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A MODELING STUDY BY RESPONSE SURFACE METHODOLOGY ON THE CULTURE PARAMETERS OPTIMIZATION OF CITRIC ACID BIOPRODUCTION FROM SWEET POTATO PEEL

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ABSTRACT

The study investigated the use of sweet potato peel hydrolysate (SPPH) as a potential substrate for citric acid (CA) production. Effect of methanol on the synthesis of CA was initially investigated. The fermentation process involved was optimized by the application of response surface methodology (RSM) to determine the effect of SPPH concentration, methanol concentration, and time, and their reciprocal interactions on CA amount produced. The highest CA concentration was observed on the 4th day of fermentation and the medium induced with methanol produced higher amount of CA (16 g/l) than medium without methanol supplementation (11.37 g/l). The results obtained for the optimization studies showed that a statistically significant ($p<0.05$) quadratic model best described the fermentation process. The highest CA predicted using the quadratic model obtained was 15.97 g/l at the optimal condition of SPPH concentration of 150 g/l, time of 3.61 days, and methanol concentration of 3 volume %. Using these optimal condition values in setting up three independent replicates, an average experimental value of 15.98 g/l was obtained, which was well within the range predicted by the model. This work demonstrated that SPPH could serve as an inexpensive and novel feedstock for CA bioproduction.

Keywords: *sweet potato peel, optimization, response surface methodology, methanol, Aspergillus niger, citric acid.*

1. INTRODUCTION

Citric acid (CA) is a weak organic acid with multiple uses in various industries. It is used in the food, beverage, pharmaceutical, chemical, cosmetic and other industries for applications such as acidulation, antioxidation, flavour enhancement, preservation, plasticizer and as a synergistic agent (Kirimura et al., 1992; Shankaranand and Losane, 1993; Suzuki et al., 1996). The global market for CA is expanding every year at the rate of 5% per year, and its current production is about 1.7 million tons per year as estimated by Business Communications Co. (http://www.bccresearch.com). In recent times, high energy, coupled with raw material costs, has squeezed CA production into an unprofitable market. The search for economical substrates as an alternative to high cost substrates is vital to reduce the production cost of CA. In recent years, considerable interest has been focused on agricultural and industrial wastes for CA production (Dhillon et al., 2011), as this will serve to solve wastes management problem, which arise from disposal of such wastes, and also to serve as a source of income for such industries. Due to the increasing demand for CA, there is need for alternative fermentation processes using cheap raw materials, such as sweet potato peel through surface fermentation.

Sweet potato (*Ipomoea batatas*) is a dicotyledonous plant that belongs to the family *Convolvulaceae*. Its large, starchy, sweet-tasting, tuberous roots are an important root vegetable. The young leaves and shoots are sometimes eaten as greens. Of the approximately 50 genera and more than 1,000 species of *Convolvulaceae*, *Ipomoea batatas* is the only crop of major importance, some others are used locally, but many are actually poisonous. China accounts for 75-80% of worldwide sweet potato production with an annual production of 78.8 Tg followed by Nigeria with about 3.3 Tg. Since sweet potatoes are rich in starch (15-25%), it is expected that the peels would also possess starch, which can be hydrolysed into fermentable sugar and subsequently converted to useful products like citric acid, oxalic acid, gluconic acid, ethanol among others (Crabb and Mitchinson, 1997 and Pandey et al., 2000). Betiku et al. (2013) has optimized the enzymatic hydrolysis of sweet potato peels using statistical approach. Whereas, Yuguo et al. (1999) reported CA production from mash dried sweet potato with dregs by *A. niger* in an external-loop airlift bioreactor without process variables optimization, Betiku and Adesina (2013) produced CA from sweet potato starch hydrolysate using *A. niger* and established optimal condition of the fermentation process variables using experimental design approach.

Many studies have been carried out on the use of inducers to improve CA production. It has been reported that lower alcohols added in pure material inhibit CA production but if added into crude carbohydrates, could enhance the production. Methanol, ethanol, n-propanol, isopropanol or methylacetate neutralize the negative effect of the metals in CA production generally in amounts of about 1 to 5

volume (Kubicek and Röhr, 1986). Methanol in particular has been shown to increase CA yields in earlier reports. Concentrations between 2-3% (v/v) have been found to produce optimum results. The effects of methanol, ethanol and butanol on citric acid production were studied by Anwar et al. (2009), where maximum CA production was observed with methanol. Roukas and Kotzekidou (1987) reported that the addition of methanol at a concentration of 1.0 to 4.0 %, which resulted in a marked increase in the amount of CA produced by *A. niger* using spent grain liquor and brewery wastes. Similar positive effect of methanol on CA production was also reported by John et al. (2012). Dhillon et al. (2011) observed significant increase in CA yields when ethanol and methanol (3 % v/v) were added individually to apple pomace juice ultrafiltration sludge.

