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Statistical optimization of process parameters for the production of citric acid from oil palm empty fruit bunches

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In this study, optimization of process parameters such as moisture content, incubation temperature and initial pH (fixed) for the improvement of citric acid production from oil palm empty fruit bunches through solid state bioconversion was carried out using traditional one-factor-at-a-time (OFAT) method and response surface methodology (RSM). The possible optimum level of moisture content, incubation temperature, and initial pH were found from the OFAT study to be 70%, 30 - 32°C and 5.5 - 8, respectively. The optimum moisture content of 70.3% (v/w) and incubation temperature of 33.1°C with initial pH of 6.5 gave the maximum production of citric acid (369.16 g/kg of dry EFB). The analysis of variance (ANOVA) of the statistical optimization using central composite design showed that moisture content ($p < 0.001$) and incubation temperature ($p < 0.0001$) as well as the interaction of these two parameters were highly significant for the citric acid production.

Key words: citric acid, process condition, optimization, solid state bioconversion, *Aspergillus niger* and central composite design (CCD).

INTRODUCTION

Citric acid (2-hydroxy-1,2,3-propanetricarboxylic acid) is extensively produced by *Aspergillus niger* and widely used in food, beverage, cosmetic, pharmaceutical, chemical, textile and electroplating industry (Crolla and Kennedy, 2001; Lofty et al., 2007). Currently, it is commercially produced by submerged (liquid state) fermentation (SmF) of starch or sucrose based media (sucrose or glucose syrups) using *A. niger* (Lofty et al., 2007; Barrington and Kim, 2008). However, the global demand of citric acid is growing faster than its production implying that more economical process is required. Solid state bioconversion (SSB) involves the growth of microorganisms on moist substrates in the absence or near-

absence of free flowing water and the solid substrate acts as a source of carbon, nitrogen, minerals and carrier necessary for microbial growth (Chahal, 1985; Robinson et al., 2001). As the microorganisms in a solid substrate are growing under the conditions similar to their natural habitat, they can produce certain enzymes, metabolites, proteins and spores more efficiently than in submerged (liquid state) fermentation (Ellaiah et al., 2004).

The major physico-chemical parameters which influence the growth of *A. niger* and its production of citric acid on a solid substrate are nutrient sources, moisture content, particle sizes, incubation temperature, pH and inoculum density (Lee and Yun, 1999; Ellaiah et al., 2004; Lofty et al., 2007). The accumulation of citric acid is strongly affected by fermentation conditions and higher production of citric acid was recorded by the optimization of fermentation process conditions besides optimization of media, (Lofty et al., 2007).

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Few researchers have optimized the fermentation conditions for citric acid production in liquid state fermentation by using statistical design (Lofty et al., 2007). However, so far our knowledge goes, there is a lack of information on optimum fermentation conditions for citric acid production through solid state bioconversion in the literature. Kumar et al. (2003b) only observed the effect of moisture content for the production of citric acid from sugarcane bagasse in solid state fermentation. Several researchers have maintained fermentation temperature at 30°C for citric acid production by solid state fermentation (Hang and Woodams, 2001; Shojaosadati and Babaeipour, 2002; Kim and Barrington, 2003). Roukas (1999) studied the individual effect of moisture content, initial pH and incubation temperature on citric acid production by solid state fermentation from carob pod. However, statistical approach, which introduces the interactive effects among the parameters, is the prime option for the optimization of fermentation process parameters.

The common parameters for submerged and solid state fermentation are initial pH and incubation temperature. Furthermore, moisture content is also important parameter for solid state fermentation. In the present study, firstly the OFAT method was used to find out the possible optimum level of the factors followed by central composite design (CCD) to develop a model which helped to determine the optimum process parameters for citric acid production from EFB through solid state bioconversion.

MATERIALS AND METHODS

Major substrate and microorganisms

The major substrate oil palm empty fruit bunches (EFB) was collected from Seri Ulu Langat Palm Oil Mill in Dengkil, Selangor, Malaysia and stored in a cold room at 4°C to avoid the unwanted bio-degradation by any microorganisms. The EFB samples were prepared by grinding to 0.5 mm d particle size and below after washing vigorously with tap water and drying at 105°C for 24 h. The ground EFB was dried at 60°C for 48 h to get constant dry weight for the experimental study.

A local isolate of *A. niger* IBO-103 MNB (IMI396649) identified by the microbial identification organization, CABI Europ-UK, was selected for this study. This strain was obtained through series experiments of isolation, purification and screening against the citric acid production by using EFB as new substrate. The fungal strain was maintained on 3.9% w/v of potato dextrose agar (PDA, Mark) slants, sub-cultured once in a month and stored at 4°C.

