]. Although there have been several arguments in support of surface fermentation over the submerged option, the objective of this study was to investigate the potential of utilizing CAJ as a carbon source for OA bioproduction using *A. niger* under submerged fermentation. The views are to optimize the fermentation variables of the process so that a preferred option of fermentation can be established based on productivity. This work is important as it provides a base case data for CAJ fermentation via submerged option. RSM was applied to determine the effects of five independent variables *viz.* CAJ concentration, fermentation time, pH, $NaNO_3$ concentration and methanol concentration, and their reciprocal interactions, which have been previously reported to affect the synthesis of the acid in *A. niger* [8]. Also, a mathematical model was developed to describe the OA fermentation process.

2. Materials and methods

2.1. Cashew apple juice (CAJ) extraction

The cashew apple fruits used for this work were purchased from the Ile-Ife market, Nigeria. The fruits were cleaned by washing with water to remove dirt and undesirable materials. The juice used was gotten by pressing

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the apple through mechanical process. The juice was then centrifuged and the supernatant obtained was sterilized using an autoclave and stored at −20 °C [8].

2.2. CAJ characterization

The pH of the CAJ was determined by direct measurement with a Uniscope pH meter (PHS-3B, Surgifriend Medicals, England). The nitrogen content of the sterilized CAJ was determined according to Kjeldahl method and the results were expressed as total protein [27]. Fe, Ca, Mg, Mn, Zn and Cu were quantified using atomic absorption spectrophotometer (Perkin-Elmer, model PG-990). Na and K were directly determined using flame photometer (Digital flame analyzer, model 2655).

2.3. Microorganism and inoculum preparation

The OA-producing strain of *Aspergillus niger* used in this work was a local strain from Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria. Spores of *A. niger* were produced on saboroud dextrose agar (SDA) for 5–7 days at 30 °C. Subsequently, for inoculum preparation, the spores were aseptically moved into a flask containing 100 ml of sterile distilled water. Inoculation of flasks were carried out as previously described by Emeko et al. [8].

2.4. Medium composition for OA bioproduction

The fermentation medium used for this work consisted of CAJ as carbon source, 1.6 g/l of yeast extract, 0.025 g/l of KCl, 1.5 g/l of $NaNO_3$, 0.025 g/l of $MgSO_4.7H_2O$ and 0.5 g/l KH_2PO_4 [11]. Before sterilization of the medium, the pH was adjusted to 6.0 using NaOH solution. Then, 20 ml of universal pH indicator solution (Burgoyne Burbidges and Co, India) was added per litre medium for observing culture pH, which was kept at 6.0 ± 0.5 during fermentation. Keeping the medium pH at 6.0 ± 0.5 during fermentation has been shown to be essential for OA accumulation in *A. niger* [4, 7]. Sterilization of the media were carried out using an autoclave at 121 °C for 15 min.

2.5. Submerged fermentation studies for OA bioproduction

pH and methanol have been established to affect OA production in *A. niger* [7, 12, 14]. In order to determine their effects on the acid production, three different experiments were set up at shake flask level. The first experiment was without methanol addition to the medium and without pH adjustment of the culture medium in the course of the experiment. The second experiment was

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with pH adjustment in the course of the investigation but without methanol addition to the medium. The third experiment was with methanol addition to the medium and with pH adjustment of the culture medium in the course of the experiment. For each experiment, 50 ml of CAJ was measured into 250 ml Pyrex flask and the nutrients were added appropriately. The pH of the media was adjusted to 6.0 ± 0.5 using appropriate buffer solution. Further, 5% volume of inoculum size was added aseptically to the flasks, which were incubated continuously in an environment-controlled incubator shaker (New Brunswick Scientific Co., USA) at 3.3Hz and 30 °C for 11 days. During fermentation, the pH of the medium was maintained at 6.0 ± 0.5. This was achieved by the addition of 4 M NaOH solution to stabilize the pH at the set point during the fermentation period. CAJ consumption and OA production rates were monitored during the course of fermentation.