Statistical-based approach optimization, like RSM, is an established tool for overcoming the limitations of the traditional "one-factor-at-a-time" method. It is a more efficient method since it has the ability to reduce number of experimental runs needed to provide sufficient information for statistically acceptable results. Besides, it is a valuable tool for determining interactions between variables and for the prediction of optimal fermentation conditions (Anand and Venkat, 2012). This method has been applied to many fermentation processes successfully. For example, Betiku and Adesina (2013), Dhillon et al. (2011) and Anand and Venkat (2012) applied this method to CA production optimization. While Wang et al. (2011) employed the method in the optimization of ethanol from sweet sorghum juice; Naveena et al (2005) used this method in direct fermentation of starch to l(+) lactic acid in SSF by *Lactobacillus amylophilus* GV6.

This present work focused on optimization of CA production via Statistical-based approach. An attempt was made to investigate the use of sweet potato peel hydrolyzate (SPPH) as an alternative substrate to conventional carbon sources like molasses. Effect of methanol as an inducer on CA synthesis was also investigated. A mathematical model was also developed to predict the CA amount produced.

2. METHODOLOGY

2.1. Materials

2.1.1. Sweet Potato Peel Starch Preparation.

Sweet potato tubers were obtained from a market in Ile-Ife, Osun state, Nigeria. They were washed thrice in clean water to remove dirt and unwanted materials. The sweet potatoes were manually peeled, with the weight of the peels taken and recorded. The peels were then dried to constant weight by sun-drying. After drying the peels to constant weight, they were milled to flour and stored in a container for further use.

2.1.2. Enzymes

Two-stage enzymatic hydrolysis was carried out using alpha-amylase (E.C.3.2.1.1) from bacterial source (*Bacillus licheniformis*) and glucoamylase (E.C.3.2.1.3) from a fungal source (*Aspergillus niger*) were used in the liquefaction and saccharification steps, respectively, for the hydrolysis. The enzymes were obtained from Federal Institute of Industrial Research Oshodi (FIIRO), Lagos.

2.2. Methods

2.2.1. Sweet potato peel hydrolysis

For the hydrolysis of the powdered sweet potato peels, the method of Betiku et al. (2013) was employed. The powdered sweet potato peel was slurried with distilled water in appropriate proportion. In order to make 35% (w/v) of slurry, 35 g of the powder was weighed into 80 ml of a 40 ppm $CaCl_2$ solution. The pH was adjusted to 6.5 using a pH meter with citrate-phosphate buffer and the solution was made up to 100 ml. The slurried powder was gelatinized by heating the mixture to 97 °C for 10 min. Liquefaction was then carried out using the alpha-amylase, based on established conditions from the work of Betiku et al. (2013). The liquefied starch was subjected to saccharification using glucoamylase at established optimal condition (Betiku et al., 2013). After inactivation of the enzymes by heating, the final mixtures were centrifuged at 11,952 g for 15 min and the supernatant was analyzed for reducing sugar concentration, which was referred to as sweet potato peel hydrolysate (SPPH).

2.2.2. Microorganism and inoculum preparation

A pure culture of *Aspergillus niger*, the organism of choice used throughout this study, was obtained from the Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria. Stock cultures were maintained on Potato Dextrose Agar (PDA) at 30 °C for 6-7 days and the spores were stored in 4°C refrigerator. For the inocula, loopful of spores were collected from the PDA slant and aseptically transferred to 250-ml Duran flask containing 100 ml of sterile distilled water. The spore suspension was shaken for uniform distribution in an environment-controlled incubator shaker (New Brunswick Scientific Co., USA) at 5 g and 30 °C for 1 h before it was used to inoculate the medium for the fermentation.

2.2.3. Media composition

The fermentation medium used for this work was the optimized medium of Sankpal et al. (2000), which composed of 3.55 g/l $(NH_4)_2HPO_4$, 2.58 g/l KH_2PO_4, 0.10 $MgSO_4.7H_2O$ (Betiku and Adesina, 2013) and the carbon source, which was SPPH. All media and flasks were sterilized in an autoclave at 121°C for 15 min.

