Original Research Article

Optimization for oxalic acid production by *Aspergillus niger* using Response Surface Methodology

.

Aims: To optimize selected process variables for oxalic acid production by *Aspergillus niger* using Response surface methodology

Study design: Central composite design

Place and Duration of Study: Department of Microbiology, University of Port Harcourt, Rivers State, Nigeria.

Methodology: Three media for the study was set up- algal biomass medium, sucrose medium and mixture of both algal biomass and sucrose medium. Inoculum of *Aspergillus niger* was prepared and subsequently inoculated into media for oxalate production by submerged fermentation. The oxalate produced after 14 days was determined by the catalytic effect of oxalic acid on the redox reaction between rhodamine B and dichromate.

Results: The predicted conditions of pH 6.838, temperature 35 ^oC and substrate algal biomass and sucrose with oxalic acid production of 8.618 g/L were reported in the study. This slightly varies with the experimental conditions of pH 6, temperature 35 ^oC, algal biomass and sucrose mixture and oxalic yield of 12.12 g/L. The R^2 value of 0.968 validates the model and adjusted R^2 of 0.9449 shows that the model is significant.

Conclusion: The study shows the feasibility of using the response surface methodology (RSM) in optimizing pH, temperature and substrate for the production of oxalic acid (g/l). It further shows the increased possibility of algal biomass as alternative feedstock for production of organics.

Keywords: (Aspergillus niger, algal biomass, response surface methodology, oxalic acid.)

1. INTRODUCTION

Oxalic acid is a dicarboxylic acid with the IUPAC formula, $H_2C_2O_4$. It is a strong acid and usually occurs as a free acid but more often as calcium salt. It has wide applications in pharmaceutical, textile, wastewater treatment and food industry as well as hydrometallurgy [1]. It is used as an anti-browning agent for apples [2] and for removal of kaolin iron as a result of its high reducing power [3, 4].

Currently, most of the oxalic acid used today is produced by synthetic methods. These chemical processes include: (i) oxidation of olefins and glycols (ii) fusion of sawdust with caustic soda (iii) fermentation of carbohydrates (iv) decomposition of formats (v) radiation processing of carbonate solutions and molasses [5, 6] Regrettably, these synthetic processes have been reported to be eco-harmful and unsustainable thus, the need for a more sustainable ecologically friendly approach.

Oxalic acid has been reported to be biosynthetically produced by bacteria, fungi, plants and animals [7]. Some microorganisms that have been reported to produce oxalic acid include: *Aspergillus ficuum* [8]; *Glyophyllum trabeum* [8]; *Paxillus involutus* [9]; *Penicillium oxalicum* [10] and *Aspergillus niger* [11]. *A. niger* has been reported to give the highest amount of yield hence its preference over other isolates. The organism is generally accepted because of ease of handling, rapid growth and its versatility in fermenting cheap raw materials [12, 13].

In a bid to synthesize oxalic acid from cheap substrates and optimize process parameters, some authors have employed experimental design and optimization tools such as Annual Neural Network (ANN) and Response Surface Methodology (RSM) for their research [11]. RSM is an experimental design and mathematical modeling tool which involves the partial regression fitting of the experimental factors [14]. It is currently preferred because it has an edge of reducing the number of experimental runs needed to give satisfactory information for statistically acceptable results. The conventional one-factor method used for optimizing fermentation media parameters one at a time is labourious, time consuming and ignores the interaction between independent process parameters [15]. In addition, [16] reported that optimization of process variables is very important because of its impact on the feasibility and economy of the fermentation process. Furthermore, optimization of fermentation variables is essential because of the complexity of the metabolic state in fungus for increased yield of the desired product [17]. RSM has been applied in research for optimizing various processes such as biodiesel production by Novozym 435 [18]; production of scleroglucan [19], production of citric acid [20]; thermostable lipase [21]; biodiesel production from alkaline transesterification of rice bran oil [22]; production of ethanol [14]; oxalic acid production from sweet potato starch hydrolyzate [23] and oxalic acid production from cashew apple juice [11].

In this study, oxalic acid production from algal biomass slurry, sucrose medium and mixture of algal biomass and sucrose medium using A. niger in submerged fermentation system was investigated. In order to optimize the process variables, the central composite design (CCD) and RSM were employed to determine the effect of three factors (substrate type, pH, and temperature) and their reciprocal interactions on oxalic acid yield.