2.6. Experimental design by central composite design (CCD)

Five parameters that have been reported to influence OA production via fermentation were chosen as independent variables: CAJ concentration, fermentation time, pH, $NaNO_3$ concentration and methanol concentration, and described as X_1, X_2, X_3, X_4, and X_5, respectively. The minimum, center point and maximum levels of each variable were coded as −1, 0, and +1, respectively (Table 1). A second-order mathematical equation, including all interaction terms, was used to calculate the predicted response:

$$Y = \alpha_0 + \sum_{i=1}^{5}\alpha_i X_i + \sum_{i=1}^{5}\alpha_{ii} X_i^2 + \sum_{i<j}^{5}\alpha_{ij} X_i X_j + e \quad (1)$$

where Y is the predicted response, i.e. oxalic acid (OA) concentration in g/l, α_o is the intercept, α_i ($i = 1, 2, 3, 4, 5$) is the first order model coefficient, α_{ij} is the interactive effect, and α_{ii} denotes the coefficients of X_i^2, and e is the random error.

Table 1. Coding of factors and levels for OA production.

			Coded factors				
Factor	**Unit**	**Symbols**	**Axial (-α)**	**−1**	**0**	**+1**	**Axial (+α)**
CAJ	g/l	X_1	109	150	200	250	291
pH	–	X_2	4.18	5	6	7	7.82
Time	day	X_3	5.36	7	9	11	12.64
Methanol	%v/v	X_4	0.18	1	2	3	3.82
$NaNO_3$	g/l	X_5	0.59	1	1.5	2	2.41

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Central composite design (CCD), which is appropriate for fitting complex surfaces when a second order model is selected, was employed for the experimental design in this work. To avoid redundancy in full factorial design [28], which would have produced 50 experimental runs using three levels and five factors, a fractional factorial design was applied to generate 26 experimental conditions used to investigate the selected factors for determination of OA production (Table 2). The levels of the factors (Table 1) were chosen based on the preliminary experiments carried out. Statistica 12 software package (StatSoft Inc., Tulsa, OK, USA) was used for designing the experiments.

2.7. Statistical analysis

The observed data were subjected to multiple regression analysis using the Statistica Program (StatSoft, Inc., Tulsa, OK, USA) to obtain the coefficients of the quadratic equation. The F-value and the probability p-value were used to appraise the significance of the model. The multiple coefficient of correlation (R) and coefficient of determination (R^2) were calculated to evaluate the performance of the regression equation. The behavior of the model in the experimental area was investigated graphically. Statistical evaluation of the model was carried out using analysis of variance (ANOVA. For the Pareto plot of standardized effects, the significant effects are visually identified. The bars relate to the absolute magnitudes of the estimated effect coefficients. Any effect that goes beyond the vertical line ($p = 0.05$) may be taken to be significant [29].

2.8. Analytical methods

2.8.1. Determination of reducing sugar concentration

A modified dinitrosalicylic acid (DNS) method [30] described by Saqib and Whitney [31], was used to quantify the CAJ, expressed as sucrose. To 1 ml of the supernatant, 3 ml of the DNS solution was added and boiled for 15 min. The resulting mixture was cooled and diluted with distilled water as required, followed by measurement of the absorbance using the UV-visible spectrophotometer (Libra 21 Model, UK) set at wavelength of 540 nm.

2.8.2. OA concentration determination

In this work, the OA produced was measured using the technique described by Jiang et al. [32]. This technique has earlier been described in our previous report on OA production [8], which is based on the catalytic effect of OA on the redox reaction between rhodamine B and dichromate.

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Table 2. CCD of five independent factors for OA production including the coded levels of each parameter.