2.2.4. Surface fermentation procedure

Sixty milliliter of SPPH was measured into 250-ml Duran flasks and the nutrients were added appropriately. The pH of the medium was adjusted using 1 M HCl and 2 M NaOH buffer solutions. All media and flasks were sterilized in an autoclave at 121 °C for 15 min. Subsequently, 5% volume fraction of inoculum size was added aseptically to the flask, which was placed on a clean table for surface fermentation.

2.2.5. Studies on the effect of methanol as an inducer

Nine flasks containing 60 ml of the medium with 3 volume % methanol each were set up and another nine flasks containing 60 ml of the medium without methanol were also set up. The initial SPPH concentration in all the flasks was approximately 100 g/l. Daily samplings were carried out to analysis for SPPH, CA and biomass concentrations.

2.2.6. CA optimization studies

The Box-Behnken design (BBD) was employed for this work by considering SPPH, methanol concentration and fermentation time in a three-level-three-factor design, which generated 17 fermentation runs. These included 6 factorial points, 6 axial points and 5 central points to provide information regarding the interior of the experimental region, making it possible to evaluate the curvature effect. RSM was used to optimize the process and regression equation analysis was used to evaluate the response surface model. Selected process variables for the fermentation process were SPPH concentration (X_1), methanol concentration (X_2), and time (X_3). The coded independent variables levels are depicted in Table 1 while Table 2 shows the 17 experimental runs generated in terms of coded variables. The independent variables used were coded according to Eq. (1):

$$x_i = \frac{x_i - x_o}{\Delta x} \quad 1, 2, \ldots, k, \tag{1}$$

where, X_i and x_i are the actual value and coded value, respectively, X_o is the value of X_i at center point, and Δx is the step change value. To

correlate the response variable to the independent variables, multiple regressions was used to fit the coefficient of the polynomial model of the response. The quality of the fit of the model was evaluated using test of significance and analysis of variance (ANOVA) using the Design Expert 8.0.3.1 software. The fitted quadratic response model is given by Eq. 2:

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_{i<j}^{k} b_{ij} X_i X_j + e \quad (2)$$

where, Y is response variable (CA concentration), bo is the intercept value, b_i (i = 1, 2,..., k) is the first order model coefficient, b_{ij} is the interaction effect, and b_{ii} represents the quadratic coefficients of X_i, and e is the random error. Pareto chart was plotted using Statistica 12 software (StatSoft Inc., Tulsa, OK, USA). In the Pareto plot of standardized effects, the important effects are visually identified. The bars correspond to the absolute magnitudes of the estimated effect coefficients. An effect that exceeds the vertical line (p = 0.05) may be considered significant.

2.3. Analytical procedures

2.3.1. Reducing sugar assay

The dinitrosalicylic acid method described by Miller (1959) and modified DNS Reagent were used to determine the SPPH produced after hydrolysis, and to follow the SPPH utilization during fermentation. To 1 ml of the supernatant, 3 ml of the DNS solution was added to the test tube and was boiled for 15 min, cooled and diluted appropriately after which their absorbance was measured at a wavelength of 540 nm using a UV-Visible Spectrophotometer (Libra 21 Model).

2.3.2. CA analytical procedure

CA produced was determined using improved pyridine-acetic anhydride spectrophotometric method (Marier and Boulet, 1958). For the assay, 10 mL of sample was withdrawn from fermentation medium and filtered with Whatman No. 1 filter paper. Subsequently, 1 mL from the filtrate was mixed thoroughly with 100 mL of distilled water and the resulting solution was used for citric acid analysis.

Table 1: Box-Behnken design for the fermentation showing the process variables and their coded values

Variable	Symbol	Coded levels		
		-1	0	+1
SPPH (g/l)	X_1	90	120	150
Methanol (vol %)	X_2	1	2	3
Time (day)	X_3	1	4	7

2.3.3. Biomass Concentration Determination

For each sample taken, a pre-weighed filter paper was used to filter the broth. The residue was washed three times with distilled water and dried at a temperature of 120 °C for 6 h to a constant weight. Afterwards, it was allowed to cool, then it was weighed, and the final weight was recorded. The weight of the biomass was determined by subtracting the weight of the filter paper from that of filter paper and the cell mass.

