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Response Surface Methodology for the Optimization of Kojic Acid Production by Aspergillus Flavus using Muntingia Calabura Fruits as a Carbon Source

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Abstract

The current study focused on the optimization of kojic acid production by a soil isolated fungal organism *Aspergillus flavus* through Surface Fermentation (SF) by utilizing a novel carbon source i.e. ripened fruits of *Muntingia calabura* L. Initially the most significant 7 physico-chemical factors were studied for the optimization process through One-Factor-At-A-Time Method (OFAT). Among these 5 influential factors were screened and five-factor-three-level Central Composite Design (CCD) matrix and Response Surface Methodology (RSM) was performed to enhance the production rate of kojic acid. Through one-factor-at-a-time method the optimal values were Substrate concentration 100g/L, Peptone concentration 4g/L, KH_2PO4 concentration 2g/L, $MgSO_4$ concentration 0.7g/L, pH 6.0, Time 28d and Temperature 30^0C and the maximum kojic acid production was 85.1g/L. The maximum production obtained at the predicted optimal conditions through response surface methodology was Substrate concentration 100g/L, Peptone concentration 4g/L, pH 6.0, Time 28d and Temperature 29^0C and the maximum kojic acid production was 88.8 g/L. These results directs to the success of the model in improving a process for the production of kojic acid, a significant organic acid with vast industrial importance.

Keywords: Kojic Acid, *Muntingia calabura* L, One-Factor-at-a-Time Method, Optimization, Response Surface Methodology

1. Introduction

From the past 30 years it was observed that, various fungal species and few specific bacteria were used for the production of an important secondary metabolite and a natural organic acid kojic acid. It has a powerful anti-oxidizing activity and used widely as preservative and flavor enhancer. Kojic acid along with the combination of vitamin 'C' was utilized as an anti-browning agent in food stuffs especially sea foods and fruits. It functions as bleaching and anti-oxidizing factor in cosmetic products. Although it is recently used for the manufacture of cosmetic products there are so many advantages roles of kojic acid in various industries. Different types of microbial genera like Aspergillus and Pencillium were capable of producing kojic acid by utilizing various industries.

Different types of microbial genera like *Aspergillus* and *Pencillium* were capable of producing kojic acid by utilizing various sources of carbohydrates under aerobic conditions. Kojic acid can also be obtained through bacterial fermentation like *Acetobacter* from carbohydrates. The biosynthesis of kojic acid through fermentation by *Aspergillus* often involves the direct conversion of glucose through multistep enzymatic reactions1. Until 2003, the optimization of medium composition and cultural conditions for the kojic acid production was not discovered[2–4]. But during the year 2006, El-Aasar[5] first reported the optimum cultural conditions for the production of kojic acid. The physical and nutritional parameters for the optimization of kojic acid production in the present study was done by utilizing a non-statistical method One-Factor-At-A-Time Method (OFAT) and a statistical design

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model called Response Surface Methodology (RSM). RSM is a sequential procedure with an initial objective to lead the experiments rapidly and efficiency to the general vicinity of the optimum. This method of optimization i.e., RSM is preferred because it can simultaneously consider several factors at many different levels and corresponding interactions among these factors using a small number of observations. During downstream processing of kojic acid fermentation, it produces pale white yellow colored needles often soluble in water, ethanol, acetone or ethyl acetate.

2. Materials and Methods

2.1 Microorganisms

Aspergillus flavus isolated from soil using serial dilution technique was used for the optimization. The isolate was maintained on CZA slants at 4^0C.

2.2 Fermentation Media and Kojic Acid Production:

Surface fermentation was done with the production medium containing 50g of M. calabura fruits, Peptone 0.5g, KH_2PO4 0.5g and $MgSO_4.7H_2O$ 0.25g was added to 250ml conical flask. After addition of 5ml of spore suspension, the flask was incubated at 25^0C for 12d. After completion of the fermentation, filtration was done and the mycelia dry weight was estimated. The supernatant was subjected to Bentley's colorimetric method[6] and crystallization[7]. Finally the weight of the crystal mass was determined.

