Statistical optimization of media components to enhance citric acid production from paddy straw using solid state fermentation

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Summary

A sequential optimization based on experimental design method was employed to optimize the concentration of media components for improvement of citric acid production from paddy straw by solid state fermentation using *Aspergillus niger*. The optimization procedure for the media components (NH_4NO_3, KH_2PO_4, $MgSO_4$, $CuSO_4$, $ZnSO_4$, $FeSO_4$ and $MnSO_4$) was carried out using the Plaket-Burman Design (PBD) for the screening and Central Composite Design (CCD) for the optimization. The result obtained from (PBD) indicates NH_4NO_3, KH_2PO_4 and $MgSO_4$ were the most significant components that affect the citric acid production. The maximum yield obtained by (CCD) was 39.5 g citric acid/kg of paddy straw. The statistical analysis showed that the optimum media concentration was 0.03 g/L for NH_4NO_3, 2.08 g/L for KH_2PO_4 and 0.015 g/L for $MgSO_4$, which gives the maximum predicted yield of citric acid (51.1 g citric acid/Kg paddy straw).

Keywords: solid-state fermentation, citric acid, experimental design, *Aspergillus niger*, agro-residual by-products

Introduction

Citric acid is one of the most important organic acids that is commercially produced through microbial fermentation. It's widely used in food and beverage as a flavor enhancer, and pharmaceutical and chemical industries as an acidifying agent (Heinzle et al., 2007). *A. niger* is the most commonly-used fungus for citric acid production due to their high yield and relatively high tolerance to the acid accumulation (Shuler and Karagi, 2002). Conventionally, it is produced through submerged fermentation (SmF) using molasses as the raw material. In recent years, considerable interest has been shown in solid state fermentation (SSF) using agro-residues as a substrate; Darani and Zoghi (2008) and Kumar et al. (2003b) have used bagasse, Lu et al. (1998) used kumara and carob pod has been used by Roukas (1999). Some researchers have used the waste of food processing industries like pineapple waste, studied by Imandi et al. (2008), Shojaosadati et al. (2002) used apple pomace and Kumar et al. (2003a) used maosmi waste. The solid-state fermentation has some advantages over submerged fermentation such as lower energy requirements, less risk of bacterial contamination, less waste water generation and less environmental concerns regarding the disposal of solid waste (Rao, 2005).

Many factors (physical and chemical) were observed to have an effect on the *A. niger* ability to produce the citric acid. These included temperature, pH, inoculums size, rate of mixing, aeration, moisture level, carbon source, nitrogen source and mineral salts in the media. Many researchers have reported the effect of chemical factors such as nitrogen sources and mineral salts on production and accumulation of citric acid in SSF. Bari et al. (2009) studied the effect of the co-substrate (sucrose), stimulator (methanol) and metal ions (Zn, Cu, Mn and Mg) on citric acid production using PBD and CCD. The optimization method increased the rate of production 2.6fold and they found the optimum conditions for the metal ions was limited to 9 % v/w, the same result that Tran et al. (1998) found through their study on the effect of metal ions (Fe, Cu, Zn, Mn and Mg) on the production of citric acid. Nitrogen affects the production through the effect of *A. niger* metabolism, where it affects the rate of accumulation of citric acid in SSF (Kun, 2006). The work of Dawson et al. (1989) has shown that the nitrogen and potassium concentrations should be limited. The excess of nitrogen and metal ions would enhance the cell to consume the carbon source towards energy and biomass instead of other metabolic products.

Paddy straw is one of the agricultural residues that are mostly left in a field to burn, causing environmental problems by realizing large amounts of heat and carbon dioxide; also, it is considered one of the cheapest cellulose sources, where its content is about 51-64 % of cellulose and hemicelluloses (Reddy and Yang, 2005). From the structural form of the paddy straw the cellulose is restricted by the lignin, so it is important to release the cellulose from

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the lignin and provide it to the microorganism to use (Graminha et al., 2008). The pretreatment process is aimed at breaking the lignin bonds to release the cellulose and hemicellulose and to improve its utilization by the microorganism. Several pretreatment processes are used, such as chemical and physical processes. Regardless of the type of pretreatment, the goal that could be achieved from the pretreatment should promote high product yields through the fermentation process with minimal costs (Palonen, 2004).
This paper reported the applications of screening of media components by the Plackett-Burman design and optimization of selected media by Central Composite Design (CCD) to improve the citric acid production from paddy straw by SSF using *A. niger*.

