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Statistical Optimization of Kojic Acid Production through Response Surface Methodology by *Aspergillus Flavus* using Sago Starch Hydrolysate as a Carbon Source

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ABSTRACT

Optimization of process conditions to achieve maximum yield of kojic acid was carried out with a soil isolated fungal culture *Aspergillus flavus*. Five physicochemical factors which significantly influence the production were screened using a non-statistical One-factor-at-a-time method (OFAT) and later statistical Central Composite Design (CCD) and Response surface methodology (RSM) were performed for designing of the experiment, analysis of results to study the interaction among the parameters. The maximum production of 82.6g/L of kojic acid was noticed at optimized conditions of Carbon source concentration i.e., starch hydrolysate of Sago starch 100ml, Peptone concentration 4g/L, KH_2PO_4 concentration 1g/L, $MgSO_4$ concentration 0.5g/L, pH 6.0, Time 28d and Temperature 28^{0}C with OFAT method whereas the production yield was enhanced significantly to 90.8g/L with response surface methodology. It was estimated from the model that the determination co-efficient was (R^2=0.9561) represented that 95.61% of the variability in the response could be interpreted by the model and the results showed an excellent adequacy of the multiple-regression model. During the last phase of the kojic acid fermentation, the entire fermented broth was subjected to evaporation followed by crystallization and the purity of the crystal was confirmed by X-ray crystallography.

Key words: Optimization, kojic acid, *Aspergillus flavus*, One-factor-at-a-time method, Response surface methodology

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INTRODUCTION

The kojic acid production through fermentation has become a remarkable prospect in the field of industrial microbiology since 1955. Interest in production of kojic acid was largely due to its wide applications in food, pharmaceutical, cosmetics, medical and chemical industries etc., Nearly 58 diverse strains of kojic acid producing microorganisms like *Aspergillus*, *Penicillium* have been used (El-Aziz, 2013) and it was reported recently that even some plants like *Kingella africana* can also produce kojic acid Eyong, et al. (2012). Optimization of process parameters is the most valuable step in industrial production methodology, during which even a slight progress may lead to success in the fermentation process commercially. Variation in the Carbon source concentration, Nitrogen concentration, Mineral salt concentration, pH, Time, Temperature shows an immense effect on the production of kojic acid. The fermentation medium composition had main effect on the production of kojic acid and generally differs for each and every microorganism and nearly 30-40% is the estimated cost of the production medium in the total production cost of a fermentation process. So it is essential to optimize the nutrient composition and fermentation conditions accordingly. The present research efforts on the optimization of the medium constituents and process conditions by a soil isolated fungal microorganism *Aspergillus flavus* and the following steps are involved in the potent statistical optimization of kojic acid production. 1. Screening of process parameters through OFAT strategy that effect the production of kojic acid. 2. Optimization of most significant factors or variables by using CCD matrix. 3. The developed model is validated underneath the optimized conditions. Though there was an available literature in using sago starch as a carbon source Rosfarizan, et al. (2002) the present research differs in using sago starch hydrolysate as a carbon source instead of using sago starch directly as a substrate and optimization of process conditions was done with a combinational statistical and non-statistical approach.

MATERIALS AND METHOD

Microorganism

Aspergillus flavus producing kojic acid was isolated using serial dilution method from the soil sample. The cultural identification is done by Lactophenol cotton blue staining through needle mount technique. The organism is cultivated at 30^{0}C in Czapek-dox agar (CZA) medium for one week and later maintained on CZA slants at 4^{0}C. The inoculum was prepared by transferring the isolate from a slant into a 250ml conical flask containing 50ml of Ariff's medium Ariff, et al. (1996). These seed cultures are cultivated at 30^{0}C in an incubator.

Kojic acid fermentation

In order to perform starch hydrolysis, 100g of sago starch powder was weighed and then 1L of 0.02M sodium phosphate buffer solution of pH 6.9 was added then the contents were thoroughly mixed. Then the beaker was subjected to starch gelatinization procedure by keeping it in shaking boiling water bath (Remi instruments Ltd.,) for 3 hrs to prevent lump formation. After that, the liquefaction was performed with the α-amylase enzyme (purchased from Coastal Chemical Enterprises Ltd., Visakhapatnam) at a concentration of 9.0KNU/100g suspension. The contents are incubated at 30^{0}C. The hydrolysis is performed for 4-5 hrs. Kojic acid fermentation is done with the production medium containing 50ml of starch hydrolysate, Peptone 0.5g, KH_2PO_4 0.5g and $MgSO_4.7H_2O$ 0.25g is added to 250ml conical flask. Now 5ml of spore suspension at a concentration of 1x10^7 prepared with Tween 80 solution is added and the flask is incubated at 25^{0}C for 12d. When fermentation was finished, filtration is done and the mycelia dry weight is estimated. The supernatant is subjected to Bentley's colorimetric method (Bentley, 1957) and crystallization (Hazzaa, 2013). At the end, weight of the crystal mass is determined.

