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Statistical Approach to the Optimization of Citric Acid Production using Filamentous Fungus Grown on Wheat Straw

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A central composite design (CCD) was used to optimize fermentation condition for the production of citric acid by *Aspergillus niger* NRRL 567 using wheat straw. The statistical model was capable of predicting optimum condition for maximum citric acid production. The pH of nutrient solution, inoculum density and moisture content were found to significantly affect citric acid production, while fermentation temperature was showed insignificant effect within tested range. The maximum citric acid production of 123 g/kg dry wheat straw (DWS) was predicted with the fermentation condition of 35!, pH 8, 3.74 x 10^6 spores/ml and 80% moisture content at 72 h. Maximum citric acid production on optimized condition represented a 1.6-fold increase compared to that obtained from control experiment. The experimental data fitted well with the model predicted values and the CCD has proved to be very effective for the optimization of the citric acid production.

Key words: Citric acid, *Aspergillus niger*, solid substrate fermentation, Wheat straw, response surface methodology.

A variety of fungi are known to produce organic acids such as citric, oxalic, succinic and malic acid. Among many groups of organic acids, citric acid is extensively produced by filamentous fungus *Aspergillus niger* and it is widely used by industries producing food, beverages, chemicals and pharmaceutical products[1-3]. Nowadays, the global demand for citric acid is growing faster than its production, implying that more economical processes are required to replace the present process[4,5]. Solid substrate fermentation (SSF) has gained renewed attention due to potential advantages in producing organic acids in high yield comparing to conventional submerged fermentation. Since the fungi in the solid substrate grows under the condition similar to their natural habitat without free flowing water, they can produce organic acid economically when growing on agricultural byproducts such as agricultural byproducts, industrial byproducts and food waste[6-9].

According to previous studies, the production of citric acid under a SSF is strongly affected by substrate composition, moisture content, particle size distribution, fermentation temperature, pH and inoculum density[10]. Even though SSF is conducted in the near-absence of water, a small variation in moisture content can produce significant changes in fungal growth and metabolite production[11-14]. Moisture contents ranging from 65 to 85% resulted in the maximum citric acid production by *A. niger* DS 1 growing on fruit waste,[15]. However, moisture content between 70 and 80% increased citric acid and lipase production by *A. niger* species growing on semi-dried figs and wheat bran[15,16].

During fermentation, a solid substrate with a low heat transfer coefficient results in

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localized heat buildup and non-homogeneous temperature distribution in solid substrate[17-18]. High temperature results in growth inhibition, enzyme denaturation and moisture loss, while low temperature leads to low metabolic activity[19]. Although most of filamentous fungi are mesophilic requiring optimal temperature between 25 and 35°C, a temperature of 40°C was identified as optimum for organic acid and enzyme productions and sugar utilization by *A. niger* ATCC 10577 and *A. niger* V. Tiegham[20-23].

The solid substrate pH strongly influences the growth of microorganisms and the accumulation of fermentation products. Most filamentous fungi grow well under acidic pHs ranging between 3 and 6, but some fungi are able to growth at pH lower than 2 [24-25]. Roulas[26] reported that *A. niger* produced maximum amounts of citric and gluconic acid at an initial substrate pH 7. While Kamini et al. reported an optimal pH between 6 and 8, for the production of citric acid and lipase by *A. niger* ATCC 10577 and *A. niger* MTCC 259 [27].

The aim of the study is to optimize the fermentation conditions for citric acid production by *A. niger* under SSF. The effects of four crucial process parameters, namely temperature, pH, inoculum density and moisture content, on citric acid production were optimized using a statistically based optimization. Among four parameters, the effective parameters were screened and then optimization of the significant parameters was carried out using CCD to maximize citric acid production from wheat straw.