Preparation of inoculum

The cultures were grown on PDA plates at 32°C for 4 days and washed with 25 ml sterilized distilled water to prepare the inoculum. Spore suspension was collected in 100 ml Erlenmeyer flask by filtering with Whatman No. 1 filter paper. A haemocytometer was used to maintain the spores density of 1×10^8 spores/ml.

Experimental procedure for solid state bioconversion

Bioconversion experiment was carried out in 250 ml Erlenmeyer flasks. Twenty grams of total substrate (wet basis) was prepared with required percentage of major substrate – EFB (particle size ≤ 0.5 mm) to maintain 30% solid substrate with 6.4% (w/w) sugar (sucrose), 2% methanol and 9% mineral solution containing 0.09 g/l $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g/l $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.4 g/l MnSO_4 and 5 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, which was optimized by previous study (Bari et al., 2009). Moisture content was adjusted with mineral solution, inoculum, methanol and distilled water. Methanol and inoculum were added after sterilization of media by autoclaving at 121°C for 15 min.

Harvesting and extraction of citric acid

Harvesting and extraction of citric acid was carried out after 6 days of bioconversion. Fifty milliliter (50 ml) distilled water was added to the fermented substrate and mixed with spatula thoroughly to dilute properly. Diluted media were shaken for 1 h at 150 rpm at room temperature ($28 \pm 1^\circ\text{C}$) in a rotary shaker (Tran et al., 1998). The supernatant was collected by filtering with Whatman no. 1 filter paper and immediately analyzed to determine the content of citric acid.

Determination of citric acid

The concentration of citric acid in extract was determined by Waters HPLC instrument equipped with a refractive index detector (RID), Shodex RSpak KC 811 column (inner dia. 8×300 mm, Shodex, Japan). The eluent used for this analysis was 0.1% phosphoric acid solution. HPLC analysis was carried out under the following operation conditions: pump flow, 1 ml/min; column temperature, 40°C; sample volume 5 μl ; integration method and peak area. Concentrations were automatically calculated by Breeze software (version 3.3), Waters Corporation, U.S.A. The production of citric acid was expressed as g/kg of dry solid substrate (EFB).

Experimental design

Experimental design for the optimization by one-factor-at-a-time method

The influence of moisture content, incubation temperature and initial pH were investigated with the traditional “one-factor-at-a-time” method for the approximate optimization. Moisture content was varied as 50, 60, 70, 80 and 90% of total substrate; incubation temperature was varied as 25, 28, 30, 32 and 35°C and initial pH was varied as 3, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8, 9, 10 and 11.

The central composite design

The central composite design (CCD) under response surface methodology (RSM) was employed in order to illustrate the nature of the response surface in the experimental region and to elucidate the optimal concentrations of the most significant independent variables. Two variables namely, moisture content and incubation temperature were included in this model. However, initial pH was not included in the CCD design due to the effect of pH on citric acid production within the range of 5.5 to 8 was found very less by the OFAT method. The factors were examined at five different levels (relatively low, low, basal, high, relatively high) coded (-2, -1, 0, +1,

Table 1. The experimental and predicted citric acid production by CCD.

Run	Moisture content (%v/w), A	Incubation temp. (°C), B	Citric acid productivity (g/kg of dry EFB)	
			Experimental	Predicted
1	70 (0)	32 (0)	364.61	365.386
2	78 (+1)	36 (+1)	246.24	248.25
3	82 (+2)	32 (0)	188.90	186.06
4	70 (0)	38 (+2)	274.33	275.51
5	58 (-2)	32 (0)	149.26	156.14
6	78 (+1)	28 (-1)	201.30	202.06
7	70 (0)	32 (0)	371.40	365.38
8	70 (0)	32 (0)	363.14	365.38
9	62 (-1)	28 (-1)	163.10	156.56
10	70 (0)	32 (0)	365.81	365.38
11	70 (0)	32 (0)	360.91	365.38
12	62 (-2)	36 (+1)	259.14	253.85
13	70 (0)	26 (-2)	165.05	167.90

+ 2) as shown in Table 1. According to the CCD for two variables, 13 experimental runs with 5 runs at center point were executed and their observations were fitted to the following second order polynomial model:

$$Y = \beta_0 + \beta_1A + \beta_2B + \beta_{11}A^2 + \beta_{22}B^2 + \beta_{12}AB \quad (1)$$

where, Y is the dependent variable (citric acid productivity); A and B are the independent variables (moisture content and incubation temperature); β_0 is the regression coefficient at center point; β_1 and β_2 are the linear coefficients; β_{11} and β_{22} are the quadratic coefficients and β_{12} is the second order interaction coefficient.