Material and methods

Microorganism

The fungal strain of *Aspergillus niger* was obtained from the culture collection laboratory in the School of Bioprocess Engineering, University Malaysia, Perlis. *A. niger* was grown and cultured in a selective agar solution containing (g/L): NH_4NO_3: 2.5; KH_2PO_4: 1.0; $MgSO_4$: 0.25; $CuSO_4$: 0.0048; $ZnSO_4$: 0.0038; $FeSO_4$: 0.0022; $MnSO_4$: 0.001 (Krishnan, 1999); it used molasses as a carbon source with 14 % w/v, and incubated for 7 days at 32 °C. The spore suspensions were suspended by using glass beads to collect the spores in distilled water. The spores were counted using a haemocytometer to maintain a density of $1\text{-}2*10^7$ spores/ml. The fungal strain was maintained on the same agar slants, stored at 4 °C and sub-cultured once every twenty days.

Pretreatments

Pretreatment of paddy straw: The paddy straw was washed first with water to clean the dust from it and dried in an oven at 70 °C overnight. After that, it was pretreated with sodium hydroxide under these conditions: molarity of sodium hydroxide: 1M; solid-liquid ratio: 10 % w/v; time of pretreatment: 1 hour, and at room temperature. Then, it was washed with water sufficiently to remove the base and neutralize it, and then it was dried in an oven at 70 °C overnight. Finally, the dried treated paddy straw was put through a series of sieves to get the desired particle size (Yang et al., 2009).
Pretreatment of Molasses: Molasses was treated with sulfuric acid in order to theoretically convert all the sucrose in the molasses to its glucose equivalent and to remove the heavy metals, where it might have an inhibitory effect on the microbial metabolism. Subsequently, the pH of the molasses was adjusted to 4.00 by adding 1N H_2SO_4. It was left for 1.5 hours at room temperature, then followed by centrifugation at 10000 rpm for 10 minutes. It was then neutralized with sodium carbonate and was left overnight. Then it was put in the centrifuge again, where the supernatant was taken and diluted to the desired concentration (Lotfy et al., 2007).

Preparation for solid-state experiments

Subsequent experiments were conducted in 500 ml Erlenmeyer flasks. Two grams (2gr) of pretreated paddy straw with a particle size > 2mm was put into the flask. The treated molasses was prepared before added to the paddy straw through adjust its pH and concentration which it was 5 and 14 % w/v, respectively. The moisture content adjusted to the level of 80 % by adding the certain amount (8 ml) of adjusted molasses to the paddy straw by using the following relationship:

$$Moisture\ Content = \frac{Weight\ of\ \ water}{Weight\ of\ \ water + Weight\ of\ \ Paddy} \quad (1)$$

The spore suspension was prepared for paddy straw inoculum. The density of the spore was measured by using the hemocytometer to make sure all the inoculum was the same in all the experiments which was $1\text{-}2 *10^7$ cell per ml. The flasks were incubated at 31 °C for 8 days. All experiments were performed in triplicate.

Analytical assay

One hundred milliliters (100 ml) of distilled water was added at the end of fermentation and was shaken at 150 rpm for 1 hour at room temperature for extraction of the citric acid. The pH of the extracted solution was determined by using a pH meter. The citric acid was determined spectrophotometrically by the acetic anhydride-pyridine method that was developed by Miller (1958). Total reducing sugars were measured by the 3-5 dinitrosalicylic acid (DNS) method of Marier and Boult (1958). The cellulose content was determined by using the Updegraff method (Updegraff, 1969).

Experimental Design

Plackett-Burman Design

The Plackett-Burman design (PBD) is a statistical method used for screening the factors (media components) and finds the most significant influence on factors in a minimum number of experiments. This design only considers on the main effect of these variables, but not their interacting effects (Plackett and Burman, 1946). It can be represented by the following first-order polynomial equation:

$$y = \beta_0 + \sum \beta_i x_i \qquad (2)$$

where y represents the response, β_0 is the model coefficient, β_i is the linear coefficient and x_i is the variables. Each variable was represented in two levels, i.e. high (+) and low (-). Table 1 represents the selected variables to be evaluated, whereas, Table 2 showed the design matrix; seven assigned variables were screened in the 12 experimental runs. The significant factors that had a level above 95 % (p-value < 0.05) were considered reliable (Montgomery, 2005). The selected factors (media components) were used for further optimization by response surface methodology (RSM). These experiments were designed and analyzed by using Minitab Software v.15.