2. MATERIAL AND METHODS

2.1. Media Preparation

2.1.1 Cultivation of Microalgae

The microalgae used for this study were obtained from the department of Microbiology, University of Port Harcourt, Choba, Nigeria. A pure culture of the organism was obtained by repeated sub-culturing of the isolate on nutrient agar using spread plate technique, supplemented with a mixture of chloramphenicol (62.5 µg/ml) and nystatin (100 µg/ml) to ensure free fungal and bacterial cultures. The algal strain was selected after preliminary screening using biochemical and morphological characteristics and was finally maintained on agar slants until when required [24]. Five milliliters of the bloomed culture was aseptically inoculated into flasks containing effluent-and freshwater medium. About 1ml of the bloomed culture was inoculated into a defined synthetic medium (0.132 g/L Potassium nitrate, 0.066 g/L sodium silicate, 0.066 g/L monosodium phosphate and 0.066 g/L EDTA. The pH of the medium was adjusted to 6.5 with 4M NaOH prior to autoclaving at 121 °C for 15 minutes [25]. The setups were maintained at 28±2 °C under natural illumination and aerated intermittently by shaking at interval for 14 days. Samples were periodically removed every 48 h to monitor changes in algal concentration, optical density and biomass as dry weight was determined [25].

Three different media were setup: algal biomass medium, sucrose medium and mixture of both algal biomass and sucrose medium. The algal biomass medium was setup by aseptically harvesting 100 ml of wet algal biomass and subsequently transferred into 250 ml conical flask. The sucrose medium (100 ml) was prepared in line with the manufacturer's specification and supplemented with 1.6 g/L yeast extract, 0.025 g/L KCL, 1.5 g/L $NaNO_3$, 0.025 g/L $MgSO_4.7H_2O$ and 0.5 g/L KH_2PO_4 [11]. The third medium was setup by measuring 50 ml of the two media previously prepared and aseptically transferred into a 250 ml conical flask. In order to maintain the pH conditions of 5,6 and 7; the pH condition of the three set-ups were then adjusted with 4.0 M NaOH and 3.0 M HCl and maintained during the study [26].

2.2 Inoculum Preparation for Oxalic Acid

The strain of *Aspergillus niger* which served as the inoculum was obtained locally from the department of Microbiology, University of Port Harcourt, Choba, Nigeria. Spores of *A. niger* were grown on Potato dextrose agar (PDA) for 5-7 days at 30 °C**.** Afterwards, the *Aspergillus niger* spores were aseptically transferred into 100 ml sterile distilled water [11]. Inoculum size of 5 % (v/v) was inoculated aseptically into the setups.

2.3 Oxalic Acid Determination

The oxalic acid produced after 14 days was measured using the technique reported by [26] which is based on the catalytic effect of oxalic acid on the redox reaction between rhodamine B and dichromate neasured at absorption wavelength of 555 nm in tetraoxosulphate (vi) acid (H_2SO_4). 10 ml of the sample was taken from the fermentation medium and filtered with Whatman No 1 filter paper. Afterwards, 1 ml from the filtrate was added to 0.5mL of 0.06M potassium dichromate. Then, 0.20 mL of 2.5M H_2SO_4 and 0.10 mL of 3.28×10^{-4} M rhodamine B were added and brought up to 10mL in a test tube and mixed vigorously. The resulting mixture was placed in a water bath at 90 °C for 8 minutes. After cooling, the absorbance of the mixture was read at 555 nm against the blank solution. The oxalic acid concentration was determined using a standard curve prepared with synthetic oxalic acid.

2.4 Experimental design by central composite design (CCD)

This study was designed to optimize some process variables for oxalic acid production *by A. niger* Three factors that have been reported to influence oxalic acid synthesis (pH, temperature and substrate) were chosen as independent variables. The factors were tested at three levels: minimum, middle and maximum (Table 1). A central composite design was used to generate 27 experimental conditions used to investigate the selected factors for optimization. A first-order polynomial was used to express oxalic acid as a function of the independent variable as follows:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_1 x_2 + \beta_5 x_1 x_3 + \beta_6 x_2 x_3 + \beta_7 x_1^2 + \beta_8 x_2^2 + e \qquad (1.0)$$

where

y is the predicted response factor (oxalic acid)

β_0, β_1, β_2, β_3, β_4, $\beta_{5,}$ $\beta_{6,}$ β_7 and β_8 are constant regression coefficients of the model, in which β_0 is the intercept term, β_1 and β_2 are linear coefficients, β_7 and β_8 are quadratic coefficients and β_3, $\beta_{4,}$ and β_5 is the interactive coefficient.