The developed regression model was evaluated by analyzing the values of regression coefficients, ANOVA (analysis of variance), *p*-values and *F*-values. The quality of fit of the polynomial model equation was expressed by the coefficient of determination, R^2 . The statistical software package Design-Expert[®]6.0.8 (Stat Ease Inc., Minneapolis, USA) was used to generate a regression model to predict the effect of the operating parameters on citric acid production. A final experiment was designed to validate the CCD model prediction.

RESULTS AND DISCUSSION

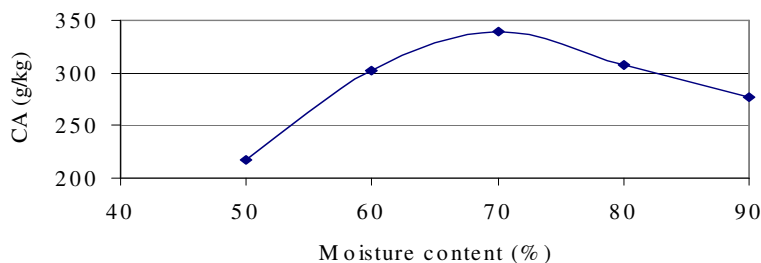
Traditional one-factor-at-a-time method

The purpose of this study was to determine the suitable levels of the parameters that would result in the highest citric acid production. Possible optimum level of moisture content of the media, incubation temperature and initial pH were optimized by one-factor-at-a-time method. The production of citric acid with the variation of moisture content is presented in Figure 1(a). The highest production of citric acid of 339.14 g/kg of dry EFB was found with 70% moisture content which was 56.5 and 22.6% higher than the production obtained with the low (50%) and with high (90%) moisture content. It was

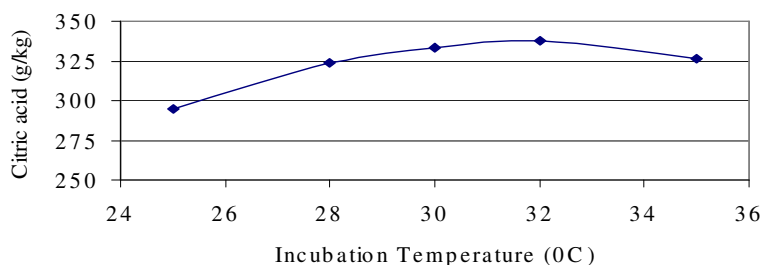
observed that high and low level of moisture content decreased the production of citric acid. The reason might be that these conditions do not provide the natural habitat which is unfavorable for citric acid production (Ellaiah et al., 2004). Moreover, the inhibitory effect might be due to the reason of lower moisture content which reduces mass transfer to the cell and increases osmotic pressure while higher moisture content reduces the inter-particle space (Khosravi-Darani and Zoghi, 2008). Thus, the optimum level of moisture content necessary to enhance the production was done by applying statistical optimization technique with the interaction of another parameter.

The incubation temperature for bioconversion was examined at different levels ranging from 25 to 35°C. The highest production of citric acid of 337.86 g/kg of dry EFB was found at 32°C incubation temperature while other parameters, moisture content and initial pH were 70 and 5.5%, respectively. The production of citric acid increased gradually until the temperature reached 32°C and then reduced slightly at 35°C. Figure 1(b) shows that the variation of citric acid production with temperature within the ranges was minimal. This result is supported by similar studies done by Szewczyk and Myszk (1994) and Khosravi-Darani and Zoghi (2008), who found that the temperature did not strongly affect the growth rate in SSF in the range of 28-34°C. Therefore, it was postulated that the optimum temperature might be around 32°C.