MATERIALS AND METHODS

Microorganism

Aspergillus niger NRRL 567 was obtained from the American Type Culture Collection (ATCC, USA). *A. niger* spores were produced on potato dextrose agar (PDA, Sigma, USA) plates at 30°C and were sub-cultured at biweekly interval. At seven to ten days of incubation on PDA plates, 10 ml of 0.1% Tween 80 (Sigma, USA) solution were added to each plate and their surfaces were scraped to correct and dilute again to gain a solution containing 1 x 10^6 spores/ml.

Solid substrate

Wheat straw was obtained from the Macdonald Campus farm of McGill University (Montreal, Canada). The air-dried wheat straw was cutting-milled to 20 mesh in a Wiley mill and was stored at -20! freezer until being used. Since the optimum water content of the solid substrate for citric acid is about 70 to 80%, additional distilled water and mineral solution were added before autoclaving. In preparation for fermentations in the Erlenmeyer flask, 10 g wheat straw and 35 mL of mineral solution were autoclaved for 15 min at 121°C.

Fermentation condition

Small-scale experiments were conducted using 250-ml Erlenmeyer flasks containing 10 g of dry substrate wetted with the solution containing glucose and mineral solution composed per kg of dry wheat straw (DWS) of: 967.9 g glucose, 15.4 g $(NH_4)_2SO_4$, 43.9 g KH_2PO_4 and 4.0 g NaCl [9,10]. The effect of initial moisture content was varied by adding deionized water into wheat straw to obtain initial levels of 65, 70, 75, 80 and 85% (Table 2). The fermentation temperature was varied from 20 to 40ºC. The initial nutrient solution pHs were varied from 2.0 to 9.0 by adding phosphate, acetate and carbonate buffer solution adding. For the inoculation, 1 ml of spore solution containing 0.5, 2.0, 3.5, 5.0, or 6.5 x 10^6 spores/ml was added to the each flask.

Analytical methods

The contents of 250-ml Erlenmeyer flasks were sacrificed each sampling point. 5 g of wet sample was used to measure the moisture content and the other 5 g of wet sample was placed in 50 ml of distilled water and shaken for 15 min at 150 rpm. The supernatant was tested for pH level and stored at –20°C for citric acid analysis. The concentration of citric acid in culture filtrates was determined by the Waters HPLC system equipped with a refractive index detector (RID) and Aminex HPX-87H column (7.8 × 300 mm, Bio-Rad, USA). The mobile phase used for the analysis was 0.005 N sulfuric acid. The HPLC analysis was carried out under the following conditions: 0.6 ml/min flow rate; 50°C column temperature[7].

Experimental design

The interactive effect of selected four fermentation parameters, including temperature, pH, inoculum density and moisture content, was tested using a statistically based optimization. As shown in Table 1, the variables were examined at five coded levels (-2, -1, 0, +1, + 2) and each parameters Xi associated with each coded level was calculated as:

$$Xi = ?i\Delta Xi + Xcp \quad ...(1)$$

where ι = 1, 2, 3 and 4 and corresponds to each one of the four parameters; i = dimensionless coded level for Xi, namely –2, -1, 0, 1 and 2; Xi = real concentration of the independent variable for the code used; Xcp = level of independent variable at the coded value 0; [11,12].

Table 1. Coded values used in CCD to optimize the fermentation conditions for citric acid production

Variables	Parameter	Unit	Coded and actual level				
			-2	-1	0	+1	+2
X_1	Temperature	°C	20	25	30	35	40
X_2	pH	-	2	4	6	8	9
X_3	Inoculum density	10^6 spores/ml	0.5	2.0	3.5	5.0	6.5
X_4	Moisture content	%	65	70	75	8	85

With actual levels from 19 combinations, CCD produced second-order polynomial equations representing a model of citric acid production based on the level of the actual values at 48 and 72 h of fermentation:

$$Y = \beta_0 + \beta_1 c_1 + \beta_2\chi_2 + \beta_3\chi_3 + \beta_{121}\chi_2 + \beta_{23}\chi_2\chi_3 + \beta_{13}\chi_1\chi_3 + \beta_{123}\chi_1\chi_2\chi_3 \quad ....(2)$$

where Y = predicted response; β_0 = intercept; β_1, β_2, β_3 = linear coefficients; β_{11}, β_{22}, β_{33} = squared coefficients; β_{12}, β_{13}, β_{23}, β_{123}= interaction coefficients;