Table 1. The levels and actual values of the factors that screened in PBD and the *p*-values of the factors

Variables		NH_4NO_3	KH_2PO_4	$MgSO_4$	$CuSO_4$	$ZnSO_4$	$FeSO_4$	$MnSO_4$
Coded		A	B	C	D	E	F	G
Low level (-)		0	0	0	0	0	0	0
High level* (+)		2.5	2.5	0.25	0.00006	0.001	0.00025	0.0013
P-value	C. A.	***0.001***	***0.050***	0.418	0.093	0.089	0.818	0.553
	R. S.	***0.001***	0.284	***0.039***	0.364	0.100	0.134	0.257
	pH	***0.003***	0.468	0.232	0.828	0.367	0.432	0.588
Significant sign		*	*	*				

*The concentration of the components is in g/L

Table 2. Twelve-trials of the PBD with the responses

Run	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**Citric Acid[a]**	**Reduce Sugar[b]**	**pH[c]**
1	+	-	+	-	-	-	+	*4.44*	*83.86*	*33.89*
2	-	+	+	+	-	+	+	*4.36*	*85.46*	*37.13*
3	+	-	-	-	+	+	+	*7.47*	*62.93*	*46.53*
4	-	-	+	+	+	-	+	*4.63*	*82.14*	*37.59*
5	+	-	+	+	-	+	-	*5.20*	*85.46*	*37.59*
6	+	+	+	-	+	+	-	*4.32*	*85.46*	*34.82*
7	-	+	+	-	+	-	-	*6.70*	*63.05*	*46.53*
8	-	-	-	+	+	+	-	*7.28*	*44.82*	*47.30*
9	+	+	-	+	+	-	+	*8.47*	*52.58*	*47.61*
10	+	+	-	+	-	-	-	*6.74*	*82.01*	*43.29*
11	-	+	-	-	-	+	+	*8.39*	*64.77*	*46.68*
12	-	-	-	-	-	-	-	*7.12*	*64.16*	*46.53*

[a](g/L) final Conc. of citric acid; [b](%) the amount of consume sugar; [c](%) reduce of acidity

Central Composite Design

After screening for the significant media components by the PBD method, the statistically-based optimization using central composite design (CCD) was carried out under response surface methodology (RSM), which is widely used in experiments where the main interest is in modeling the relationship between a number of quantitative factors and one or more response variables, and locating the combination of the factor levels that yields the best response (Devies and Box, 1971). The benefits from this method were from the decreasing number of experiments, and in using time and material resources. Furthermore, the analysis performed on the results is easily realized, and experimental errors are minimized (Cox, 1958).

A CCD consists of a 2^n factorial augmented by 2n axial points and n_c center points that are explained through the following equation:

$$A = 2^n + 2n + C_o \qquad (3)$$

where A is the total number of experiments, n number of variables that are used and C_o is the number of center points (Ali and Al-Azzawi, 2010).

The corresponding factors are coded according to the following equation and summarized in Table 3:

$$X_{coded} = \frac{\left[x_{act} - x_{cen}\right]}{\left[\dfrac{x_{cen} - x_{\min}}{\sqrt{n}}\right]} \qquad (4)$$

The second-order polynomial equation that describes the relationships between the factors and the dependent variable and gives information about interaction between variables (factors) in their relation to the dependent variable is:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n} \sum_{j=1}^{n} \beta_{ij} X_{ij} \qquad (5)$$

where Y is the predicted response, X_i to X_n the input variables, β_0 the intercept term, β_i the linear effects, β_{ii} the squared effects, and β_{ij} the interaction term. In this study, three operating factors were chosen (from previous PBD) as independent variables, namely, (NH_4NO_3, KH_2PO_4 and $MgSO_4$). The "Design Expert" Software v. 7 was used for the regression analysis and graphical analysis of the data obtained.