3. RESULTS AND DISCUSSION

3.1. Effect of methanol as an inducer on CA production

Figure 1 shows the results of the preliminary work carried out to investigate the effect of methanol on CA production. The results showed that methanol had a positive effect on the production of CA. The CA concentration was observed to increase steadily from 1st day to 4th day after which it started to decline up to the final day of fermentation. This may be due to the depletion of the SPPH, reduction in medium nutrients, formation of other metabolites and decrease in the pH of the medium, all of which inhibits CA formation. The highest CA concentrations as observed on the 4th day of fermentation were 16 g/l and 11.37 g/l for fermentation with and without methanol supplementation, respectively. Hence, methanol amount was included in the variables investigated for the optimization studies. This observation is corroborated with the reports of several authors that methanol enhances CA synthesis (Kubicek and Röhr, 1986; Roukas and Kotzekidou, 1987; Anwar et al., 2009; Dhillon et al., 2011).

Table 2: Box-Behnken Design Showing Varied Parameters for the CA fermentation.

Run	X_1	X_2	X_3
1	0	1	1
2	0	0	0
3	-1	-1	0
4	-1	1	0
5	1	1	0
6	1	0	1
7	0	0	0
8	0	1	-1
9	0	0	0
10	0	0	1
11	-1	0	0
12	0	-1	1
13	0	-1	0
14	1	-1	-1
15	0	0	-1
16	-1	0	0
17	0	0	-1

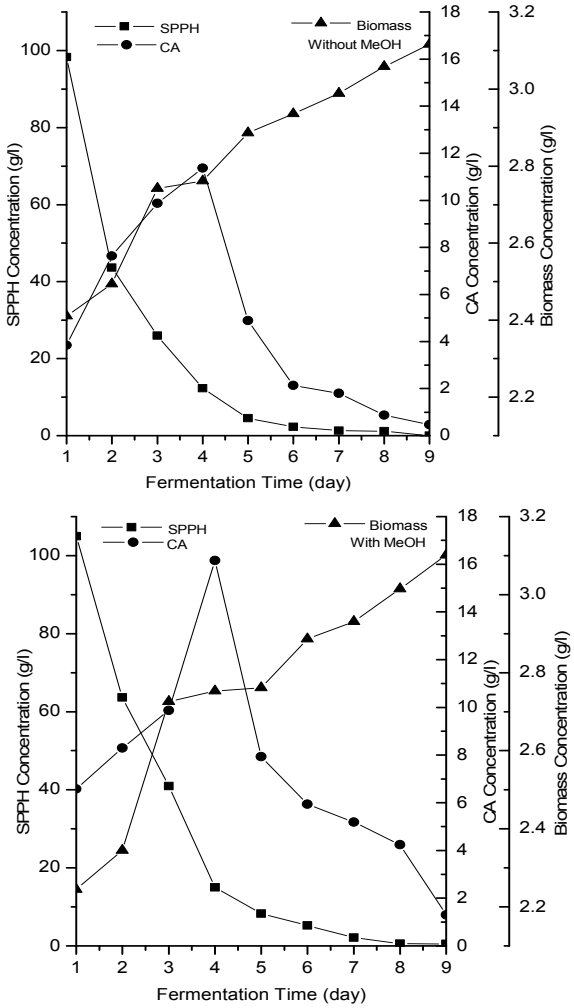

Figure 1: Plots of SPPH, CA and biomass concentrations versus fermentation time

3.2. CA Production Optimization

The results of test of significance for every regression coefficient are displayed in Table 3. The results showed that the p-values of the model terms were significant, i.e. p<0.05. In this case, the two linear terms (methanol (X2) and time (X3)), one cross-product (methanol x time (X2X3)) and the three quadratic terms (SPPH x SPPH (X12), methanol x methanol (X22) and time x time (X32)) were all remarkably significant model terms at 95% confidence level. The model F-value of 101.05 with corresponding low p-value (p<0.0001) implied the model obtained was significant. p-value indicates whether a term in a model is significant or not and the Fisher test (F-values) shows the level of significance for the model terms but they do not make a distinction between a positive and a negative significance effect on the model. Thus, the standardized effects of the SPPH, methanol and time and their interactions on the CA production were investigated by preparing the Pareto chart (Figure 2). The positive coefficients for the model terms (methanol, methanol x time and time x time) showed a favourable or synergistic effect on CA production but the negative coefficients for the model terms (time, SPPH x SPPH and methanol x methanol) indicated an unfavourable effect on CA production. The quadratic of time was the most significant effect and followed by linear time, quadratic methanol, linear methanol and quadratic SPPH (Figure 2). The data obtained fitted best to a second-order mathematical model and exhibited low standard deviation. The value of coefficient of determination (R2) gives an indication of consistency between the experimental values and the predicted values. Guan and Yao (2008) suggested that R2 should be at least 0.80 for the good fit of a model. In this case, R2 of the model obtained was 0.9924. The value obtained for the R2, indicates that the sample variation of 99.24% for the CA production was attributed to the independent variables and only 0.66% of the total variations are not explained by the model.