RESULTS AND DISCUSSION

Optimization using statistically based optimization

The statistical software package Design-Expert 8 (Stat Ease Inc., USA) was used to generate a regression model to predict the optimum conditions considering the effects of linear and interaction on citric acid production. Four major variables, namely temperature, pH, inoculum density and moisture content, were included in this model. Table 2 presents the 19 combinations of input variables and the actual levels of citric acid. Then, actual values were fitted to the following second order regression models:

$$Y_{48\,h} = 18.69 + 0.94\chi_1 + 13.86\chi_2 + 2.73\chi_3 + 7.15\chi_4 - 2.86\chi_1^{\ 2} + 3.46\chi_2^{\ 2} - 1.55\chi_3 + 0.33\chi_4^{\ 2} - 6.66\chi_1\chi_2 + 1.49\chi_1\chi_3 + 21.53\chi_1\chi_4 + 0.23\chi_2\chi_3 - 11.93\chi_2\chi_4 + 1.27\chi_3\chi_4 \quad ...(3)$$

$$Y_{72\,h} = 78.19 + 4.60\chi_1 + 23.0\chi_2 + 7.24\chi_3 + 25.51\chi_4 - 15.94\chi_1^{\ 2} - 0.81\chi_2^{\ 2} - 1.05\chi_3^{\ 2} - 5.62\chi_4^{\ 2} - 10.36\chi_1\chi_2 - 1.93\chi_1\chi_3 + 18.22\chi_1\chi_4 + 2.36\chi_2\chi_3 + 4.40\chi_2\chi_4 - 3.77\chi_3\chi_4 \quad ...(4)$$

where χ_1, χ_2, χ_3 and χ_4 correspond to the coded values of physico-chemical parameters of temperature, a nutrient solution initial pH, inoculum density and initial moisture content.

The corresponding analysis of variance (ANOVA) is presented in Table 3. The quality of fit of the regression model is expressed by the coefficient of determination, R^2. In this experiment, the value of and R^2 were 0.981 and 0.987, respectively, for the citric acid productions after 48 and 72h. The correlation between the actual and predicted values is better when the value of R^2 is closer to 1. Such high coefficients indicate good agreement between the observed and predicted response, as only 1.9 and 1.3% of the total response variation was not explained by the model[28]. The regression models were highly significant, as an evident from the Fisher's *F*-test with a very low probability value (p_{model}> F = 0.0098). The linear effect of pH (X_2), inoculum density (X_3) and moisture content (X_4) were significant at the level of $p < 0.05$ at 72 h.

The 3D response surface curve is the graphical representation of the second-order regression equation used to determine the optimum values of the variables within the tested ranges[29]. Using the model presented in equations (3) and (4), Figs. 1a and 1b predict the interactive effects of fermentation temperature and initial pH on citric acid production at 48 and 72 h, respectively, fixing an inoculum density of 3.5 x 10^6 spores/ml and initial wheat straw moisture content of 75%. Both plots indicate that citric acid production significantly increased with nutrient solution pH, but not with temperature within tested levels. Citric acid production showed a steep increase as initial pH increased from 4.0 to 8.0. A maximum citric acid

Table 2. Actual and predicted citric acid production optimization after 48 and 72 h of fermentation.