Table 3. The CCD matrix for the selected variables in coded and actual values with the responses

Exp. no	Coded and real Values				Responses			Yield [a]
	NH_4NO_3 [*]	*KH_2PO_4* [*]	*$MgSO_4$* [*]		*Citric acid* [*]	*Reduce sugar* [*]		
1	(-1) *0.528*	(-1) *0.528*	(-1) *0.052*		8.714	47.32		35.0
2	(1) *1.972*	(-1) *0.528*	(-1) *0.052*		2.677	41.88		10.5
3	(-1) *0.528*	(1) *1.972*	(-1) *0.052*		9.292	50.20		37.0
4	(1) *1.972*	(1) *1.972*	(-1) *0.052*		3.228	42.59		13.0
5	(-1) *0.528*	(-1) *0.528*	(1) *0.197*		8.970	46.72		36.0
6	(1) *1.972*	(-1) *0.528*	(1) *0.197*		5.218	45.37		21.0
7	(-1) *0.528*	(1) *1.972*	(1) *0.197*		8.929	50.00		35.5
8	(1) *1.972*	(1) *1.972*	(1) *0.197*		3.242	41.17		13.0
9	(-1.681) *0*	(0.0) *1.25*	(0.0) *0.125*		9.817	71.53		39.5
10	(1.681) *2.5*	(0.0) *1.25*	(0.0) *0.125*		2.112	42.27		8.50
11	(0.0) *1.25*	(-1.681) *0*	(0.0) *0.125*		5.823	44.59		23.5
12	(0.0) *1.25*	(1.681) *2.5*	(0.0) *0.125*		5.390	44.59		21.5
13	(0.0) *1.25*	(0.0) *1.25*	(-1.681) *0*		5.702	50.00		28.0
14	(0.0) *1.25*	(0.0) *1.25*	(1.681) *0.25*		5.662	43.24		22.5
15	(0.0) *1.25*	(0.0) *1.25*	(0.0) *0.125*		5.46	42.46		22.0
16	(0.0) *1.25*	(0.0) *1.25*	(0.0) *0.125*		5.299	47.88		21.0
17	(0.0) *1.25*	(0.0) *1.25*	(0.0) *0.125*		5.702	48.65		23.0
18	(0.0) *1.25*	(0.0) *1.25*	(0.0) *0.125*		5.702	54.07		23.0
19	(0.0) *1.25*	(0.0) *1.25*	(0.0) *0.125*		6.186	47.49		24.5
20	(0.0) *1.25*	(0.0) *1.25*	(0.0) *0.125*		5.944	58.71		24.0

Values within parenthesis indicate the coded values; [*]Conc. measured in (g/L); [a]Yield as [g citric acid/ kg paddy straw used]

Results and Discussion

Paddy straw that was treated with sodium hydroxide was analyzed to find the amount of cellulose content according to a known weight of paddy straw by using the Updegraff method, where it was found the cellulose content increased about 15 %. The sodium hydroxide extracted some of the lignin and silica during the treatment. Fig. 1 shows the comparison between the treated paddy straw and the non-treated straw according to the concentration of citric acid. The fungus was able to produce, after seven days of fermentation, about 7.5 g/L when it used the treated paddy, and 5.3 g/L when it used the non-treated paddy straw.

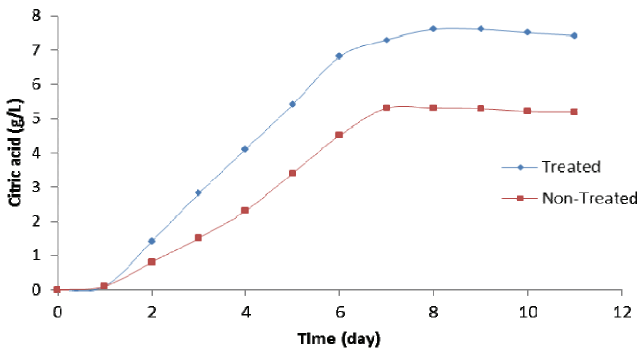

Fig. 1. Concentration of citric acid for treated and non-treated paddy straw over time

Fig. 2 shows the concentration of reduced sugar and citric acid over time in order to find the minimum time to end the fermentation. The fungus consumed almost all the molasses in the first four days (79 %) for germination and building up of the hyphae, after that the fungus will start to change the cellulose to citric acid by producing enzymes that are able to convert the cellulose to glucose, and then to citric acid. The benefits of using low concentrations of molasses at the beginning, enhances the cell into growing and building the hyphae. Citric acid is considered as a secondary metabolic product, so it is important to enhance the cell to produce the citric acid, rather than to grow and produce biomass. The concentration of citric acid appears to rise after the second day (0.1 g/L) and keeps rising till it reaches a constant value in the 8^{th} day (7.6 g/L); this value stayed constant for 3 days, then the concentration started to decrease because of the oxidization of citric acid, which means the maximum concentration for citric acid was reached on the 8^{th} day, and no need to make the time of fermentation more than eight days. *A. niger* was able to consume 82 % of the molasses that was used during the fermentation time.