2.3 Optimization by OFAT Strategy

Through OFAT method different physico-chemical parameters which influence the kojic acid production were tested. The parameters tested were Substrate concentration (10g/L – 100g/L), Peptone concentration (1 – 5g/L), KH_2PO_4 (0.5 – 2.5g/L), $MgSO_4$ concentration (0.1 – 0.9g/L), pH (4 – 8), Time (11 – 37d) and Temperature (20 – 35^0C). The screened optimized fermentation conditions obtained by OFAT method were further optimized by central composite design and RSM analysis.

2.4 Central Composite Design Matrix

The process parameters substrate concentration, peptone concentration, pH, time and temperature were selected as significant variables for CCD analysis while the optimum phosphate concentration by OFAT method was kept constant throughout the experiment. Based on the following equation, the testing variables were coded and represented in Table 1.

The kojic acid concentration (g/L) with 32 experimental runs comprising different combinations of five variables was shown in Table 2. Minitab version 16.0 was used for the statistical analysis. For calculating the predicted response for the model terms the second order polynomial equation was used.

ANOVA was employed for determination of significant variables. ANOVA implicated Fischer's test to estimate the overall significance of the model, associated 'p' values and determination coefficient R^2 is used to determine the regression model's goodness of fit. Then fitted polynomial equation was furthermore represented in terms of 3D and contour plots where the interactions can be shown in the form of graphs. At last, the model was also validated.

3. Results and Discussion

The preliminary studies of the present research was conducted using one-factor-at-a-time technique and the results revealed that the optimum process parameters Substrate concentration 100g/L, Peptone concentration 4g/L, KH_2PO_4 concentration 2g/L, $MgSO_4$ concentration 0.7g/L, pH 6.0, Time 28d and Temperature 30^0C causes the maximum production of kojic acid of 85.1g/L. Among these the phosphate concentration was kept constant and the remaining five variables were screened to statistical optimization through RSM to study the combined effects of the factors on the response. Table 2. depicted the experimental and predicted values of kojic acid concentrations in 32 experimental runs with various combinations of the factors. The highest production obtained at Run 32 in the predicted response and the optimal conditions were Substrate concentration 100g/L, Peptone concentration 4g/L, pH 6.0, Time 28d and Temperature 29^0C and the maximum kojic acid production was 88.8 g/L. The second order regression equation presents the concentration of kojic acid as the function of five variables substrate concentration, peptone concentration, pH, time and temperature which can be expressed as a coded terms in the following equation.

$$Y = 10904.3 + 788.5 x_1 + 28.3 x_2 - 36.3 x_3 + 401.3 x_4 - 1106.4 x_5 + 2.5 x_1 x_2 - 0.1 x_1 x3 - 0.4 x_1 x_4 - 1.4 x_1 x_5 + 0.1 x_2 x_3 - 0.6 x_2 x_4 - 0.6 x_2 x_5 + 0.2 x_3 x_4 + 0.7 x_3 x_5 + 1.3 x_4 x_5 + 41.8 x_{12} + 9.7 x_{22} - 0.9 x32 + 31.6 x_{42} - 20.0 x_{52}$$

Table 4 showed the ANOVA for the response surface. The 'F' value for the model is 2.98 and it is greater than probability 'p' value 0.03 proves that the model terms are significant. The results of multiple regression analysis in Table 3 reveals that the model terms x_1, x_1^2 and x_4^2 were significant for the production of kojic acid. The calculated determination coefficient R^2 for the kojic acid production was 84.43%. R^2 was generally used to measure the goodness-of-fit of the model. The R^2 value implies that 84.43% of the variability in the response could be explained by the model and only15.57% of the variation was not explained. The adj R^2 value was 56.13%.