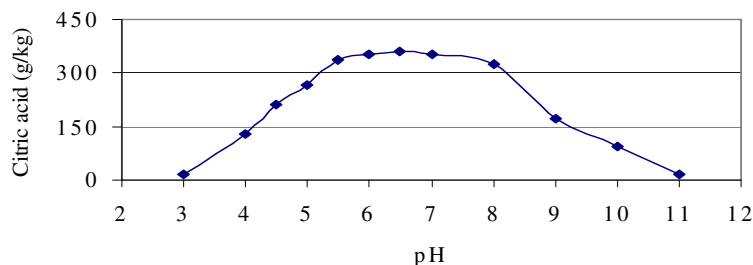
On account of the effect of pH on citric acid production, different random levels of initial pH as 3, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8, 9, 10 and 11 were investigated. The effect of initial pH on citric acid production is shown in Figure 1(c). The highest level of citric acid production was obtained at pH of 6.5. The production was increased up to 6.5, while any further increase and/or decrease of pH showed an inhibitory effect. It is also observed from Figure 1(c) that



(a) pH and incubation temperature were maintained 5.5 and 32°C, respectively



(b) pH and moisture content were maintained 5.5 and 70% (v/w), respectively



(c) Incubation temperature and moisture content were maintained 32°C and 70% (v/w), respectively

Figure 1. Production of citric acid with the variation of (a) moisture content, (b) incubation temperature and (c) initial pH when parameters were fixed as: moisture content, 70% (v/w); incubation temperature, 32°C and initial pH, 5.5.

the production of citric acid below and above the pH level of 5 and 8 decreased significantly and citric acid production was almost steady within the pH from 5.5 to 8. The production of citric acid decreased considerably at pH lower than 5 and higher than 8 due to its inhibitory effects on spore germination and cell growth (Papagianni, 1995). Several researchers also reported that the optimum level of pH was within the range of 5 to 8 (Tran et al., 1998; Roukas, 1999; Lofty et al., 2007; Khosravi-Darani and Zoghi, 2008).

Optimization of process conditions by the CCD

The process conditions of solid state bioconversion such as moisture content and incubation temperature as independent variables were optimized for maximum produc-

tion of citric acid from EFB. Another parameter (pH) was not included in this statistical optimization because of its effects which varied in the range from 5.5 to 8 was observed to be steady in terms of citric acid production in the one-factor-at-a-time study. Independent variables were investigated at five levels according to the CCD (Box and Draper, 1987). The design matrix of the coded and actual values of the variables together with the experimental and predicted (using Equation 2) results for citric acid production are presented in Table 1. Experiments were carried out as per the design, and the cultures were performed in triplicates and the average of the obtained citric acid after 6 days of bioconversion was used.

For predicting the optimal point mathematically within the experimental constraints, a second order polynomial model was fitted to the experimental results of citric acid

Table 2. Analysis of variance (ANOVA) for response surface quadratic model.

Source	Sum of Squares	DF	Mean sq.	F-value	P-value > F
Model	94063	5	18813	651	< 0.0001**
Moisture content (%v/w), A	845	1	845	29	0.0010**
Incubation temp. (°C), B	10937	1	10937	379	< 0.0001**
A ²	61763	1	61763	2138	< 0.0001**
B ²	33775	1	33775	1169	< 0.0001**
AB	653	1	653	23	0.0021**
R-square	0.9979				
Adj R-square	0.9963				
Adequate Precision	57.304				

** P < 0.01 indicate the model terms are highly significant.

productivity by applying Design Expert software.

$$Y = -11600.53 + 202.90A + 292.32B - 1.35A^2 - 3.99 B^2 - 0.40AB \quad (2)$$

The regression equation for the optimization of medium constituents showed that citric acid production (Y, g/kg of dry EFB) is a function of the moisture content (A, v/w) and the incubation temperature (B, °C).

A second order quadratic model was developed with the effect of linear, quadratic and interactive terms on the response. It was necessary to perform test for significance of the regression model, test for significance on individual model coefficients and test for lack of fit. At the model level, the correlation measures for the estimation of the regression equation are the multiple correlation coefficient, *R* and the determination coefficient, *R*². The closer the value of *R* to 1, the better the prediction of the model is.

In this study, the value of *R* and *R*² were 0.9989 and 0.9979 respectively for the citric acid production. This value indicates a high degree of correlation between the experimental and the predicted values. The value of *R*² indicates that 99.79% of the variables: moisture content and incubation temperature were supported by the response. The value of *R*² is also a measure of fit of the model and it can be mentioned that only 0.21% of the total variations are not explained by the citric acid productivity.

The value of the adjusted coefficient of determination was also very high (99.63%) to indicate a high significance of the model (Khuri and Cornell, 1987; Alam et al., 2008). The adjusted *R*² value is particularly useful when comparing models with different number of terms. This comparison was done in the background when model reduction was taking place. Adequate precision (signal to noise ratio) compares the range of the predicted values at the design points to the average prediction errors. The value of adequate precision (signal to noise ratio) of

57.30 is very high compared to desirable value (greater than 4) which indicates this model can be used to navigate the design space.