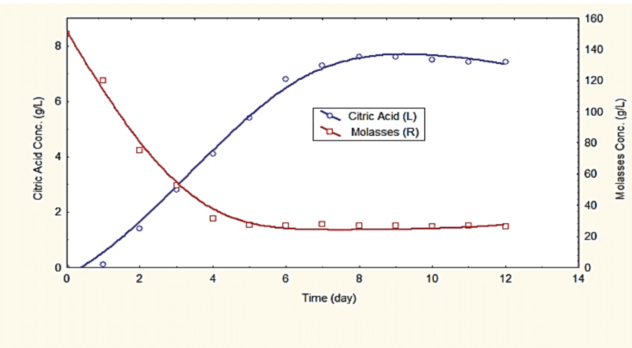

Fig. 2. Concentration of citric acid and molasses during fermentation time

Screening of Media Components

The nutrient conditions are shown to have an important role in the fermentation of citric acid. PBD was used for screening the parameters (nutrients). The *p*-value was considered as a tool for evaluating the significance of each of the coefficients. The parameters with confidence levels greater than 95 % were considered as influencing the response significantly.

Table 1 shows the ANOVA for the measured responses (pH, citric acid concentration, and reduced sugar). It was obviously seen that NH_4NO_3 was the most significant nutrient due to the low *p*-value in all the responses where it noted (0.001, 0.001 and 0.003) in (citric acid, reduced sugar and pH) responses, respectively. KH_2PO_4 was observed to affect the citric acid fermentation where its *p*-value was 0.05; $MgSO_4$ was also a significant nutrient where it affected the reduced sugar response, where its *p*-value was 0.039. The maximum yield obtained from these experiments was 33.88 g citric acid/kg of paddy straw (Experiment no. 9).

Optimization of significant selected media components

The selected media components that have significant effects on citric acid production were optimized to find the maximum yield. Experiments were carried out using CCD; the responses obtained are in Table 3. For predicting the optimal values within the experimental results, a second-order polynomial equation was established to explain the citric acid production and fitted to the experimental results using Design Expert software. The model developed in terms of real value is as follows:

$$\begin{aligned} \text{Yield} = {} & 42.58 - 17.57{*}NH_4NO_3 + 4.5{*}KH_2PO_4 - \\ & - 41.9{*}MgSO_4 + 1.13{*}NH_4NO_3^2 + 0.17{*}KH_2PO_4^2 + \\ & + 193.17{*}MgSO_4^2 - 1.58{*}NH_4NO_3{*}KH_2PO_4 + \\ & + 24.89{*}NH_4NO_3{*}MgSO_4 - 29.41{*}KH_2PO_4{*}MgSO_4 \end{aligned} \tag{6}$$

The results of the analysis of variance (ANOVA) are summarized in Table 4, and indicate that the model was significant, where its *p*-value (<0.0001), and the determination coefficient (R^2), which provides an indicator for the variability of the predicted response and the experimental data, the closer R^2 is to 1, the better the model predicts the response (Aghaiea et al., 2009 and Wang, 2006). The R^2 of the following model was 0.9633, which indicates that 96.33 % of the variability in the experimental response could be explained by the model; also, the adjusted determination coefficient (R^2 adj) was 0.9302, which conformed that the model was highly significant. Fig. 3 shows the variability between the values that were predicted by the above model versus the observed data obtained from the experimental work. It shows the total determination coefficient (R^2) was 96.33 %, indicating a reasonable fit of the model to the experimental data.