Table 1. Independent variables in the experimental plan

Independent Variables	Symbols	Coded Levels -1 0 +1		
Substrate Concentration (g/100ml)	X_1	9	10	11
Peptone Concentration (g/L)	X_2	3	4	5
Incubation time (d)	X_3	27	28	29
pH	X_4	5	6	7
Temperature (^{0}C)	X_5	29	30	31

Table 1. CCD matrix having real values along with the experimental and predicted values of kojic acid concentration

Run Order	Substrate Concentration	Peptone Concentration	Incubation Time	pH	Temperature	Experimental Values	Predicted Values
1	9	3	27	7	27	11.69	9.293
2	11	4	28	6	28	18.42	28.544
3	9	5	29	7	27	9.58	2.703
4	10	4	28	6	28	86.75	69.052
5	10	4	28	6	28	86.75	69.052
6	10	4	28	5	28	42.06	39.448
7	11	3	29	5	29	6.38	4.381
8	11	5	27	5	29	15.15	19.786
9	10	3	28	6	28	40.5	58.082
10	10	5	28	6	28	51.64	60.604
11	11	5	27	7	27	12.94	12.407
12	9	5	27	5	27	5.16	7.812
13	10	4	28	6	28	86.75	69.052
14	11	3	27	5	27	4.91	7.272
15	10	4	28	6	28	86.75	69.052
16	11	5	29	7	29	13.06	8.167

17	9	4	28	6	28	9.49	25.912
18	10	4	28	6	27	71.06	89.121
19	11	3	29	7	27	7.65	0.483
20	11	3	27	7	29	5.53	5.117
21	9	3	27	5	29	10.16	12.931
22	10	4	29	6	28	31.5	67.002
23	9	5	29	5	29	4.47	2.761
24	10	4	28	6	28	86.75	69.052
25	9	3	29	7	29	3.52	-3.237
26	11	5	29	5	27	9.42	7.302
27	10	4	28	6	28	86.75	69.052
28	9	3	29	5	27	12.04	8.057
29	10	4	28	7	28	6.31	35.469
30	9	5	27	7	29	3.66	3.537
31	10	4	27	6	28	81.9	72.944
32	10	4	28	6	29	80.4	88.886

Table 3. Model coefficients estimated by multiple linear regression (significance of regression coefficients)

Term	Coefficient	Standard Error Coefficient	T-value	P-value
Constant	10904.3	14066.6	0.775	0.455
Substrate Concentration	788.5	362.6	2.175	0.052
Peptone Concentration	28.3	257.0	0.110	0.914
Incubation Time	-36.3	814.9	-0.045	0.965
pH	401.3	285.7	1.405	0.188
Temperature	-1106.4	814.9	-1.358	0.202
Substrate Concentration*Substrate Concentration	-41.8	14.2	-2.939	0.013
Peptone*Peptone	-9.7	14.2	-0.682	0.509
Incubation Time*Incubation Time	0.9	14.2	0.065	0.950
pH*pH	-31.6	14.2	-2.220	0.048
Temperature*Temperature	20.0	14.2	1.402	0.188
Substrate Concentration *Peptone Concentration	2.5	5.6	0.455	0.658
Substrate Concentration *Incubation Time	-0.1	5.6	-0.011	0.992
Substrate Concentration *pH	0.4	5.6	0.075	0.942
Substrate Concentration *Temperature	1.4	5.6	0.245	0.811
Peptone Concentration *Incubation Time	0.1	5.6	0.026	0.980
Peptone Concentration *pH	0.6	5.6	0.114	0.912
Peptone Concentration *Temperature	0.6	5.6	0.111	0.913
Incubation Time*pH	0.2	5.6	0.034	0.973
Incubation Time*Temperature	-0.7	5.6	-0.124	0.904
pH*Temperature	-1.3	5.6	-0.232	0.821