Table 1: Coding of factors and levels for oxalate production

Factor	Symbols	Coded Factors		
		-1	0	+1
pH	A	5.00	6.84	7.00
Temperature	B	25	35	35
Substrate	C	Algal biomass	Algal biomass+sucrose	Sucrose

2.4 Statistical analysis

Data generated for CCD was subjected to regression analysis using the Design Expert 10.0 in order to generate parameters required for the optimization of oxalic production. The *f*-value and *p*-value were determined and used to appraise the significance of the model. The multiple coefficient of determination R^2, the adjusted R^2 and predicted R^2 were used to evaluate the performance of the regression equation. The statistical evaluation of the model was evaluated with the analysis of variance (ANOVA).

3. RESULTS AND DISCUSSION

3.0 Results and Discussion

3.1 Effluent-freshwater characteristics and wet algal biomass composition

The results of analysis of the medium for growth of algal biomass used in this study and the composition of the algal biomass has been reported in previous study by authors [25].

3.2 Modelling and optimization of oxalate production

After the preliminary studies, response surface methodology with central composite design was used to model for oxalic acid production. Table 2 below shows the conditions, predicted and experimental oxalic acid yields.
The data obtained were fitted into the second-order equation for the three different substrates as shown below.

Algal medium

$$Y = -78.19259 + 18.88333A + 1.895*B - 0.017500*A*B - 1.56389*A^2 - 0.028889*B^2 \quad (2.0)$$

Algal biomass-sucrose medium

$$Y = -74.98704 + 19.35000*A + 1.82833*B - 0.017500*A*B - 1.56389*A^2 - 0.028889*B^2 \quad (3.0)$$

Sucrose medium

$$Y = -78.84259 + 19.15000*A + 1.8650*B - 0.017500*A*B - 1.56389*A^2 - 0.028889*B^2 \quad (4.0)$$

Where Y is oxalic yield in g/L, A is pH and B is temperature in ^oC.

Table 2 shows the predicted and experimental oxalic yields for the different factors considered in the study. Table 3 shows the analysis of variance, test of significance of the regression equation model results, coefficient of determination (R^2), adjusted R^2 and predicted R^2. The R^2 of the model demonstrate good correlation between the predicted and observed values. The R^2 value of 0.9682 showed that 96.82% sample variation for oxalic acid production is attributable to the independent factors and 3.18% of the total variation are not accounted for by the model [1]. The R^2 value reported in this work varies with 0.9964 as reported by [1] and this could be attributed to the different independent variables employed in the study. The adjusted R^2 of 0.9449 indicated that the model was significant as it has been suggested that R^2 should be greater than 80% for the good fit of a model [27]. The results showed that the p-values of the model were significant at $p<0.05$ and the observed p-value of 0.0001 and the corresponding high F-value of 41.51 obtained was significant. Also, it is evident from table 3 that the linear effect of substrate have significant ($p<0.05$) effect on oxalic acid production. Also, there is significant ($p<0.05$) effect of the square of pH and temperature on the production of oxalic acid. However, the linear terms of pH and temperature were reported as not having a significant effect ($p<0.05$) on the oxalic acid production. This disagrees with [1] who reported that pH has significant effect on oxalic acid production.

Table 2: Experimental design for the processing conditions for oxalic acid production (g/l)