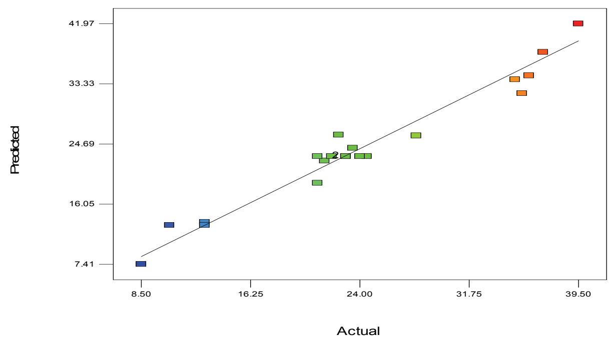

Fig. 3. Compression between predicted and observed citric acid yield

It was observed from the (ANOVA) listed in Table 4 that each of the model parameter coefficients was significant by using the Student *F* test and *p*-value. It was observed that the variables that have high significant effects was the linear term of the nitrogen source (A) and the interactive term between potassium and magnesium (BC), and also the two terms have the highest *F*-value.

Table 4. ANOVA of the response surface model

Source	Sum of Squares	DF	*F*-value	*P*-value
Model	1463.98	9	29.14	< 0.001
NH_4NO_3	1397.21	1	250.29	< 0.001
KH_2PO_4	3.97	1	0.71	0.4187
$MgSO_4$	0.041	1	7.3E-003	0.9332
NH_4NO_3* KH_2PO_4	6.13	1	1.10	0.3195
NH_4NO_3* $MgSO_4$	15.13	1	2.71	0.1308
KH_2PO_4 * $MgSO_4$	21.13	1	3.78	0.0804
(NH_4NO_3)^2	5.63	1	1.01	0.3388
(KH_2PO_4)^2	0.13	1	0.023	0.8819
$(MgSO_4)$^2	16.41	1	2.94	0.1172

R^2: 0.9633, adj R^2: 0.9302

The 3D response surface plots are the graphical representation of the regression equation used to determine the optimum concentration for used media components within the considered ranges (Tanylidizi et al., 2005). The 3D response plots showed that the interaction between two parameters and keeping the others at center point. Fig. 4 shows the interaction effect of NH_4NO_3 and KH_2PO_4 on the citric acid production; it seems that the production increased with the decrease in NH_4NO_3 and the increase in KH_2PO_4. Fig. 5 shows the decreasing of NH_4NO_3 and $MgSO_4$ will increase the rate of citric acid production. Fig. 6 indicates and confirms the results obtained from Fig. 4 and 5, where it shows the increasing of citric acid production with the increasing of KH_2PO_4 and the decreasing of $MgSO_4$. Nitrogen plays a major role in the metabolism of citric acid. The cell needs nitrogen in the form of ammonium to build up cell substances. On the other side, too much nitrogen inhibits the production of citric acid, as it will enhance the cell to grow and produce biomass (Wieczorek and Brauer, 1998). Potassium and phosphate in (KH_2PO_4) is considered also a growth-enhancing nutrient and as a buffering agent, where it acts to keep the pH at the desired value. Magnesium affects the rate sugar utilization by the cell that relates to rise in mycelia weight (biomass rise) (Shu, 1948). As is shown in Figs. 4-6, statistical analysis of the experimental design and analyzing of the evaluated model for the optimal concentrations of the selected media components were: 0.05 g/L of NH_4NO_3, 2.46 g/L of KH_2PO_4, 0.03 g/L; $MgSO_4$, with a predicted yield; 50.98 g citric acid/Kg paddy straw.

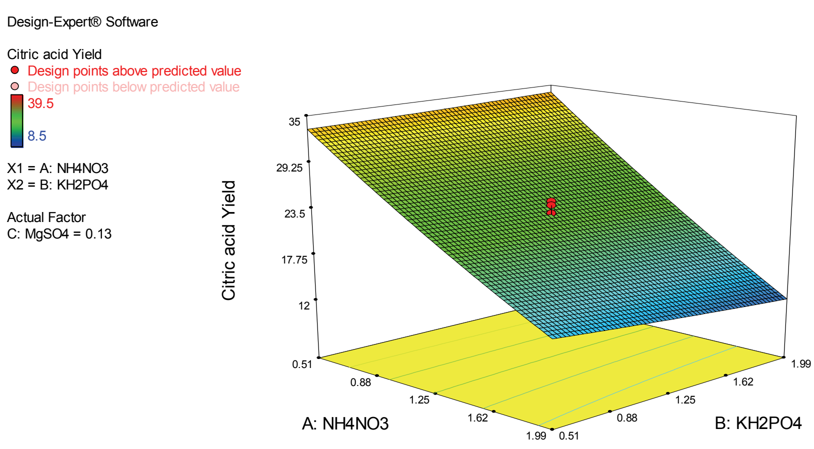

Fig. 4. Dimensional Response surface curve for citric acid Yield as a function to NH_4NO_3 and KH_2PO_4