Optimization by OFAT strategy

The following physicochemical parameters effecting the kojic acid production are examined for optimization with OFAT method. The parameters include Substrate concentration (10ml – 100ml), 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). Among these, the most significant factors which influence the production are further optimized by central composite design and RSM analysis.

CCD and RSM

CCD and RSM is used to optimize the five important variables for increasing the kojic acid production rate which is examined at three different coded levels (Table I) and thirty two experimental runs are performed (Table II). The Minitab version 16.0 is employed for the result analysis of the experimental design. By using second order polynomial equation (Equation 1), the effect of individual variable, their interactions and statistical analysis to calculate predicted responses are interpreted.

$$Y = b_o + \sum b_i X_i + \sum b_i{}^2 X_i{}^2 + \sum b_{ij} X_i X_j \qquad - \qquad (1)$$

Y is the concentration of kojic acid (g/L)

b_o is the intercept

b_i is the coefficient for linear direct effect

$b_i{}^2$ is the coefficient for quadratic effect and is responsible for curvatures of the model

b_{ij} is the coefficient for interaction effect a positive (or) negative significant value indicates possible interactions between the variables.

For validation of the experimental model, the fermentation is carried out at the optimized conditions, for verifying the results from the response surface.

Table I: Independent variables in the experimental plan

Independent		**Coded levels**		
Variables	Symbols	**-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	27	28	29

Result and Discussion

A non-statistical procedure and a statistical based experimental methodology are used cooperatively for the optimization of medium constituents and cultural conditions for getting opulent yields of kojic acid. A group of experiments are performed serially to study the effect of physical factors pH, Time, Temperature and chemical factors like Carbon source concentration, Nitrogen source concentration, KH_2PO_4 and $MgSO_4$ concentration on kojic acid production by *A.flavus* with OFAT experiments. It is observed that the production is higher at carbon source concentration 100ml (71.2g/L), peptone concentration 4g/L (82.6g/L), KH_2PO_4 1g/L (4.62g/L), $MgSO_4$ concentration 0.5g/L

(12.05g/L), pH 6.0 (50.6g/L), Time 28d (66.8g/L) and Temperature 28^{0}C (52.9g/L). The kojic acid yield obtained in a conical flask when these optimized conditions were maintained is 78.9g/L through colorimetric method. From the OFAT results it was found out that five variables Carbon source concentration, Peptone concentration, pH, Time, Temperature plays a significant role in the kojic acid production. Hence, these variables are chosen for further optimization studies by RSM using 5-factor-3-level-CCD. Hence, a set of thirty two experiments were done with different combinations of five variables Table II. The highest production 90.8g/L was observed at Run 2. The predicted response was 82.142g/L. A second order polynomial function was fitted to the experimental kojic acid production may results in the generation of the above regression equation in terms of actual factors.

$Y = -1469.08 + 97.29\, x_1 + 211.10\, x_2 - 357.31\, x_3 + 220.47 x_4 + 354.73\, x_5 + 0.85 x_1 x_2 + 1.73 x_1 x_3 + 2.82\, x_1 x_4 + 0.80 x_1 x_5 + 0.50 x_2 x_3 - 1.63\, x_2 x_4 + 1.53 x_2 x_5 + 0.28\, x_3 x_4 - 0.02 x_3 x_5 - 3.65 x_4 x_5 + 0.33\, x_1^2 + 25.83 x_2^2 - 6.62 x_3^2 + 26.38 x_4^2 + 6.48 x_5^2$