Run	X_1 (g/l)	X_2	X_3 (day)	X_4 (%v/v)	X_5 (g/l)	Observed OA (g/l)	Predicted OA (g/l)	Residual
1	200 (0)	6 (0)	9 (0)	0.18 (-α)	1.5 (0)	83.83	84.71	−0.88
2	150 (-1)	7 (1)	7 (-1)	3 (1)	2 (1)	95.67	95.18	0.49
3	150 (-1)	5 (-1)	7 (-1)	1 (-1)	1 (-1)	65.8	64.83	0.97
4	150 (-1)	5 (-1)	11 (1)	3 (1)	2 (1)	87.83	87.34	0.49
5	200 (0)	6 (0)	9 (0)	2 (0)	0.59 (-α)	76.33	77.21	−0.88
6	200 (0)	6 (0)	9 (0)	2 (0)	1.5 (0)	98.33	97.87	0.46
7	150 (-1)	7 (1)	11 (1)	3 (1)	1 (-1)	81.17	80.68	0.49
8	200 (0)	6 (0)	9 (0)	2 (0)	1.5 (0)	98.33	97.87	0.46
9	200 (0)	6 (0)	5.36 (-α)	2 (0)	1.5 (0)	70.84	71.72	−0.88
10	108.94 (-α)	6 (0)	9 (0)	2 (0)	1.5 (0)	101.33	102.21	−0.88
11	200 (0)	4.18 (-α)	9 (0)	2 (0)	1.5 (0)	70.67	71.55	−0.88
12	150 (-1)	7 (1)	11 (1)	1 (-1)	2 (1)	85.83	85.34	0.49
13	150 (-1)	7 (1)	11 (1)	1 (-1)	1 (-1)	76.83	76.34	0.49
14	200 (0)	6 (0)	12.64 (α)	2 (0)	1.5 (0)	78	78.88	−0.88
15	291.06 (α)	6 (0)	9 (0)	2 (0)	1.5 (0)	77.67	78.55	−0.88
16	250 (1)	5 (-1)	11 (1)	3 (1)	1 (-1)	70.5	70.01	0.49
17	200 (0)	6 (0)	9 (0)	2 (0)	1.5 (0)	98.67	97.87	0.80
18	200 (0)	6 (0)	9 (0)	2 (0)	1.5 (0)	98.33	97.87	0.46
19	200 (0)	6 (0)	9 (0)	3.82 (α)	1.5 (0)	83.83	84.71	−0.88
20	250 (1)	7 (1)	7 (-1)	1 (-1)	2 (1)	75.33	74.84	0.49
21	250 (1)	5 (-1)	7 (-1)	3 (1)	2 (1)	83.17	82.68	0.49
22	200 (0)	6 (0)	9 (0)	2 (0)	1.5 (0)	98.67	97.87	0.80
23	200 (0)	7.82 (α)	9 (0)	2 (0)	1.5 (0)	79.33	80.21	−0.88
24	200 (0)	6 (0)	9 (0)	2 (0)	2.41 (α)	109.5	110.38	−0.88
25	250 (1)	5 (-1)	11 (1)	1 (-1)	2 (1)	64.17	63.68	0.49
26	250 (1)	7 (1)	7 (-1)	3 (1)	1 (-1)	78.5	78.01	0.49

α represents the axial point with a coded level of 1.821.

3. Results and discussion

3.1. CAJ physiochemical properties

Table 3 shows results of the physiochemical properties of the CAJ used in this work. The results showed that the CAJ was very high in reducing sugars (805 g/l). The observation is similar to the value of 788 g/l reported by Osho [22] but different from the observed values of 90.45 and 100.23 g/l by Honorato et al. [21] and Fontes et al. [23], respectively. Also, the values of pH, total nitrogen and crude protein of the CAJ observed in this present work were

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Table 3. Physiochemical properties of CAJ.

Parameter	Value
Total reducing sugar (g/l)	805.00
pH	4.06
Total nitrogen	0.04
Crude protein	0.26
Potassium (mg/l)	175.50
Sodium (mg/l)	18.10
Calcium (mg/l)	2.59
Magnesium (g/l)	16.28
Iron (mg/l)	0.51
Manganese (mg/l)	0.26
Copper (mg/l)	ND
Zinc (mg/l)	4.21
Tannin (g/100 ml)	0.72

ND – not detected.

comparable to the reported values by Osho [22]. The Na, Ca, Mg and Zn contents were higher in the CAJ used by Osho [22] than CAJ used in this work. The mineral compositions of the CAJ varied widely from the values reported by Honorato et al. [21]. These observtions may be attributed to the location where the cashew apple fruits were obtained. Whereas cashew apple fruits used in this work and that of Osho [21] were obtained from Nigeria, Honorato et al. [21] and Fontes et al. [23] worked with ones obtained from Brazil. The total tannin detected in the CAJ used was very low; hence there was no need to clarify the juice before use.

3.2. Preliminary evaluation of the effects of methanol and pH on OA production

The results of the investigation of CAJ as carbon source for OA production under submerged fermentation are presented in Fig. 1. The profiles of CAJ and OA concentrations against time without methanol addition to the culture medium and without pH adjustment of the culture medium in the course of the investigation are depicted in Fig. 1a. The results showed that the strain of *A. niger* used in this work was able to metabolize the CAJ without difficulty. The fungus was able to utilize 99.34% of the CAJ in 6 days. The highest concentration of OA obtained was 64.59 g/l (product yield of 0.32 g/g) and was observed on the 9th day of fermentation. Fig. 1b shows the profiles of CAJ and OA concentrations against time without methanol addition to the culture medium but with pH adjustment of culture medium in the course of the investigation. The fungus was able to consume 97.27% of the CAJ in 10 days. The highest OA concentration obtained on the 9th day of fermentation was