Run	Temp	pH	ID	MC	Response at 48 h		Response at 72 h	
No	(X_1)	(X_2)	(X_3)	(X_4)	Observed	Predicted	Observed	Predicted
1	35	8	5.0	70	15.70	12.63	28.27	34.39
2	35	8	2.0	70	8.68	6.29	15.49	13.50
3	35	4	5.0	80	60.75	57.68	78.05	84.17
4	25	8	2.0	80	10.98	8.59	90.50	88.51
5	35	4	2.0	80	49.55	47.16	89.80	87.81
6	25	4	5.0	70	1.86	-1.20	17.31	23.43
7	25	8	5.0	80	17.13	14.07	95.93	102.04
8	25	4	2.0	70	1.70	-0.69	6.24	4.25
9	20	6	3.5	75	2.63	5.36	7.29	5.23
10	40	6	3.5	75	6.41	9.14	25.70	23.64
11	30	2	3.5	75	2.07	4.79	30.86	28.80
12	30	10	3.5	75	57.50	60.23	123.15	121.09
13	30	6	0.5	75	5.00	7.04	55.45	61.49
14	30	6	6.5	75	14.55	17.95	96.64	86.47
15	30	6	3.5	65	2.98	5.71	6.75	4.68
16	30	6	3.5	85	31.58	34.31	108.78	106.71
17	30	6	3.5	75	17.84	18.69	77.41	78.19
18	30	6	3.5	75	19.27	18.69	82.58	78.19
19	30	6	3.5	75	18.96	18.69	74.58	78.19

Note: ID – inoculum density (spores/ml); MC – initial moisture content.

Table 3. Regression analyses for the model and ANOVA results after 48 and 72h of fermentation

	Citric acid production					
	48 h			72 h		
	Sum of Squares	*F* value	*P* level	Sum of Squares	*F* value	*P* level
Model	6147.42	14.41	0.0098*	27122.21	21.30	0.0047*
X_1	7.14	0.23	0.6536	169.48	1.86	0.2440
X_2	1536.76	50.42	0.0021*	4258.45	46.82	0.0024*
X_3	119.03	3.91	0.1193	723.85	7.86	0.0489
X_4	408.93	13.42	0.0215*	5204.89	57.22	0.0016*
X_1^2	169.82	5.57	0.0776	5273.76	57.98	0.0016*
X_2^2	247.86	8.13	0.0463*	13.68	0.15	0.7179
X_3^2	49.73	1.63	0.2706	23.02	0.25	0.6413
X_4^2	2.26	0.074	0.7988	656.43	7.22	0.0549
X_1X_2	177.38	5.82	0.0734	429.72	4.72	0.0954
X_1X_3	17.69	0.58	0.4886	29.89	0.33	0.5972
X_1X_4	1854.40	60.85	0.0015*	1328.43	14.60	0.0188*
X_2X_3	0.41	0.013	0.9132	44.59	0.49	0.5224
X_2X_4	569.39	18.68	0.0124	77.37	0.85	0.4086
X_3X_4	12.94	0.42	0.5502	113.82	1.25	0.3259

*significant levels at a 95% confidence level.
X_1 – fermentation temperature; X_2 – initial nutrient solution pH; X_3 – inoculum density; X_4 – initial MC.

production of 101.0 g/kg DWS was obtained with the pH and temperature combination of 8.0 and 30°C at 72 h of fermentation.

Figs. 2a and 2b predict the interactive effects of temperature and inoculum density on citric acid production at 48 and 72 h at fixed levels of pH 6.0 and moisture content of 75%. A fermentation temperature of 32.5 and 31°C combined with the inoculums level of 5.0 x 10^6 spores/ml produced the maximum citric acid at 48 and 72 h, respectively. Inoculum density linearly affected citric acid production at all ranges of fermentation temperature, which maximized at 83.5 g/kg of DWS at 72 h of fermentation.