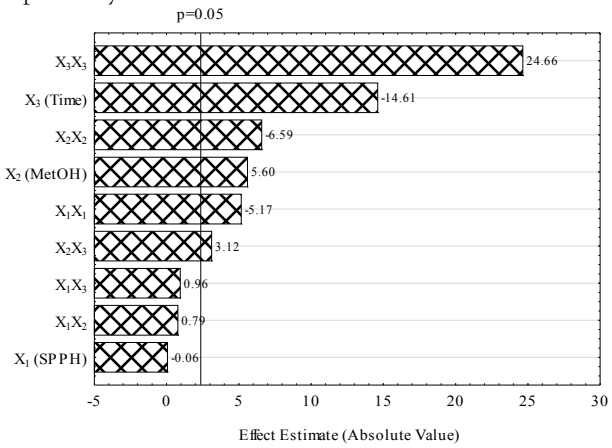

Figure 2: Standardized effects of SPPH (X_1), methanol (X_2) and time (X_3) on CA production by A. niger.

Figure 3 shows the results of experimental CA concentrations and the predicted CA concentrations of the surface fermentation by the Box-Behnken design (BBD). The figure demonstrated that there was good agreement between the predicted CA and the observed CA concentrations. Therefore, the model could be used in theoretical prediction of fermentation of SPPH for CA production. The low values of standard error observed in the intercept and model terms showed that the regression model fits the data well and that the prediction made by the software was good (Table 4). The final equation in terms of coded factors is expressed in Eq. 3. The variance inflation factor (VIF) obtained in this study showed that the center points are orthogonal to all other factors in the model.

$$Y = 11.86 - 0.011X_1 + 0.98X_2 - 2.56X_3 + 0.20X_1X_2 + 0.24X_1X_3 + 0.77X_2X_3 + 1.25X_1^2 + 1.59X_2^2 - 5.96X_3^2 \quad (3)$$

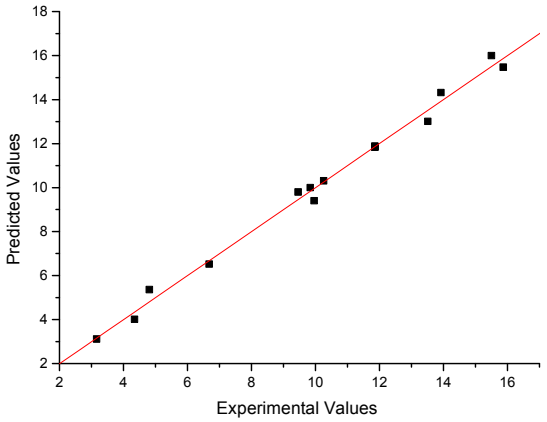

Figure 3: Plots of predicted CA concentration versus experimental CA concentration

Figure 4(a-c) shows the response surface plots representing the effect of the varied parameters on the CA concentration, and their reciprocal interactions. Figure 4a shows the response surface plots representing the effect of methanol and SPPH concentrations on CA produced while keeping fermentation time constant. The results showed that methanol increase leads to an increase in the CA concentration. The curvature nature of the surface showed that there is mutual interaction between methanol and SPPH concentrations. The response surface plot representing the effect of methanol concentration and time on CA concentration while keeping SPPH concentration constant is presented in Figure 4b. It can be seen from this plot that the CA concentration increases with fermentation time up till the 4th day, where the highest amount of CA was observed, and then it declined while the CA picked at the highest level of methanol. Figure 4c shows the response surface plot representing the effect of fermentation time and SPPH concentration and their reciprocal interaction, while keeping methanol concentration constant, on CA concentration. CA increases as methanol concentration increases. However, CA increases as the fermentation time increases up to the 4th day after which CA declines.