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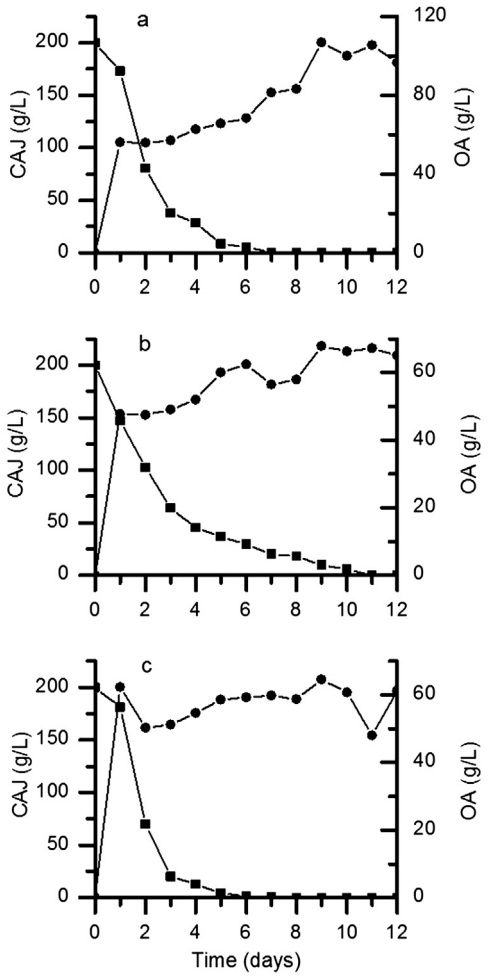

Fig. 1. Plots of OA and CAJ against time under submerged fermentation. (a) without methanol addition and without culture medium pH adjustment; (b) without methanol addition but with culture medium pH of 6.0; and (c) with methanol addition (2% volume) to the culture medium and with culture medium pH of 6.0.

67.92 g/l, representing a product yield of 0.34 g/g. Fig. 1c describes the profiles of CAJ and OA concentrations against time with methanol addition (2% volume) to the culture medium and with pH adjustment of culture medium in the course of the investigation. The fungus was able to utilize 97.27% of the CAJ within 6 days of fermentation. The highest OA concentration observed on the 9th day of fermentation was 106.75 g/l (product yield of 0.53 g/g). These results demonstrated the synergistic effect of pH adjustment of the culture medium and addition of methanol, which led to increase in the OA produced. Mandal and Banerjee [7], Ruijter et al. [16] and Bohlmann et al. [12] reported that pH has strong influence on the amount of OA produced in *A. niger*, with pH of 6.0 as the best [7]. Rymowicz and Lenart [14] and Betiku et al. [18] established that the addition of methanol to culture medium for OA production using *A. niger* increases the amount of the acid produced. The highest OA production in

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A. niger has been reported to occur on the 7th and 9th day of fermentation [7, 11, 14], which corroborated the results observed in the present work.

3.3. Modelling and optimization of OA production

After the preliminary studies, RSM coupled with CCD was used to model and optimize the fermentation process in order to increase the OA production. Table 2 shows the experimental conditions investigated together with the observed and predicted values. The data were fitted using the following second-order mathematical equation:

$$\begin{aligned} Y = {} & 97.87 - 6.50X_1 + 2.38X_2 + 1.97X_3 - 3.14 \times 10^{-16}X_4 + 9.11X_5 \\ & + 4.21X_1X_2 - 0.26X_1X_3 + 6.87X_1X_4 - 4.61X_1X_5 - 1.05X_2X_3 \\ & + 3.79X_2X_4 - 4.94X_2X_5 + 0.25X_3X_4 - 9.28X_3X_5 - 0.10X_4X_5 \\ & - {X_1}^2 - 6.63{X_2}^2 - 6.81{X_3}^2 - 3.97{X_4}^2 - 1.23{X_5}^2 \end{aligned} \quad (2)$$

where Y is the oxalic acid (OA) produced in g/l and X_1 is CAJ concentration, X_2 is medium pH, X_3 is fermentation time, X_4 is methanol concentration and X_5 $NaNO_3$ concentration.