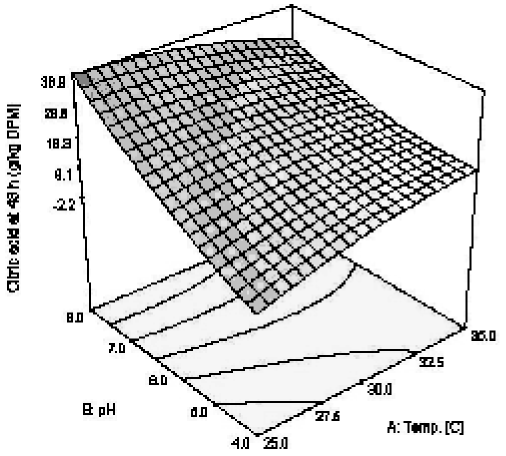

Fig. 1(a). Response surface curve representing the interactive effect of fermentation temperature and a nutrient solution initial pH on citric acid production at 48 h, using an inoculum density of 3.5 x 10^6 spores/ml and an initial moisture content of 75%.

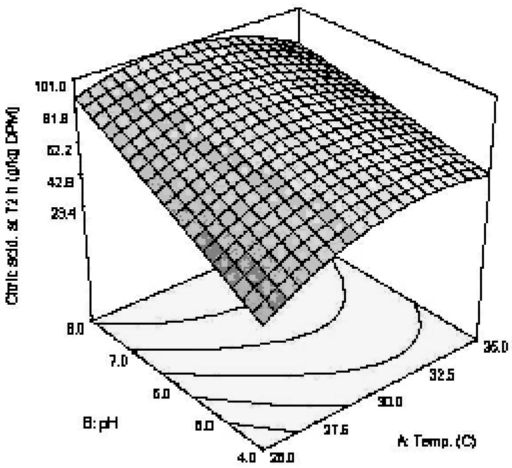

Fig. 1(b). Response surface curve representing the interactive effect of fermentation temperature and a nutrient solution initial pH on citric acid production at 72 h, using an inoculum density of 3.5 x 10^6 spores/ml and an initial moisture content of 75%

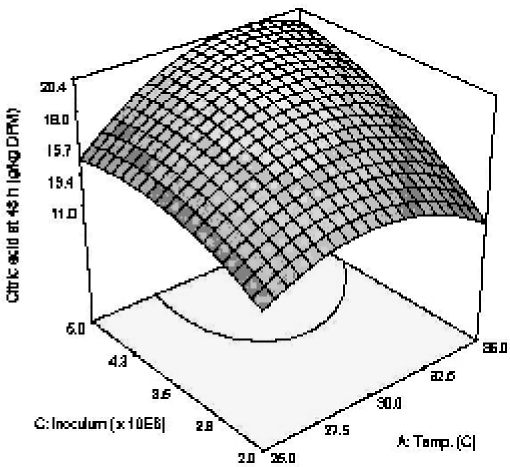

Fig. 2(a). Response surface curve representing the interactive effect of fermentation temperature and inoculum density on citric acid production at 48 h, using a nutrient solution initial pH of 6.0 and an initial moisture content of 75%

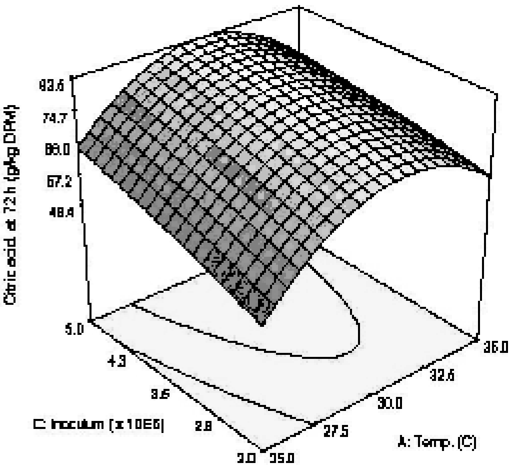

Fig. 2(b). Response surface curve representing the interactive effect of fermentation temperature and inoculum density on citric acid production at 72 h, using a nutrient solution initial pH of 6.0 and an initial moisture content of 75%

Because the moisture content of wheat straw during the fermentation had strong correlation with fermentation temperature, the interactive effect of these two conditions was examined in Figs. 3a and 3b fixing a nutrient solution initial pH of 6.0 and an inoculum density of 3.5 x 10^6 spores/ml. The maximum citric acid production was obtained under the fermentation temperature of 35°C with the moisture content of 80% at 72 h, respectively. High temperatures lead to low wheat straw moisture content due to evaporation. The loss of water in solid substrate can only be compensated by high initial moisture content. Thus, a high fermentation temperature accompanied by high initial moisture content resulted in a maximum citric acid production of 106.2 g/kg DWS at 72 h.