Randomization	Run	pH	Temperature (^{0}C)	Substrate	Oxalic Acid (g/l) (RSM predicted)	Oxalic acid (g/L) (Experimental)
3	1	5	35	Algal biomass	5.05	6.14
4	2	7	35	Algal biomass	3.75	5.38
10	3	5	25	Algae+Sucrose	8.25	8.98
1	4	5	25	Algal biomass	4.4	5.96
22	5	7	35	Sucrose	4.45	6.62
12	6	5	35	Algae+Sucrose	8.45	9.34
21	7	5	35	Sucrose	4.7	5.9
18	8	6	30	Algae+Sucrose	11.5	11.9
2	9	7	25	Algal biomass	3.85	5.45
8	10	6	35	Algal biomass	6.45	8.59
17	11	6	35	Algae+Sucrose	9.05	12.2
14	12	5	30	Algae+Sucrose	8.65	9.88
23	13	5	30	Sucrose	5.1	7.3
7	14	6	25	Algal biomass medium	5.3	6.6
26	15	6	35	Sucrose	5.75	8.48
13	16	7	35	Algae+Sucrose	8.3	10.1
27	17	6	30	Sucrose	6.3	7.2
24	18	7	30	Sucrose	4.9	5.66
25	19	6	25	Sucrose	5.5	6.42
20	20	7	25	Sucrose	4.1	5.77
9	21	6	30	Algal biomass medium	7.25	8.31
5	22	5	30	Algal biomass medium	4.7	5.32
15	23	7	30	Algae+Sucrose	8.85	10.11
11	24	7	25	Algae+Sucrose	8.55	10.03
16	25	6	25	Algae+Sucrose	9.3	9.75
6	26	7	30	Algal biomass medium	4.1	5.22
19	27	5	25	Sucrose	4.5	4.79

Table 3: ANOVA for response surface quadratic model

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob> F**	
Model	114.72	11	10.43	41.51	< 0.0001	Significant
A-pH	*0.48*	*1*	*0.48*	*1.92*	*0.1856*	
B-Temperature	*0.27*	*1*	*0.27*	*1.07*	*0.3173*	
C-Substrate	*95.08*	*2*	*47.54*	*189.24*	*< 0.0001*	
AB	*0.092*	*1*	*0.092*	*0.37*	*0.5544*	
AC	*0.66*	*2*	*0.33*	*1.31*	*0.2992*	
BC	*0.33*	*2*	*0.17*	*0.67*	*0.5285*	

A^2	14.67	1	14.67	58.41	< 0.0001
B^2	3.13	1	3.13	12.46	0.0030
Residual	3.77	15	0.25		
Cor Total	118.49	26			

R-squared (R^2)=0.9682
Adjusted R squared=0.9449
Predicted R squared=0.9191

3.3 Model Prediction

Figures 1-2 show the optimal response surface and contour plot of oxalic acid production as a function of pH, temperature and substrate and their reciprocal interactions on the oxalic acid yield. The predicted optimal values by the software were pH of 6.8, temperature of 35 °C and substrate of algal biomass-sucrose mixture with the desirability of 0.833 and oxalic acid production of 8.618 g/l. These values disagree with [1] whose model predicted a pH value of 5.4 and [23] who predicted pH value of 6.2 for optimal production of oxalic acid by *A. niger*. The differences in optimal conditions could be attributed to the different fermentation media and study designs used. The oxalic acid yield reported disagrees with [28, 29] who reported an oxalic acid yield of 21.5 g/L and 68 g/L. This disagreement is attributable to the different substrates employed for the study. The oxalic yield reported in this work also is lesser than that of [8] who reported a yield of 38 g/L. The relatively lower oxalic acid yield as reported in this work could be ascribed to the unavailability of the nutrients in the fermentation medium as the algal biomass used in this work was not pretreated prior to its usage as main carbon source.