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

Run order	Substrate Conc.	Peptone Conc.	Incubation Time	pH	Temperature	Experimental values	Predicted values
1	9	3	27	7	27	11.58	8.969
2	11	4	28	6	28	90.8	82.142
3	9	5	29	7	27	9.61	6.759
4	10	4	28	6	28	82.6	76.073
5	10	4	28	6	28	82.6	76.073
6	10	4	28	5	28	60.5	57.552
7	11	3	29	5	29	41.2	43.176
8	11	5	27	5	29	28.3	29.338
9	10	3	28	6	28	53.7	56.240
10	10	5	28	6	28	37	44.251
11	11	5	27	7	27	14.3	14.488
12	9	5	27	5	27	16.49	15.251
13	10	4	28	6	28	82.6	76.073
14	11	3	27	5	27	49.2	51.938
15	10	4	28	6	28	82.6	76.073
16	11	5	29	7	29	17.9	17.326
17	9	4	28	6	28	50.9	69.349
18	10	4	28	6	27	68.7	69.151
19	11	3	29	7	27	15.6	16.726
20	11	3	27	7	29	29.4	29.066
21	9	3	27	5	29	30.1	28.339
22	10	4	29	6	28	79.8	83.299
23	9	5	29	5	29	14.5	12.499
24	10	4	28	6	28	82.6	76.073
25	9	3	29	7	29	29.4	26.027
26	11	5	29	5	27	36.1	38.598
27	10	4	28	6	28	82.6	76.073
28	9	3	29	5	27	33.5	33.199
29	10	4	28	7	28	29.1	41.839
30	9	5	27	7	29	11.58	7.269
31	10	4	27	6	28	75.8	82.092
32	10	4	28	6	29	60.7	70.040

The F-value of the model is 10.87 and the probability value (P ~ 0) indicates the significant nature of the model. Subsequent ANOVA analysis and generation of a regression equation implies that the R^2 value of 95.18% (R^2adj: 86.43%) assured an adequate adjustment of the quadratic model to the experimental data Table IV. The Lack of fit F-value is not evident, and the adequate precision value, 10.0 specifies that an adequate signal to noise ratio. As the precision value is greater than 4.0 so the model could be used to navigate the design space. The estimated regression coefficients for the model terms are represented in Table III which revealed that, two interaction or cross product term (Peptone concentration*Peptone concentration and pH*pH) showed significant effect on kojic acid production ($p<0.05$).

Table III: Model coefficients estimated by multiple linear regressions (significance of regression coefficients)

Term	Coefficient	Standard error coefficient	T-value	P-value
Constant	-1469.08	6342.88	-0.232	0.821
Substrate Conc. (g/100ml)	97.29	163.49	0.595	0.564
Peptone Conc. (g/L)	211.10	115.88	1.822	0.096
Incubation time (d)	-357.31	367.46	-0.972	0.352
pH	220.47	128.82	1.711	0.115
Temperature (^{0}C)	354.73	367.46	0.965	0.355
Substrate conc*Substrate conc	-0.33	6.42	-0.051	0.960
Peptone*Peptone	-25.83	6.42	-4.025	0.002
Incubation time*Incubation time	6.62	6.42	1.032	0.324
pH*pH	-26.38	6.42	-4.111	0.002
Temperature*Temperature	-6.48	6.42	-1.010	0.334
Substrate conc*Peptone Conc.	0.85	2.52	0.338	0.742
Substrate conc*Incubation time	-1.73	2.52	-0.687	0.506
Substrate conc*pH	-2.82	2.52	-1.122	0.286
Substrate conc*Temperature	-0.80	2.52	-0.318	0.756
Peptone Conc.*Incubation time	0.50	2.52	0.199	0.846
Peptone Conc.*pH	1.63	2.52	0.646	0.531
Peptone Conc.*Temperature	-1.53	2.52	-0.607	0.556
Incubation time*pH	0.28	2.52	0.110	0.914
Incubation time*Temperature	0.02	2.52	0.009	0.993
pH*Temperature	3.65	2.52	1.450	0.175

Table IV: ANOVA for the entire quadratic model

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	20	22020.6	22020.6	1101.0	10.87	0.000
Linear	5	2504.5	979.7	195.94	1.93	0.168
Square	5	19021.0	19021.0	3804.20	37.550	0.00
Interaction	10	495.1	495.1	49.51	0.49	0.865
Residual Error	11	1114.3	1114.3	101.30		
Lack-of-Fit	6	1114.3	1114.3	185.72	*	*
Pure Error	5	0.0	0.0	0.00		
Total	31	23134.9				
R-Sq:95.18%, R-Sq (pred): 0.00%, R-Sq (adj): 86.43% **DF:** Degree of freedom, SS: sum of squares						