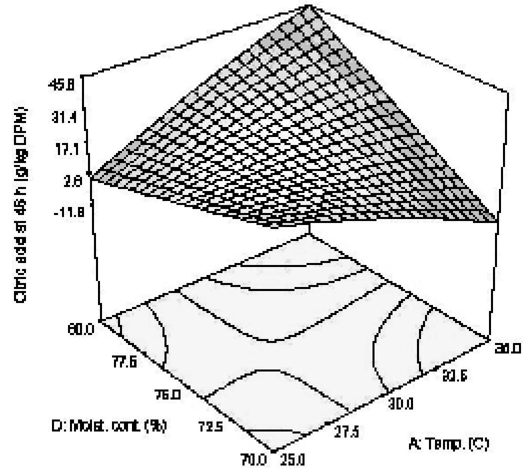

Fig. 3(a). Response surface curve representing the interactive effect of fermentation temperature and initial moisture content on citric acid production at 48 h, using a nutrient solution initial pH of 6.0 and an inoculum density of 3.5 x 10^6 spores/ml

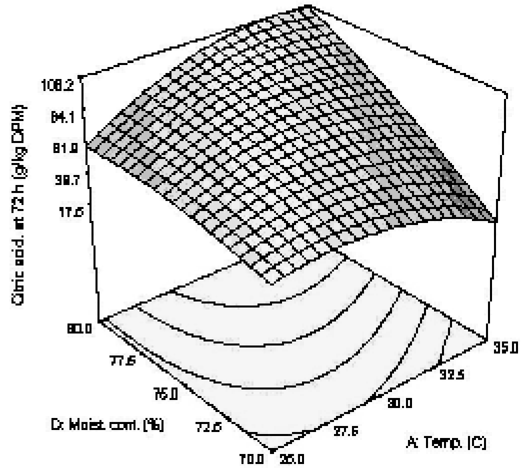

Fig. 3(b). Response surface curve representing the interactive effect of fermentation temperature and initial moisture content on citric acid production at 72 h, using a nutrient solution initial pH of 6.0 and an inoculum density of 3.5 x 10^6 spores/ml.

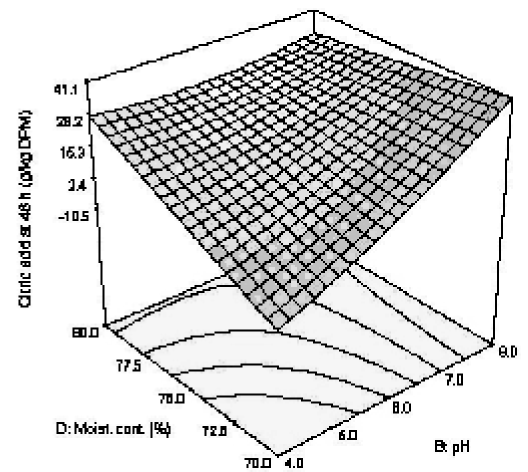

Fig. 4(a). Response surface curve representing the interactive effect of a nutrient solution initial pH and initial moisture content on citric acid production at 48 h, using a fermentation temperature of 30°C and an inoculum density of 3.5 x 10^6 spores/ml