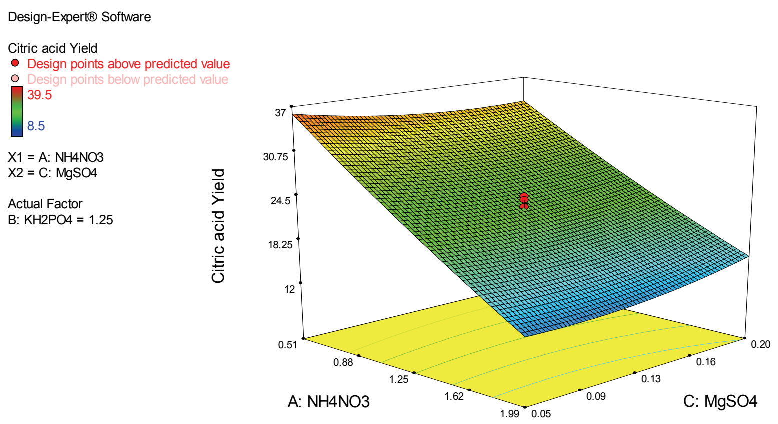

Fig. 5. Dimensional Response surface for citric acid Yield as a function to NH_4NO_3 and $MgSO_4$

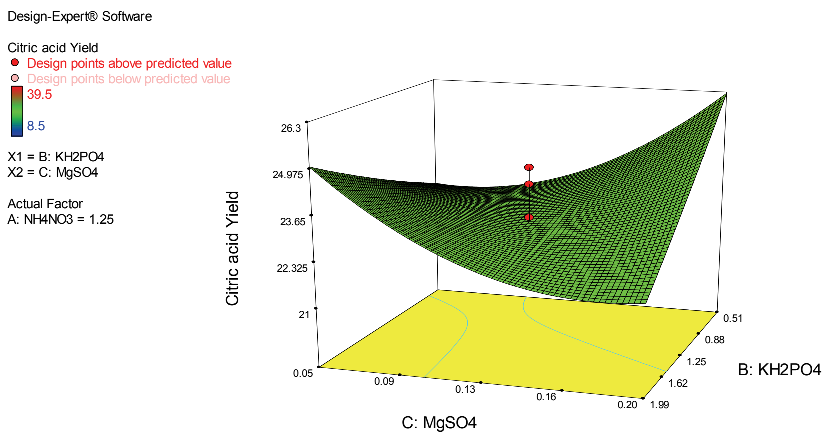

Fig. 6. Dimensional Response surface for citric acid Yield as a function to KH_2PO_4 and $MgSO_4$

Confirmative tests

The suitability of the model equation for predicting the optimum response value was tested using the recommended optimum conditions. When optimum values of the independent variables (0.05 g/L of NH_4NO_3, 2.46 g/L of KH_2PO_4, 0.03 g/L of $MgSO_4$) were incorporated into the regression equation, 50.98 g citric acid/Kg paddy straw was obtained, whereas actual experiment at above optimum conditions gave a citric acid yield of 50.23 g citric acid/Kg paddy straw. Thus, predicted values from fitted equations and observed values were in very good agreement.

Conclusions

In this study, three media components (NH_4NO_3, KH_2PO_4, and $MgSO_4$) were selected by Plackett-Burman design as a significant parameter for citric acid production from paddy straw. The selected parameters were optimized using CCD under RSM. The maximum yield that was obtained from PBD experiments was 33.88 g citric acid/Kg paddy straw, which increased about 1.12fold more than the basal experiment. The maximum yield obtained in CCD was 39.5 g citric acid/Kg paddy straw, which was an increase of 1.30fold higher. After optimization of the selected parameter the yield reached 50.23 g citric acid/kg paddy straw (a 1.65fold higher). This indicates that the RSM technique proved to be a useful tool in establishing optimum conditions for citric acid production.

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