Table 4 displays the test of significance and ANOVA of the regression equation model results. Both multiple coefficient of correlation (R) and coefficient of determination (R^2) were used to assess the goodness of fit of the regression equation. R of 0.9982 of the model demonstrated a good correlation between the observed and predicted values. While the R^2 of the model was 0.9964, which showed that 99.64% sample variation for OA produced is attributable to the independent factors and just 0.36% of the total variation are not described by the model [33, 34]. The adjusted R^2 of 0.9822 proved that the model was significant. It has been suggested that R^2 should be $\geq$ 80% for the good fit of a model [35]. The residuals between the observed and predicted values in this work revealed good fit of the model (Table 2). This was also confirmed by both the bias factor ($B_f = 1.09$), which is a measure of the agreement between observed and predicted values [36] and the correspondence plot (Fig. 2). These observations implied that the model developed for the fermentation process adequately described the actual relationship among the selected factors.

Except for methanol concentration (X_4), CAJ concentration $\times$ time (X_1X_3), pH $\times$ time (X_2X_3), time $\times$ methanol concentration (X_3X_4), and methanol concentration $\times$ $NaNO_3$ concentration (X_4X_5), the p-values of the model terms were significant at $p < 0.05$ (Table 4). Also, the observed low p-value of 0.0001 together with the corresponding high F-value of 69.97 showed that the model obtained was significant. Both the F-value and p-value do not differentiate between negative and positive significant effects of each term in the model [25, 34]. Therefore, the standardized effects of CAJ concentration, pH, time, methanol concentration

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Table 4. Test of significance for every regression coefficient and ANOVA.

Factor	Sum of squares	df	Mean square	*F*-value	*p*-value
X_1 (CAJ)	279.90	1	279.90	105.37	0.0001
X_1^2	105.27	1	105.27	39.63	0.0014
X_2 (pH)	37.50	1	37.50	14.12	0.0131
X_2^2	910.24	1	910.24	342.68	0.0000
X_3 (Time)	25.63	1	25.63	9.65	0.0266
X_3^2	958.92	1	958.92	361.01	0.0000
X_4 (Methanol)	0.00	1	0.00	0.00	1.0000
X_4^2	325.66	1	325.66	122.60	0.0001
X_5 ($NaNO_3$)	550.12	1	550.12	207.11	0.0000
X_5^2	31.05	1	31.05	11.69	0.0188
X_1X_2	51.90	1	51.90	19.54	0.0068
X_1X_3	0.20	1	0.20	0.08	0.7948
X_1X_4	144.53	1	144.53	54.41	0.0007
X_1X_5	64.88	1	64.88	24.43	0.0043
X_2X_3	3.36	1	3.36	1.26	0.3120
X_2X_4	44.02	1	44.02	16.57	0.0096
X_2X_5	74.65	1	74.65	28.10	0.0031
X_3X_4	0.19	1	0.19	0.07	0.8005
X_3X_5	263.13	1	263.13	99.06	0.0001
X_4X_5	0.03	1	0.03	0.01	0.9150
ANOVA					
Model	3717.09	20	185.85	69.97	0.0001
Error	13.28	5	2.6562		
Total sum of squares	3730.37	25			
$R^2 = 0.9964$, Adjusted $R^2 = 0.\ 9822$					

and $NaNO_3$ concentration, and their interactions on the OA production were examined by preparing a Pareto chart (Fig. 3). The model terms ($NaNO_3$, CAJ × methanol, CAJ × pH, pH × methanol, pH and time) with positive coefficients indicated a favourable or supportive effect on OA fermentation while the model terms (time × time, pH × pH, methanol × methanol, CAJ, time × $NaNO_3$, CAJ × CAJ, pH × $NaNO_3$, CAJ × $NaNO_3$, $NaNO_3$ × $NaNO_3$) with negative coefficients demonstrated an unfavourable effect on OA fermentation [29, 34, 37]. $NaNO_3$ concentration was the most positive significant model term on OA production followed by CAJ × methanol concentrations, CAJ concentration × pH, pH × methanol concentration, linear effects of pH and time (Fig. 3). pH × time, CAJ concentration × time, time × methanol concentration, methanol concentration × $NaNO_3$ concentration, and linear effect of methanol

