Original Research Article

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

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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 350C 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 **35oC**35°C, 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.

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

1. INTRODUCTION

Oxalic acid is a dicarboxylic acid with the IUPAC formula, H2C2O4. 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 (Betiku et al., 2016). It is used as an anti-browning agent for apples (Son et al., 2000) and for removal of kaolin iron as a result of its high reducing power (Aghaie et al., 2009, Musial et al., 2011).

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 formates (v) oxidation of carbohydrates with nitric acid and (vi) radiation processing of carbonate solutions and molasses (Mandal and Banerjee, 2005; Nakata and He, 2010). 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 (Hodgkinson, 1977). Some microorganisms that have been reported to produce oxalic acid **include:**include *Aspergillus ficuum* (Strasser et al., 1994); *Glyophyllum trabeum* (Strasser et al., 1994); *Paxillus involutus* (Lapeyrie et al., 1980); *Penicillium oxalicum* (Ikotum, 1984) and *Aspergillus niger* (Emeko et al., 2015). *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 (Nadem et al., 2010; Pandey et al., 2013).

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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 (Emeko et al., 2015). RSM is an experimental design and mathematical **modeling**modelling **tool which**tool, which involves the partial regression fitting of the experimental factors (Wang et al., 2011). 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**laborious, time consuming and ignores the interaction between independent process parameters (Vishwanatha et al., 2010). In addition, Lie and Tzeng (1998) 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 (Dhillon et al., 2008). RSM has been applied in research for optimizing various processes, such as biodiesel production by Novozym 435 (Chang et al., 2005); production of scleroglucan (Desai et al., 2008), production of citric acid (Imandi et al., 2008); thermostable lipase (Ebrahimpour et al., 2008); biodiesel production from alkaline transesterification of rice bran oil (Rashid et al., 2009); production of ethanol (Wang et al., 2011); oxalic acid production from sweet potato starch hydrolyzate (Adesina et al., 2014) and oxalic acid production from cashew apple juice (Emeko et al., 2015).

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.

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2. MATERIAL AND METHODS

2.1. Media Preparation

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 w*et al*gal biomass and subsequently transferred into 250 ml conical flask. The sucrose medium (100ml) 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 (Emeko *et al*., 2015). The third medium was setup by measuring 50ml of the two media previously prepared and aseptically transferred into a 250ml 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 3.0M HCl and maintained during the study (Adesina *et al*., 2014).

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 (Emeko *et al*., 2015). 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 Jiang *et al*., 1996, **which is** based on the catalytic effect of oxalic acid on the redox reaction between rhodamine B and dichromate.

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. 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, β_5, β_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, β_4, and β_5 is the interactive coefficient.

Table 1: 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

2.4 Statistical analysis

Data generated for CCD was subjected to regression analysis using the Design Expert 10.0 (from which company) in order to generate parameters required for the optimization of oxalic production.

3. RESULTS AND DISCUSSION

3.0 Results and Discussion

3.1 Optimization of oxalic acid production

Table 1 depicts the experimental design for the parameters that affect oxalic acid production under consideration. Table 2 shows the regression coefficients for response surface quadratic model in terms of oxalic acid yield. Table 3 shows the results of test of significance for every regression coefficient. The results showed that the p-values of the model were significant at $p<0.05$. Furthermore, the linear terms (A, B, C), cross-products (AB, AC, BC) and the quadratic terms (A^2,B^2) were all significant model terms at 95% confidence (Table 3). Results of the analysis of variance of regression equation model are shown in table 3. The model reported an f-value of 41.51 while the value of coefficient of determination (R^2) of the model was reported to be 0.9682. Fig 1-2 shows 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 6.838, temperature of **35°C** 35°C and substrate of algal biomass-sucrose mixture with the desirability of 0.833 and oxalic acid production of 8.618 g/l.

Table 2: Regression coefficients for response surface quadratic model in terms of oxalic acid

Final Equation in Terms of Actual Factors:	
Substrate	Algae+Aspergillus
Oxalic Acid	=
-78.19259	
+18.88333	* pH
+1.89500	* Temperature
-0.017500	* pH * Temperature
-1.56389	* pH^2
-0.028889	* $Temperature^2$
Substrate	Algae+Sucrose+Aspergillus
Oxalic Acid	=
-74.98704	
+19.35000	* pH
+1.82833	* Temperature
-0.017500	* pH * Temperature
-1.56389	* pH^2
-0.028889	* $Temperature^2$
Substrate	Sucrose+Aspergillus
Oxalic Acid	=
-78.84259	
+19.15000	* pH
+1.86500	* Temperature
-0.017500	* pH * Temperature
-1.56389	* pH^2
-0.028889	* $Temperature^2$