It is required to test the significance and adequacy of the model through analysis of variance. The Fisher variance ratio, the *F*-value ($=S_r^2/S_e^2$), is a statistically valid measure of how well the factors describe the variation in the data about its mean. The greater the *F*-value is from unity, the more certain it is that the factors explain adequately the variation in the data about its mean, and the estimated factor effects are real. The corresponding analysis of variance (ANOVA) is presented in Table 2. The ANOVA of quadratic regression model demonstrated the model was highly significant, as an evident from the Fisher's *F*-test with a very low probability value ($P_{\text{model}} > F = 0.0001$).

It can be seen that the linear term, incubation temperature (B) and square term of moisture content (A²) and incubation temperature (B²) were the most significant effect over the other model term as p-value <0.0001. Furthermore, the *p*-value of the linear term, moisture content (A) was lower than 0.01 indicated the significance at 99% confident interval. The interactive terms between moisture content and incubation temperature (AB) shown in the ANOVA analysis was also highly significant (*p*<0.01) at 99% confidence level. Linear, quadratic and interactive effects of parameters were significant, meaning that they could act as limiting conditions and little variation in their magnitude would alter either growth rate or the product formation rate or both to a considerable extent (Imandi et al., 2008). From these observations, it could be concluded that interactive effects are important for true optimization rather than the OFAT method (Kumar and Satyanarayana, 2007).

Along with the significance of all model terms, lack of fit of the model was found to be insignificant which is strongly desirable for the adequate model. The lack of fit test denotes whether the selected model is adequate to describe the observed data or a more complicated model

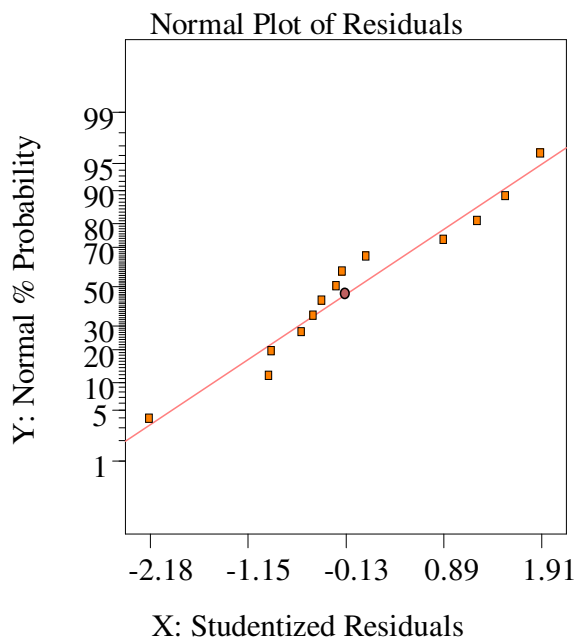


Figure 2. Normal probability plot for the residuals from the citric acid production model.

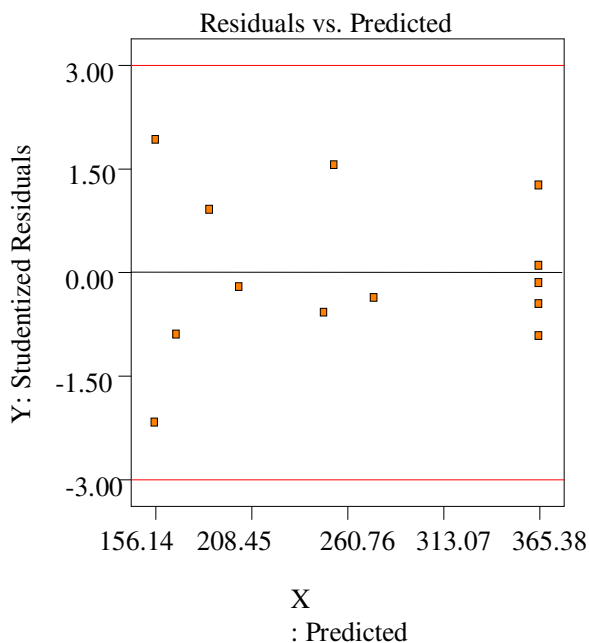


Figure 3. Plots of the residuals vs predicted response for citric acid production.

is required to be used. This test is performed by comparing the variability of the current model residuals to the variability between observations at replicate settings of the factors. Since the *p*-value for lack of fit in ANOVA is