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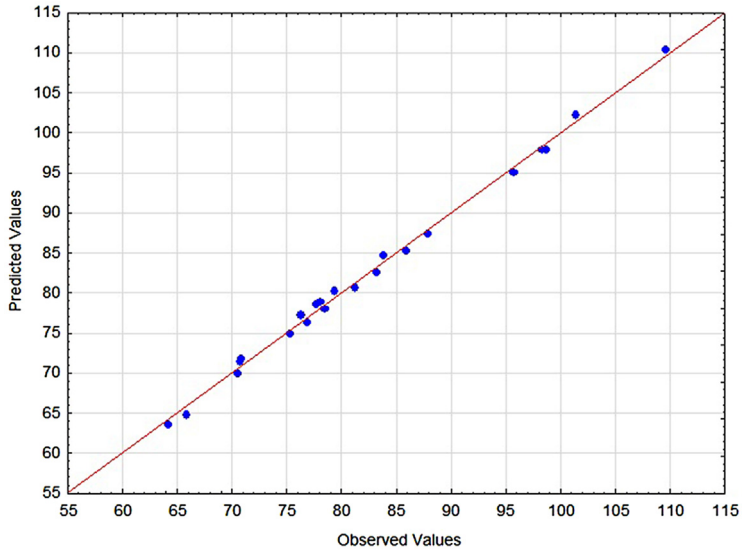

Fig. 2. Parity plot of predicted values against observed values.

concentration remained inside the reference line (Fig. 3), which confirmed insignificance of the model terms [29, 34, 37]. This observation is corroborated by the *p*-values (Table 4) of these model terms ($p > 0.05$). The results showed that the linear effect of methanol concentration contributed the least to the OA production.

3.4. Relationship among the selected factors for OA fermentation

Fig. 4 shows the contour and surface response plots for the optimization of fermentation process for the OA production. The 3-dimensional response surface is the visual illustration of the regression equation for the OA production [33, 34]. The shape of all the 3-dimensional surfaces in Fig. 4 shows significant interactions among the factors considered in this work. In particular, the dome shape of the plots in Fig. 4 (e, f and h) shows that there are shared interactions between time and pH, methanol concentration and pH, and methanol concentration and time. But in the response surfaces of Fig. 4 (a, b, c, d, g, i and j), there were striking significant interactions among the factors investigated. Additionally, 2-dimensional contour plot is the visual description of the regression equation and assists in the identification of the interactions among selected factors [38]. Each contour curve denotes an unlimited number of combinations of the two test factors. The elliptical contour plots in Fig. 4 (a, b, c, d, g and i) indicate marked significant interactions between CAJ concentration and pH, CAJ concentration and time, CAJ and methanol concentrations, $NaNO_3$ and CAJ concentrations, $NaNO_3$ concentration and pH as well as $NaNO_3$ and methanol concentrations. While the circular contour plots of the response surfaces in Fig. 4 (e, f and h) suggest the interactions between time and pH,

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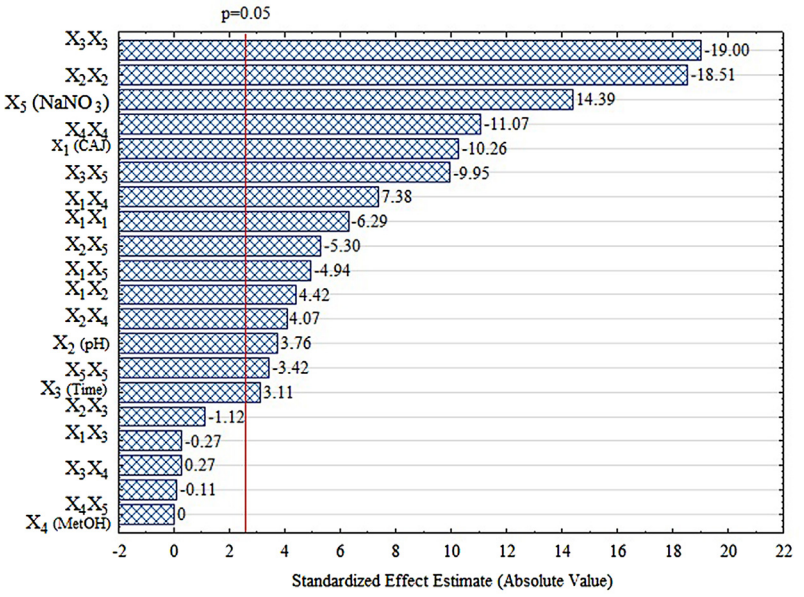

Fig. 3. Pareto chart of standardized effects for OA production.

methanol concentration and pH, and methanol concentration and time are negligible. The hyperbolic contour plot for the interaction between $NaNO_3$ concentration and time in Fig. 4j (saddle or minimax contour) indicated that the centre was neither a maximum nor a minimum point [38].