Table 4. ANOVA for the entire quadratic model

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	20	29724.0	29724	1486.20	2.98	0.033
Linear	5	290.2	4095.1	819.01	1.64	0.228
Square	5	29249.9	29249.9	5849.98	11.74	0.000
Interaction	10	184.0	184.0	18.40	0.04	1.000
Residual Error	11	5480.4	5480.4	498.22		
Lack-of-Fit	6	5480.4	5480.4	913.40	*	*
Pure Error	5	0.0	0.0	0.00		
Total	31	35204.4				

R-Sq: 84.43%, R-Sq (pred): 0.00%, R-Sq (adj): 56.13%
DF: Degree of Freedom, SS: sum of squares.

3.1 Response Surface Plots

The 3D graphs and contour plots describe the interaction effects occur in between the physicochemical factors with relation to the kojic acid production. The 3D response surface plot of kojic acid production against substrate concentration and peptone concentration Figure 1(a) showed the kojic acid production increases with increase in the substrate concentration (10g/L) and reached a maximum of 60g/L. In the mid-value region of peptone concentration of 4g/L, Figure 1(b) represents the surface plot for the interaction between pH and peptone concentration. The concentration of kojic acid increases 60g/L at the mid-value at pH 6.0. The interaction between the substrate concentration and pH Figure 1(c) depicted that while the mid-value of substrate continue to give maximum production, high pH 6.0 was required to produce maximum yield 60g/L. The concentration of kojic acid reached to maximum 80g/L at the mid-value of substrate concentration with increase in incubation temperature 28^{0}C Figure 1(d). In order to validate the experimental model, surface fermentation in triplicates was performed under optimal operation conditions and kojic acid production obtained was 88.8 g/L which indicates that 3% increase in the production yield was observed with the statistical optimization strategy. The experimental values were compared with the actual predicted values obtained by regression model which proved the satisfactory validity of the model.

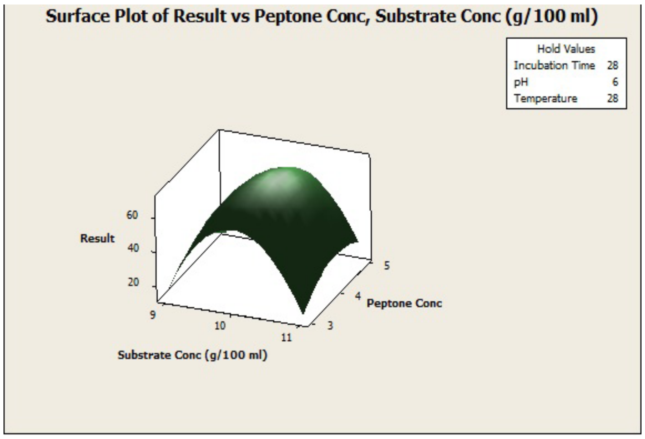

Figure 1(a). Response surface plot for the concentration of kojic acid versus peptone and substrate concentration (g/100ml).

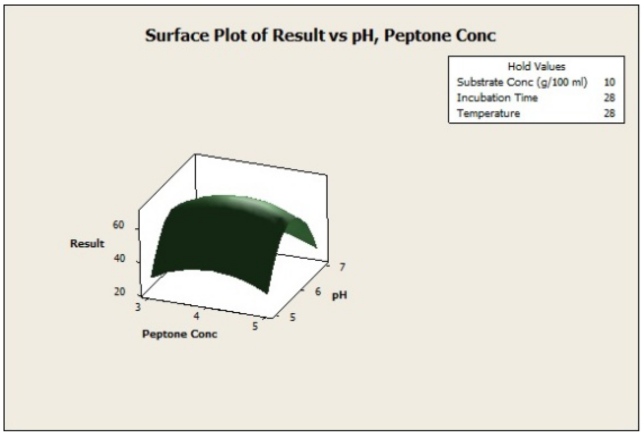

Figure 1(b). Response surface plot for the concentration of kojic acid versus pH and peptone concentration.