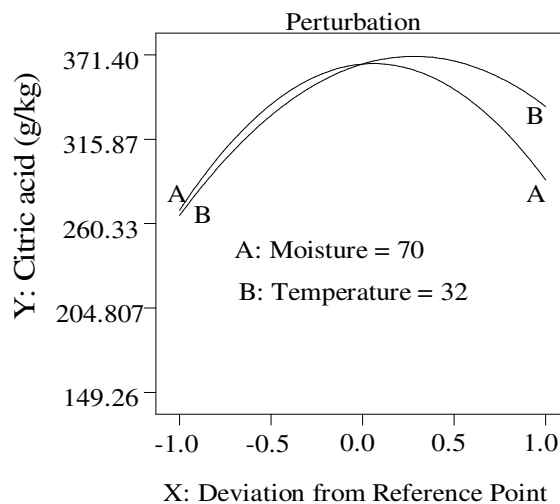


Figure 4. Perturbation plot for citric acid production.

greater than 0.05, the model appears to be adequate for the observed data at 95% confidence interval.

The normal probability plot of residuals and the plot of residuals versus predicted values of the response for citric acid production are shown in Figures 2 and 3 respectively. The result in Figure 2 indicates that the residuals can be considered to fall on a straight line implying that the errors follow a normal distribution. The data in the graph follow the line representing a normal distribution and support the assumptions of the empirical model. Furthermore, Figure 3 indicates a constant variance. The equal scatter of the residual data above and below the x-axis indicates that the variance was independent of the value of the citric acid which again supports the assumptions of the model. In a similar manner, the plot in Figure 3 revealed no unusual structure. It can be concluded from these analyses that the proposed model is adequate and there is no reason to infer any violation of the independence or constant variance assumption.

Figure 4 shows the trace or perturbation plot. The perturbation plot compares the effects of the various factors in the design space. The intersection of the lines is at the reference point (where, $X = 0.00$) and the actual conditions for the factors at the said point are as indicated in the Figure 4. In case of factor A, for instance, as it moves to the right of the reference point i.e. towards the +1.00 of the deviation from the reference point axis, the production of citric acid decreases. The opposite phenomenon is observed when it moves to the left of the reference point. Similar observation can be made for parameter B. This was also been observed in the prediction model of Equation 2.

The 3D response surface and 2D contour plots are the graphical representation of the regression equation in order to determine the optimum values of the variables

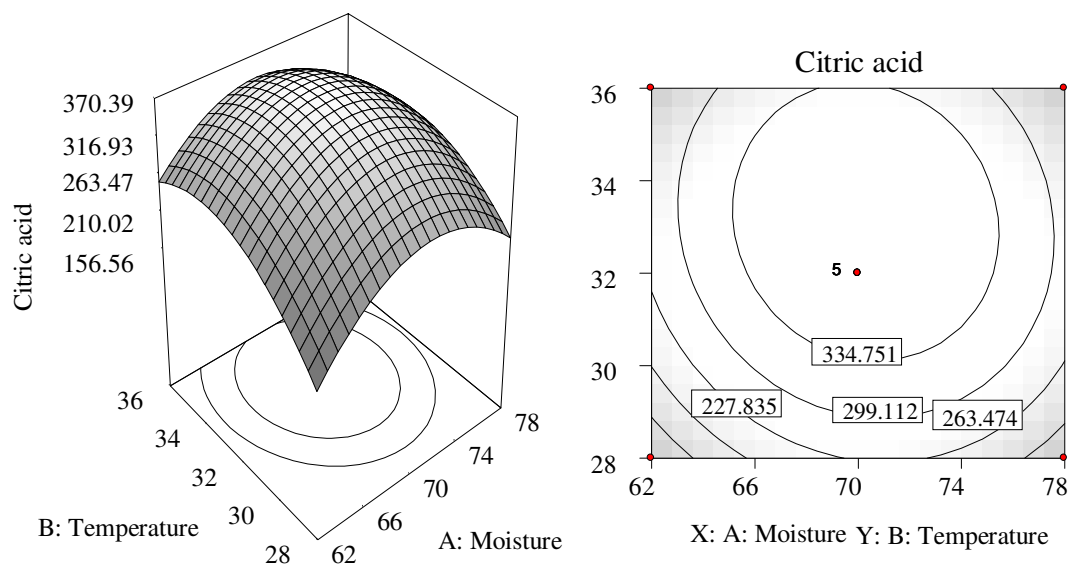


Figure 5. 3D response surface and 2D contour plots shows the effect of moisture content (% v/w) and incubation temperature ($^{\circ}\text{C}$) on the production of citric acid (g/kg of dry EFB).

within the ranges considered (Tanyildizi et al., 2005). The 3D and 2D plots for the interaction between two variables are presented in Figure 5. The principal target of response surface is to hunt efficiently for the optimum values of the variables such that the response is maximized (Tanyildizi et al., 2005). Each contour curve represents an infinite number of combinations of two test variables. The maximum predicted value is identified by the surface confined in the smallest ellipse in the contour diagram. Elliptical contours are obtained when there is a perfect interaction between the independent variables (Muralidhar et al., 2001).