Response surface plots: 3D response surface plots aided in recognizing the main and the interaction effects of five factors. The plots were illustrated with pair wise combination of five different variables, whereas the rest are held at middle level. Figure 1 shows the kojic acid production as a result of interaction between Temperature and pH with substrate concentration 10g/100ml, peptone concentration 4g/L and incubation time 28d respectively. The production was increased with increase in pH to 5.0 and temperature to 28^{0}C and later decreased. The similar fashion was also exhibited in Figure 2, when peptone concentration increases to 4g/L and Temperature to 28^{0}C the production enhances significantly. Figure 3 showed the interaction between pH and peptone concentration. The kojic acid production reached maximum of 80g/L at the mid-value of peptone concentration and 4g/L with increase in pH 6.0. A verification experiment in triplicates was done to validate the statistical results using predicted optimal medium in conical flasks under static conditions. The experimental yield 90.8g/L was in reasonable agreement with the predicted yield 82.14g/L. Finally from the results of combinational approach it was assessed that, the optimized medium which can yield maximum production was Substrate concentration 110ml, peptone concentration 4g/L, KH_2PO_4 1g/L, $MgSO_4$ concentration 0.5g/L, pH 6.0, Time 28d and Temperature 28^{0}C. After crystallization of fermented broth, 21g/L of dry crystals were produced. The X-ray diffraction spectrum of kojic acid crystal shows seven characteristic peaks at 2θ angles of 9^0, 22^0, 26^0, 31^0, 33^0,38^0 and 42^0 (Figure 4).

Figure 1: Response surface plot for kojic acid production versus Temperature and pH

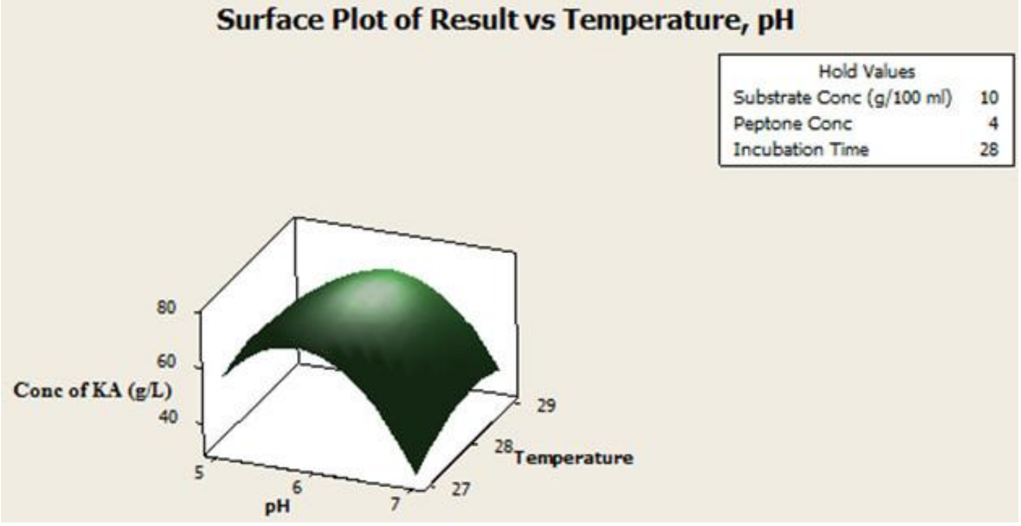


Figure 2: Response surface plot for kojic acid production versus Temperature and Peptone concentration

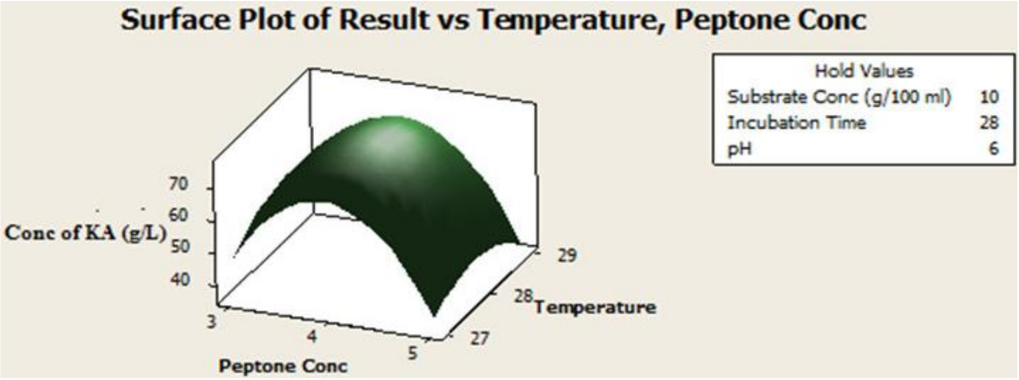


Figure 3: Response surface plot for kojic acid production versus pH and Peptone concentration