3.5. Model validation

The optimum values of the five factors selected for the fermentation process were obtained by solving Eq. (2) using the Design-Expert software package (version 9.0.2.0). The optimal condition was statistically predicted as CAJ concentration of 150.0 g/l, pH of 5.4, time of 7.31 days, methanol concentration of 1% volume and $NaNO_3$ concentration of 2 g/l. Under this condition, the OA concentration predicted was 122.19 g/l. In order to validate the model, the optimal condition values were applied to five independent experimental replicates and the average value of OA produced was 122.72 ± 1.8 g/l. In comparison to the preliminary studies, CAJ concentration required was reduced by 50 g/l, methanol concentration was reduced from 2 to 1% volume and fermentation time from 9 to 7.31 days, which led to increased OA production by margin of 15.93 g/l.

OA fermentation using *A. niger* has been reported by several workers (Table 5). Strasser et al. [11] reported OA production of 38 g/l from molasses. Bohlmann et al. [12] produced OA from milk whey with maximum production

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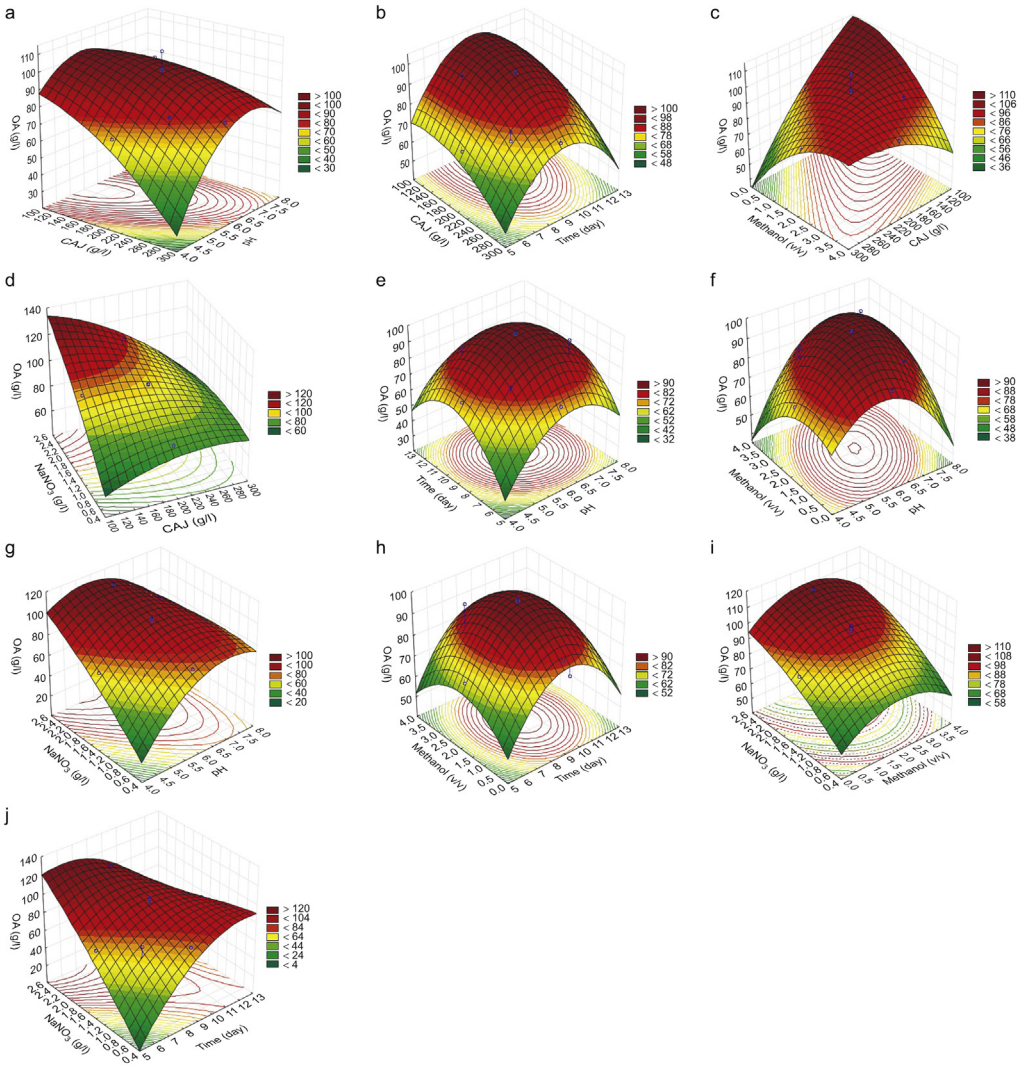