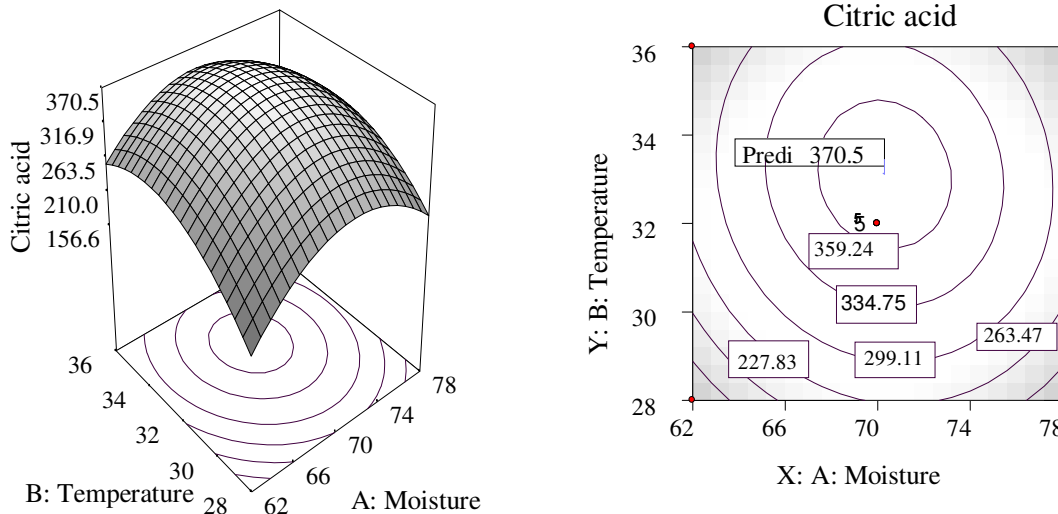
An elliptical response surface in the entire region was found from the second order quadratic equation for citric acid production with interaction of moisture content and incubation temperature (Figure 5). Plot shows that citric acid production is considerably affected by varying the moisture content and incubation temperature. The maximum production was obtained at the point of intersection of major and minor axes of the ellipse. The maximum production of citric acid was predicted at given ranges of both moisture content and incubation temperature (Figure 5). The production decreased at the maximum and minimum values of ranges considered in both parameters. About 370.3 g citric acid /kg of dry EFB was obtained from the response surface as maximum production at the moisture content level of about 70% (v/w) and incubation temperature of 33°C .

Only one numerical solution suggested by using Design Expert software within the experimental range of parameters for the maximum production of citric acid at the desirability level of 99.6%. The numerical solution of

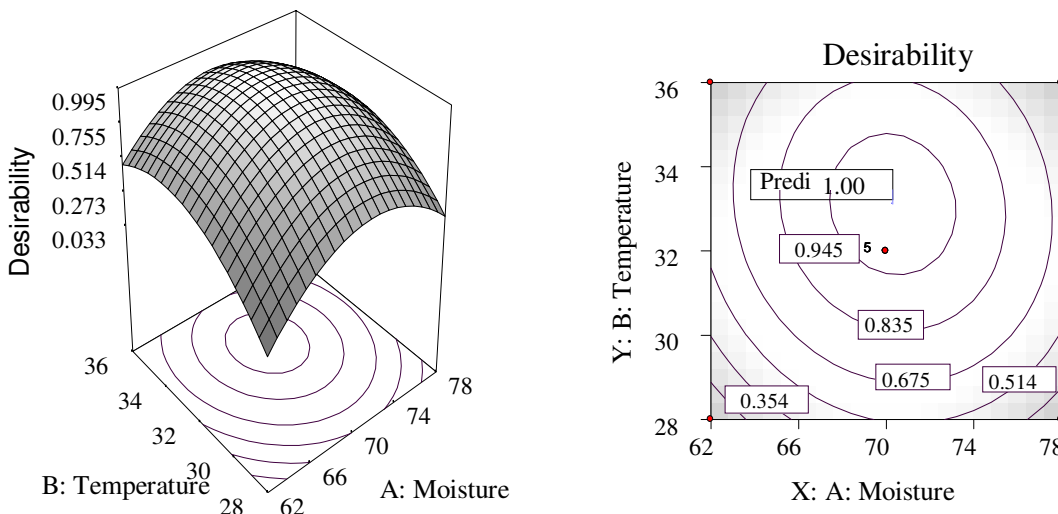
the developed model predicts the highest production of citric acid of 370.5 g/kg of dry EFB at 70.3% (v/w) moisture content and 33.1°C incubation temperature. The suggested single solution revealed that the parameters were optimized at a single point which is at the center of the plot that can be explained by the Figure 6 (a). The desirability (0.996) of the solution for the maximum production is also at the center of the plot (Figure 6 b).