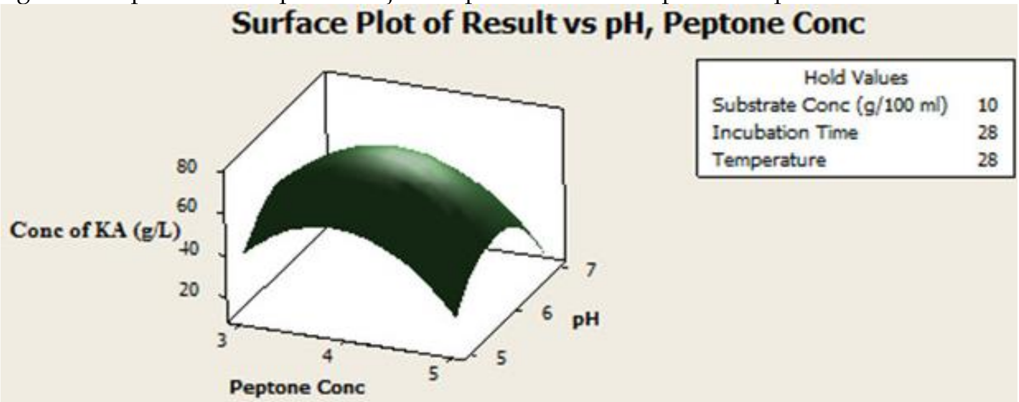
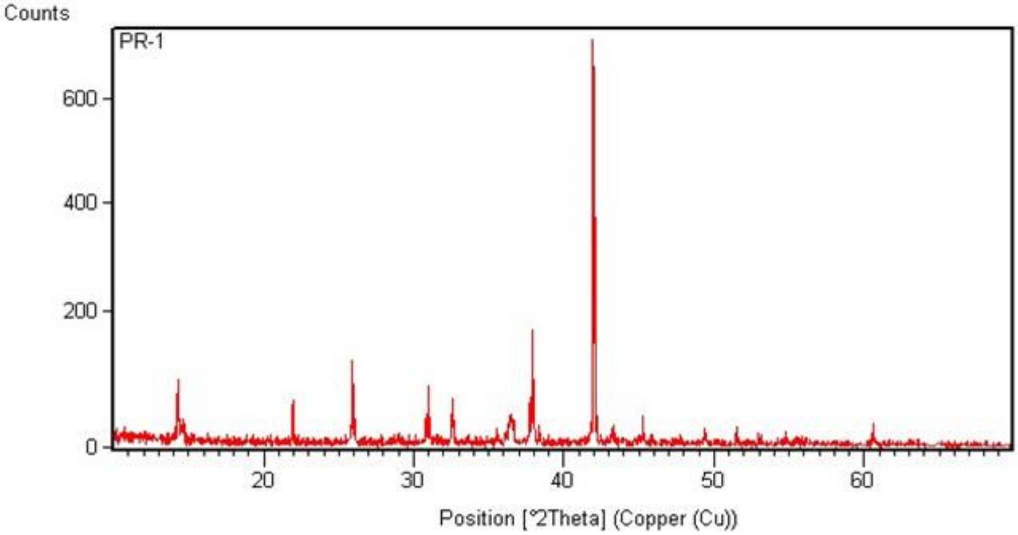


Figure 4: X-ray crystallography of kojic acid crystals



CONCLUSION

Response surface statistical analysis used in the current research had proved that, optimization of kojic acid production by *A.flavus* resulted in the significant enhancement of yield by 12% higher than the result obtained by one-factor-at-a-time traditional method. The carbon source used is economical, soil isolated culture and cost-effective down-stream processing. The optimized parameters determined by the study may really helpful for large-scale production of kojic acid.

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The authors declare "No conflict of interest".

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