Fig. 4. Response surface plots for OA production. a: interaction between CAJ concentration and pH, b: interaction between CAJ concentration and time, c: interaction between CAJ concentration and methanol concentration, d: interaction between $NaNO_3$ concentration and CAJ concentration, e: interaction between time and pH, f: interaction between methanol concentration and pH, g: interaction between $NaNO_3$ concentration and pH, h: interaction between methanol concentration and time, i: interaction between $NaNO_3$ concentration and methanol concentration, j: interaction between $NaNO_3$ concentration and time.

of 41.4 g/l. Also, Podgorski and Lesniak [13] used sugar beet molasses as carbon source and obtained OA of 38.7 g/l. Musiał et al. [5] and André et al. [17] employed biodiesel-derived waste glycerol as substrate for *A. niger* with OA production of 48.9 and 21.5 g/l, respectively. Whereas Rymowicz and Lenart [13] made use of post-refining fatty acids supplemented with methanol as substrate for *A. niger* and produced OA of 75 g/l. Betiku et al. [18] applied

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Table 5. Summary of OA production by *A. niger* using various substrates.

Substrate	OA concentration (g/l)	Reference
Molasses	38	Strasser et al. [11]
Milk whey	41.4	Bohlmann et al. [12]
Sugar beet molasses	38.7	Podgorski and Lesniak [13]
Lipids	68	Rymowicz and Lenart [15]
Post-refining fatty acids supplemented with methanol	75	Rymowicz and Lenart [14]
Biodiesel-derived waste glycerol	21.5	André et al. [17]
Biodiesel-derived waste glycerol	48.9	Musiał et al. [5]
Sweet potato starch hydrolyzate	103.88	Betiku et al. [18]
Cashew apple juice	122.68	Present work

RSM by evaluating the effects and interactions of sweet potato starch hydrolyzate as the sole carbon source, pH and time on OA production and reported acid production of 103.88 g/l from *A. niger*. In our previous work using surface fermentation [8], the highest OA production observed was 120.66 g/l under optimum values of a CAJ concentration of 291 g/l, pH of 6.9, time of 10.82 days, methanol concentration of 2.91% (v/v), and $NaNO_3$ concentration of 1.05 g/l (Table 6), which were statistically predicted by the RSM quadratic model developed. Although the results of the optimized parameters obtained in this study as compared to the results of surface fermentation option (Table 6) suggest that submerged fermentation had a marginal edge over surface fermentation based on productivity, in economic term, it may be argued that submerged option may indeed not be attractive in an industrial context if the additional costs associated with submerged option such as bioreactors, compressors, electricity etc. were to be considered [39].

Table 6. Evaluation of fermentation techniques for OA production.

Variable	Submerged	Surface [8]
CAJ concentration (g/l)	150	291
pH	5.4	6.9
Time (days)	7.31	10.82
Methanol concentration %(v/v)	1.00	2.91
$NaNO_3$ concentration (g/l)	2.00	1.05
Oxalic acid concentration (g/l)	122.68	120.66

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4. Conclusions

The prospect of submerged fermentation was explored for the utilization of CAJ as a carbon source for OA production in this work. The results showed that pH of the culture medium and addition of methanol to the medium had profound effects on the amount of OA produced in *A. niger*. The best model that described the OA fermentation process was a second-order mathematical model with R^2 of 0.9964. The most significant positive factor for the process was $NaNO_3$ concentration while methanol concentration was an insignificant factor in the OA fermentation. Optimal condition predicted for the five independent factors were CAJ of 150.0 g/l, pH of 5.4 time of 7.31 days, methanol of 1% volume and $NaNO_3$ of 2 g/l, which were validated experimentally with OA concentration of 122.72 ± 1.8 g/l. In comparison to the preliminary studies, optimization by application of RSM led to improved OA production. The required CAJ concentration to achieve this was reduced by 50 g/l, methanol concentration was reduced from 2 to 1% volume and fermentation time from 9 to 7.31 days with increased OA production from 106.75 to 122.72 g/l. These reductions may make the fermentation process cost-effective. Thus, CAJ could serve as inexpensive raw material for OA production.

Declarations

Author contribution statement

Eriola Betiku: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Harrison Emeko: Performed the experiments; Analyzed and interpreted the data.

Bamidele Solomon: Contributed reagents, materials, analysis tools or data; Wrote the paper.

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