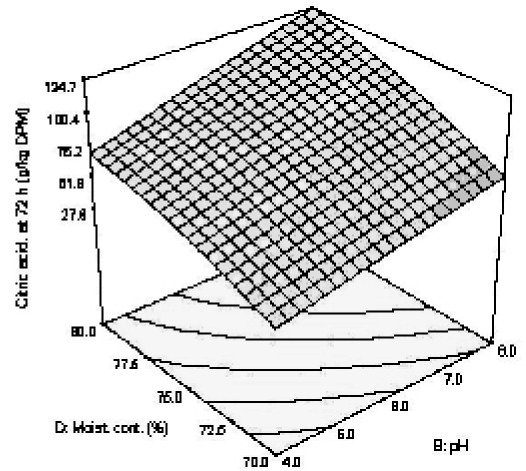

Fig. 4(b). Response surface curve representing the interactive effect of a nutrient solution initial pH and initial moisture content on citric acid production at 72 h, using a fermentation temperature of 30°C and an inoculum density of 3.5 x 10^6 spores/ml

Figs. 4a and 4b show the interactive effects of nutrient solution pH and initial moisture content on citric acid production at 48 and 72 h with fixed levels of temperature and inoculum density. High citric acid productions were predicted with high pH levels at 48 h. While the effect of initial moisture content was more pronounced and a maximum citric acid production of 124.7 g/kg DWS was predicted at 72 h under a nutrient solution pH of 8.0 and an moisture content of 80%.

Predicting optimum conditions

In order to predict optimum fermentation conditions, the numerical optimization method of CCD were employed (Table 4). Estimated optimum fermentation condition for the production of citric acid is 35°C, pH 8.0, inoculum density of 3.94 x 10^6 spores/ml and moisture content 80%. Under this condition, citric acid productions of 46.1 and 122.0 g/kg DW were predicted. In order to verify the optimization results and to validate the model developed, the control and CCD optimized fermentation conditions were compared (Fig. 5). The optimized fermentation conditions produced maximum citric acid concentrations of 130.1 g/kg DWS at 72 h of fermentation, which is slightly higher than the predicted value. From the validation experiment, it is clearly showed that the citric acid production of 130.1 g/kg DWS was obtained under optimum condition. The result from CCD optimization was 1.6-fold higher than the production (82.0 g/kg DWS) obtained by control test.

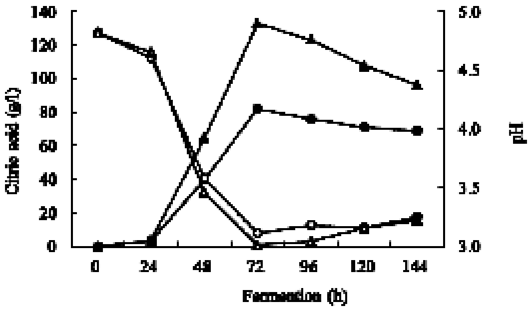

Fig. 5. Time course behavior of citric acid production and solid substrate pH by A. niger grown on wheat straw using the control conditions (30°C, pH 4.28, moisture content 80%, 1.0 x 10^6 spores/ml) and optimization condition (35°C, pH 8.0, moisture content 80%, 4 x 10^6 spores/ml), ●:control (citric acid production); ▲: Optimized by CCD (citric acid production); ○:control (pH); △: Optimized by CCD (pH)

CONCLUSIONS

The response surface methodology based on a four-variable CCD was used to determine the effect of temperature, pH, inoculum density and moisture content on citric acid production by *A. niger* under SSF. Statistical analysis showed pH, inoculum density and moisture content had significant positive effect on the responses, while temperature (25 – 35°C) had no significant effect. Using CCD, citric acid production maximized at 130.1 g/kg DWS with 35°C, nutrient solution pH of 8, 3.94 x 10^6 spores/ml and moisture content of 80%. The present study has shown a promising potential for utilization of wheat straw as a solid substrate for the production of citric acid by *A. niger*. Also, the statistically based optimization procedure using CCD was proved to be an effective technique in optimization of fermentation conditions.

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