Lofty et al. (2007) has statistically optimized the level of initial pH and incubation temperature at 4 and 31.5°C , respectively for the production of citric acid in liquid state by using CCD. Kumar et al. (2003a) obtained maximum production of citric acid from pineapple waste with 70% moisture content in presence of methanol using solid state fermentation. Roukas (1999) obtained the highest production of citric acid from carob pod by solid state fermentation at the moisture content of 65%. He also found the initial pH level of 6.5 and incubation temperature of 30°C for the maximum citric acid production. Tran and Mitchell (1995) reported that a maximum citric acid concentration was obtained from pineapple waste at a moisture level of 70% when *A. foetidus* ACM 3996 was grown in solid state fermentation. The optimum process conditions of this study agree with those of researchers who studied the effect of moisture content, initial pH and incubation temperature. On the other hand these results difference from Lofty et al. (2007) might be due to the different state of fermentation, substrate, media composition and strain used.

In order to verify the optimization results and to validate the developed second order quadratic model, an experiment was performed according to the process conditions



(a) Maximum predicted production of citric acid from the numerical solution



(b) Desirability of solution for the maximum predicted production of citric acid

Figure 6. 3D response surface and 2D contour plots shows the numerical solution for the maximum production of citric acid with the ranges of moisture content and incubation temperature at highest desirability, (a) maximum predicted production of citric acid from the numerical solution and (b) desirability of solution for the maximum predicted production of citric acid.

presented in Table 3. From the experiment, the highest citric acid of 369.16 g/kg of dry EFB was obtained in optimum conditions, which is slightly less (0.36%) than the predicted value. The effect of initial pH was checked once again varying from 5.5 to 8 at an interval of 0.5 maintaining the optimum level of moisture content and incubation temperature.

The literature showed that highest production of citric

acid of 603.5 ± 30.9 g/kg of dry corn cobs was observed by using high activity of commercial enzyme, Rapidase Pomaliq (Gist-Brocades) through liquid state fermentation (Hang and Woodams, 2001). The second highest citric acid production of 354.8 g/kg dry peat moss (DPM) was achieved mention through solid state fermentation with moisture content of 80%, initial pH of 8 and incubation temperature of 35°C (Barrington and Kim, 2008). Other

Table 3. Validation of developed quadratic model and optimum process conditions.

Run	Moisture content (%v/w), A	Incubation temp. (°C), B	Citric acid productivity (g/kg of dry EFB)		
			Predicted	Experimental	Error (%)
1	70.3	33.1	370.50	369.16	-0.36
2	65	28	217.70	223.63	+2.70
3	70	32	365.37	364.18	-0.33
4	75	30	307.97	310.11	+0.70
5	80	34	236.91	238.37	+0.62

higher yields of citric acid are 269 g/kg of dry cassava bagasse (Prado et al., 2005), 264 g/kg of dry carob pod (Roukas, 1999), 259 ±10 g/kg of dry matter of corn husks (Hang and Woodams, 2000) and 202 g/kg of dry sugarcane bagasse (Kumar et al., 2003b). Therefore, it is observed from the above discussion that a significant amount of citric acid production was achieved from EFB through solid state bioconversion with optimum of process conditions.

Conclusions

The possible optimum level of initial pH, moisture content and incubation temperature were determined with the OFAT method. The optimum level of initial pH of substrate was optimized at 6.5 from the result of the OFAT method. The CCD under the RSM was employed for further optimization of moisture content and incubation temperature through the development of second order regression model. The high adequacy of the developed second order regression model was proven by fitting the experimental and predicted values. The variables were tested for the correlation between their level and the production of citric acid, and both variables showed a significant influence on the production. The significant interacting effect between the variables proved the superiority of the RSM over the traditional the OFAT method. The predicted maximum production of citric acid from EFB was validated to be 369.16 g/kg of dry EFB at the optimum process conditions of moisture content, 70.3% (v/w); incubation temperature, 33.1°C and initial pH, 6.5.

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