The Design Expert suggested a model with optimal condition of the CA fermentation process. The suggested parameters were SPPH concentration of 150g/l, time of 3.61days and methanol concentration of 3 volume %. The statistically predicted CA concentration was 15.967 g/l. To verify this prediction, the optimal condition was applied to the three independent experimental replicates and the average CA concentration obtained was 15.98 g/l. This is obviously very close to the estimated value of the model equation. Thus, the model for the CA concentration fermentation as predicted was validated.

Table 3: Analysis of Variance (ANOVA) of Regression Equation for the Fermentation Model and Parameters.

Source	Sum of Squares	Df	Mean Square	F-value	p-value
Model	224.80	9	24.9	101.05	‹0.0001
X_1	0.001013	1	0.001013	0.004109	0.9507
X_2	7.72	1	7.72	31.34	0.0008
X_3	52.58	1	52.58	213.42	‹0.0001
X_1X_2	0.15	1	0.15	0.62	0.4578
X_1X_3	0.23	1	0.23	0.92	0.3705
X_2X_3	2.40	1	2.40	9.75	0.0168
X_1^2	6.59	1	6.59	26.73	0.0013
X_2^2	10.69	1	10.69	43.68	0.0003
X_3^2	149.78	1	149.78	607.90	‹0.0001
Residual	1.72	7	0.25		
Lack of Fit	1.72	3	0.57	174.78	‹0.0001
Pure Error	0.00132	4	0.00033		
Cor Total	225.80	16			
R^2 = 0.9924					

Table 4: Regression coefficients and significance of response surface quadratic for the fermentation

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	11.86	1	0.22	11.33	12.3	-
X_1	-0.011	1	0.18	-0.43	0.40	1.00
X_2	0.98	1	0.18	0.57	1.40	1.00
X_3	-2.56	1	0.18	-2.98	-2.15	1.00
X_1X_2	0.20	1	0.25	-0.39	0.78	1.00
$X_1 X_3$	0.24	1	0.25	-0.35	0.82	1.00
$X_2 X_3$	0.77	1	0.25	0.19	1.36	1.00
X_1^2	1.25	1	0.24	0.68	1.82	1.01
X_2^2	1.59	1	0.24	1.02	2.17	1.01
X_3^2	-5.96	1	0.24	-6.54	-5.39	1.01

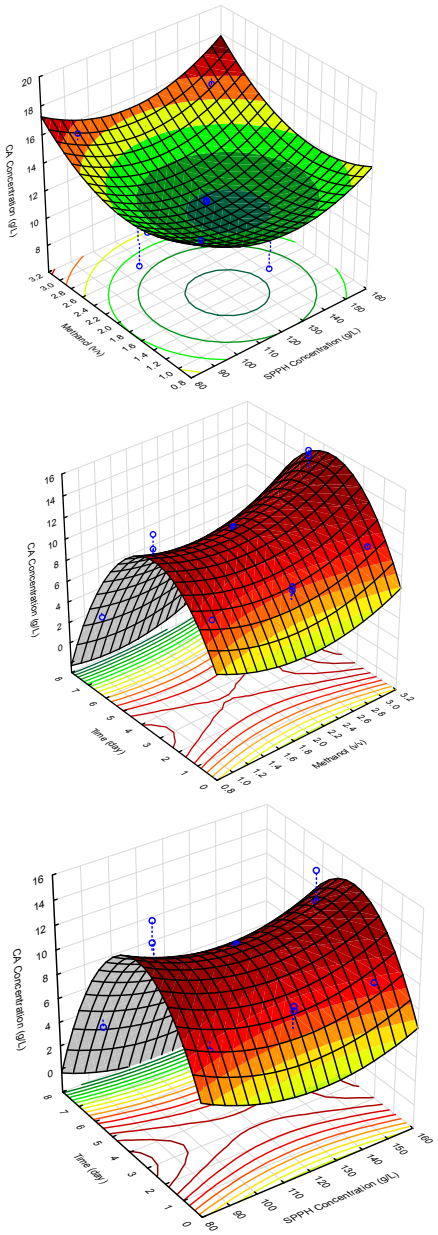

Figure 4: The response surface plots for CA production from SPPH induced with methanol

4. CONCLUSIONS

Response Surface Methodology (RSM) was successfully applied to the optimization of CA production from sweet potato peel hydrolysate supplemented with methanol by surface fermentation using *Aspergillus niger*. The optimal condition established for CA production was SPPH concentration of 150 g/l, methanol concentration of 3 volume %, and time of 3.61 days with a CA concentration of 15.98 g/l. Hence, this work demonstrated that SPPH could serve as the sole carbon source for CA production using *A. niger*.

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