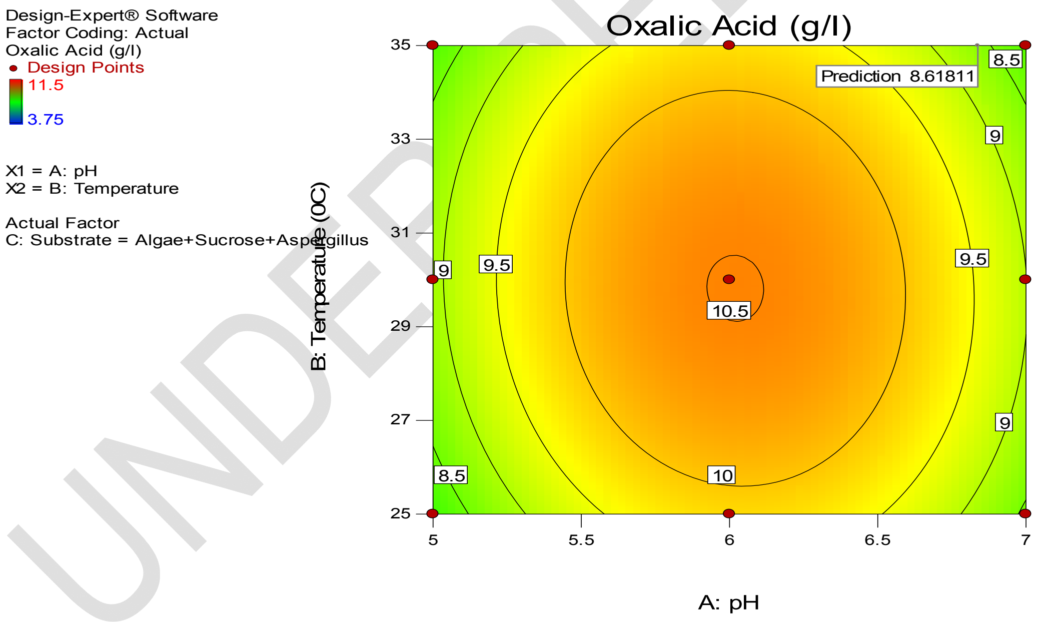

Fig. 1: Optimal response surface plot of oxalic acid production as a function of pH, temperature and substrate

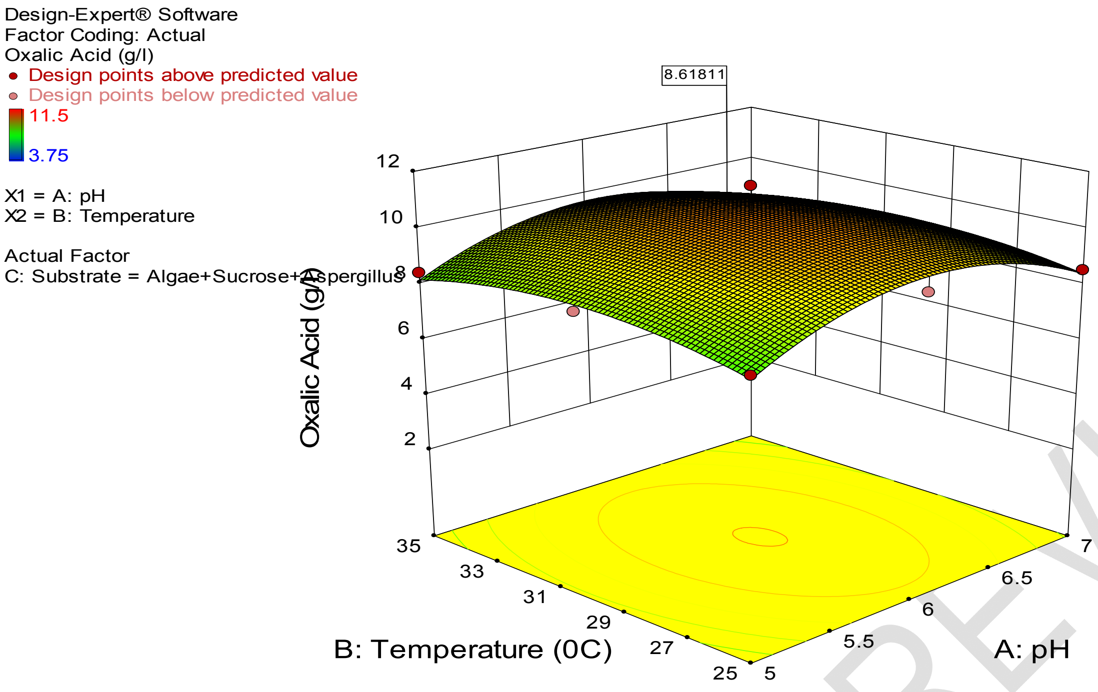

Fig 2: Optimal response contour plot of oxalic acid production as a function of pH, temperature and substrate

4. CONCLUSION

The study has shown the feasibility of using the response surface methodology (RSM) in optimizing pH, temperature and substrate for the production of oxalic acid (g/l). The study also highlights the prospect of using algal biomass as a source of carbon for oxalic acid production. The model predicted the conditions: pH value of 6.8, temperature value of 35 ^{o}C and algal biomass-sucrose substrate as optimal conditions for oxalic acid production.

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