Table 3: ANOVA for response surface quadratic model

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						

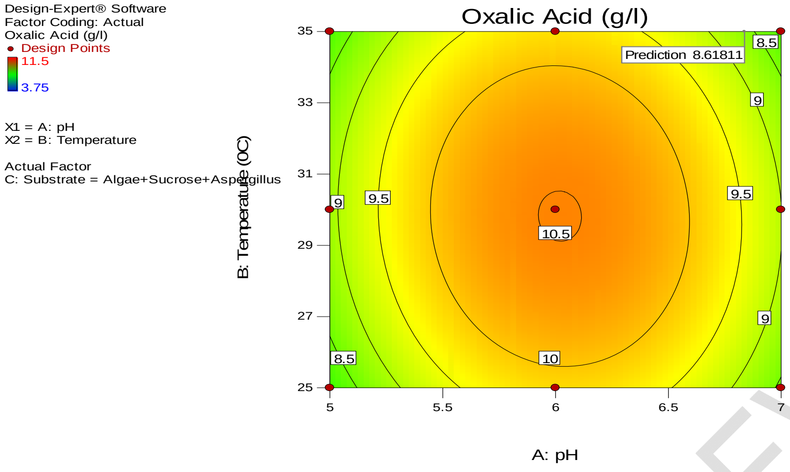

Fig. 1: Optimal response surface plot of oxalic acid production as a function of pH, temperature and substrate

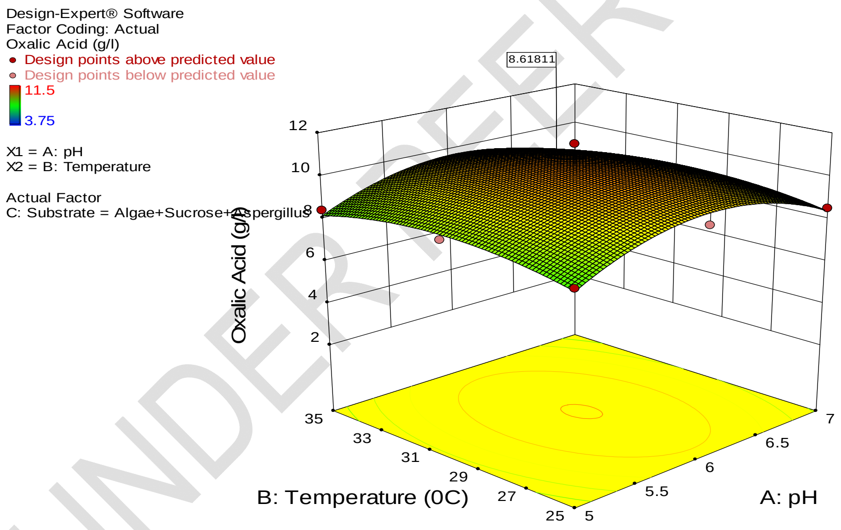

Fig 2: Optimal response contour plot of oxalic acid production as a function of pH, temperature and substrate

3.2 Discussion

The term optimization has been commonly used as a means of deciphering best conditions with which to apply to procedure that produces best possible outcome (Bezerra *et al*., 2008). The choice of central composite design in this work was based on the fact that it has been shown to be appropriate for fitting complex surfaces when a second order model is chosen and it reduces the number of experimental runs in a complete three-level factorial design involving more than two variables (Bezerra *et al*., 2008, Betiku *et al*., 2016). The design method chosen in this work does not agree with that of Adesina *et al*., 2014 who used the Box-behnken design (BBD) to optimize process variables of oxalic acid production from sweet potato hydrozylate. However, the design method used in this **work was**work was employed by Betiku *et al*., 2016 in the production of oxalic from cashew apple juice; Senthikumar *et al*., 2005 in the optimization of xylanase production Muthvelayhudam and Viruthagiri (2010) on cellulose production from agricultural waste.

Comparison of the experimental and predicted values indicated that there is an excellent agreement between the predicted and experimental data as represented in Table 1. Analysis of variance (ANOVA) showed that the resultant quadratic polynomial model adequately represented the experimental data with the coefficients of multiple determinations (R^2) for the response is 0.968. This implies that 96.8% of variations of oxalic acid produced is attributable to independent factors while only 3.2% of the total variations are not described by the model (Betiku and Adesina, 2013, Betiku and Taiwo, 2015). This further indicated that the quadratic model obtained was adequate to describe the influence of the independent variables (pH, temperature and substrate) on the response (oxalic acid) as shown in Table 3. The adjusted R^2 of 0.9449 shows the model is significant. In addition, Joglekar and May, 1987 notes that R^2 of a model should be ≥80% for the good fit of the model. The model f-value of 41.51 with its corresponding low p-value ($p<0.0001$) implied the significance of the model obtained (Dhillon *et al*., 2011). The predicted conditions of pH 6.838, temperature 35^0C and substrate algal biomass and sucrose with oxalic acid production of 8.618 g/L 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.

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 sequential optimization of oxalic acid (g/l) was studied using CCD of design of experiments. Results show that Substrate (X_3) and the square of pH (X_1^2) and temperature (X_2^2) has significant effect on oxalic acid production. These variables were optimized using CCD and second order polynomial model, well fitted to represent the effect of these three variables on oxalic acid (g/l) production. The experimental oxalic acid production of 91.9% shows a good agreement with predicted values with a high degree of accuracy ($R^2 = 0.96$) of the model. The proposed optimum condition for the production of oxalic acid is pH 6.838, temperature 35^0C and substrate Algal biomass-sucrose using RSM. The oxalic acid (g/l) produced using this optimized conditions is 8.618.

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