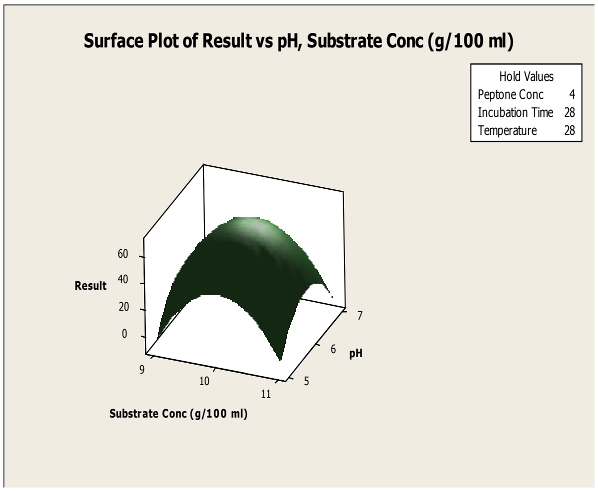

Figure 1(c). Response surface plot for the concentration of kojic acid versus pH and substrate concentration (g/100ml)

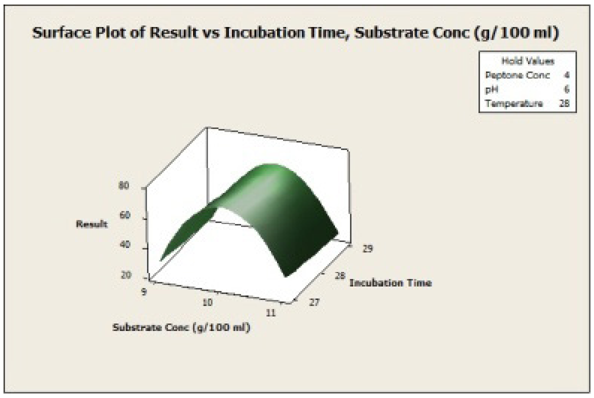

Figure 1(d). Response surface plot for the concentration of kojic acid versus Incubation time and substrate concentration (g/100ml).

At the end of the fermentation, when the concentrated fermented broth was subjected to crystallization it yields 24.3g/L of dry kojic acid crystals. X-ray crystallography was used to determine the structure of kojic acid crystal. XRD diffractometer (Shimadzu, Tokyo, Japan) was used for the analysis with in 2θ angle 70^0 with Cu Kα radiation. The kojic acid X-ray diffraction spectrum was shown in the Figure 2. The spectrum reveals that seven distinctive peaks were appeared at 2θ angles 8^0, 19.27^0, 21.71^0, 27.73^0, 31.15^0, 36.13^0, 39.09^0. The highest appeared at 19.27^0 with a count number 649.92

4. Conclusion

The present research was aimed to enhance the yield of kojic acid by employing a combinational optimization methodology i.e. OFAT and RSM. From the results it was To the top of our data, this was the first report on kojic acid production by using a novel and inexpensive carbon source Muntingia calabura fruits by fermentation with a soil isolate and the study will be useful for the large-scale fermentation process.

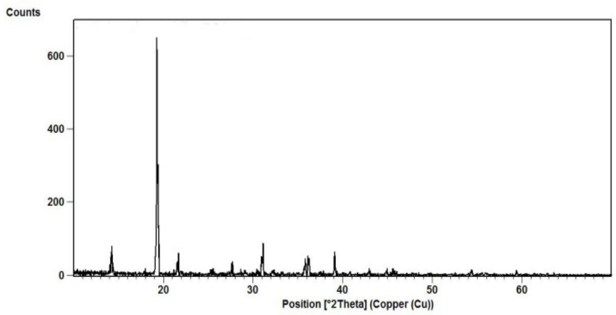

Figure 2. X-ray crystallography of kojic acid crystal.

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