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Submerged Fermentation of Whey for Citric Acid Production by *Aspergillus niger* NRRL 567: Optimization of Medium Composition by Statistical Experimental Design

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Citric acid production by *Aspergillus niger* NRRL 567 from whey-based medium with different concentrations of supplementing nutrients was studied. The effect of nutrients including glucose, $(NH_4)_2SO_4$ and KH_2PO_4 on citric acid production was evaluated using statistically-based optimization method and found that glucose and KH_2PO_4 supplementation significantly influenced citric acid production, while $(NH_4)_2SO_4$ had insignificant effects. The statistical analysis showed that the optimum medium containing 60 g/L of glucose, 1.5 g/L of $(NH_4)_2SO_4$ and 8.1 g/L of KH_2PO_4 predicted the maximum citric acid production of 12.0 g/L at 240 hr. The predicted citric acid production matched well that measured citric acid production (12.4 g/L) of the validation experiment. The results showed the effectiveness of the statistically-based optimization method and the optimized level of nutrients increased citric acid production by 2 folds, compare with the basal whey medium with 60 g/L glucose.

Key words: Citric acid, Optimization, Fermentation condition, Submerged fermentation.

Citric acid (2-hydroxy-1, 2, 3-propanetricarboxylic acid) is commercially produced by submerged fermentation (SmF) of starch or sucrose based medium using *Aspergillus niger* and widely used in food, pharmaceutical, beverage, cosmetic, chemical and textile industries[1]. Because citric acid is biodegradable, ecofriendly, economical, safe and a versatile chemical, global production of citric acid has reached 1.7 million ton/yr. and the demand of citric acid is growing faster. Nowadays, the food industry is the major consumer of citric acid, using almost 70% of the total production, followed by 12% by the pharmaceutical industry and 18% for other applications[2-4].

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Citric acid is an intermediate of the TCA cycle and its production by *A. niger* is strongly influenced by the fermentation conditions and the nutrient balance[5,6]. The major nutrients which influence the growth and production of citric acid are mainly the carbon, nitrogen, potassium, phosphorus sources and minerals[1, 6]. In our previous studies with solid state fermentation, the carbon sources enhanced the production of citric acid, the nitrogen and potassium limitation showed significantly negative effects on citric acid production by *A. niger* [7, 8]. Also, excessive nitrogen and potassium resulted in a higher growth and consequently splits the carbon flux towards energy and biomass production which restrict the production of citric acid[9, 10]. Under optimal fermentation conditions, over 90% yield was obtained on the basis of the amount of sugar consumed by submerged and solid substrate fermentations[11, 12].

Various carbon sources such as starch hydrolysate, sugarcane juice and molasses can be

used in citric acid production[13]. In recent years, the use of agro-industrial byproducts is economically favorable and can reduce various environmental problems[14,15]. Whey is a byproduct of the dairy industry representing 80 to 90% of the total milk volume processed. The world production of cheese and casein yields annually 4.0 x 10^7 tons having a biological (BOD) and chemical oxygen demand (COD) of 30 to 60 g/L and 60 to 70 g/L, respectively[16]. Disposal of whey as waste generates serious pollution problems for the environment and the process utilization of the whey waste saves cost of waste treatment[17].

Whey could be used as a fermentation substrate for the production of citric acid by *A. niger*[18]. The production of citric acid by *A. niger* using submerged fermentation of whey is known to be affected by the process conditions and nutrients[6,19-22]. The aim of the study was to optimize the composition of whey-based medium for citric acid production. To achieve the optimum medium composition, central composite design (CCD), one class of statically based optimization, was applied to find the most significant medium composition and optimized to produce maximum citric acid from whey-based medium.

MATERIALS AND METHODS

Microorganism

The white rot fungus *A. niger* NRRL 567 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and the stock was stored in tubes containing glycerol (30% v/v) at -76°C. *A. niger* stock culture was reactivated and cultivated by streaking a loop full of the culture on potato dextrose agar (PDA, Sigma, St. Louis, MO, USA) plates. After 5 days of incubation at 30°C, 10 ml of 0.1% Tween 80 were added to each plate to harvest spores. Diluted spore suspensions of 1.0x10^6 spores /ml were prepared as inoculums.

Medium composition

Cheese whey from the dairy plant of Montreal was used as basal fermentation medium after autoclaving at 121°C for 15 min. The proximate composition of cheese whey was found to be 4.8% lactose, 1.5% crude protein, 0.5% ash, 0.4% fat, 5.5% total soluble solid and 92.1% water. By adding glucose, $(NH_4)_2SO_4$ and KH_2PO_4, the final concentrations were adjusted for the 15 different experimental sets according to the CCD table. The pH of the whey-based medium was adjusted to pH 7.0±0.1 by adding 1 N NaOH or 1N HCl.

Fermentation

Flask test was performed with 49 mL of whey-based medium in 250 mL Erlenmeyer flasks. Each flask was inoculated with 1 mL of spore suspension (1.0x10^6 spores/ml) of *A. niger* incubated at 30°C on a rotary shaker (230 rpm). With 24 hours' time interval, fermented broths were harvested from flask and stored at -20! for the analysis of citric acid.

Analytical methods

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.

Experimental design

The purpose of the optimization was to identify the significant medium constituents for the production of citric acid. Statistically-based optimization (CCD) is very useful and widely used in the screening of significant constituents of the medium. For the 3 factor CCD, the medium constituents selected were glucose, $(NH_4)_2SO_4$ and KH_2PO_4 as indicated in Table 1. The statistically-based optimization procedure required to test 17 experimental sets representing the combination of 5 different levels of nutrient supplements (Tables 1 and 2). For statistical purposes, a triplicate center point was tested and the nutrient supplement level X_i associated with each coded level, –1.68, -1.0, 0, 1.0 and 1.68, was calculated as:

$$X_i = \chi_i \bullet \Delta X_i + X_{cp} \quad ...(1)$$

where χ = 1, 2, and 3 corresponds to each one of the three nutrient supplement; c_i = dimensionless coded level for X_i, namely –1.68, -1, 0, 1 and 1.68; X_i = real concentration of the independent variable for the code used; X_{cp} = concentration of independent variable at the coded value 0; ΔX_i = step change in concentration calculated as 20 g/L for glucose, 0.5 g/L for $(NH_4)_2SO_4$ and 5 g/L of KH_2PO_4. The "X_i value was determined from preliminarily unreported experimentation[23, 24, 28].

The actual levels of citric acid production obtained from 17 experimental sets were used to develop a second-order equation in order to predict citric acid production as a function of nutrient levels at 196 and 240 hr as follows[25]:

$$Y = \beta_0+\beta_1\chi_1+\beta_2\chi_2+\beta_3\chi_3+\beta_{11}\chi_1\beta+\beta_{22}\chi_2\beta+\beta_{33}\chi_3\beta+\beta_{12}\chi_1\chi_2+\beta_{23}\chi_{23}+\beta_{13}\chi_1\chi_3 \quad \ldots (2)$$

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} = interaction coefficients.

RESULTS AND DISCUSSION

Optimization of citric acid production

The medium constituents including glucose, $(NH_4)_2SO_4$ and KH_2PO_4 as independent variables were optimized for the maximum citric acid production at 196 and 240 hr of fermentation. In order to predict the optimal levels of citric acid produced, second-order polynomial equations were fitted to the results and developed as a function of the nutrient levels, respectively:

$$CA_{196h}=7.18+2.82\chi_1+0.19\chi_2-1.35\chi_3+0.28\chi_1^2+0.66\chi_2^2+0.042\chi_3^2+0.68\chi_1\chi_2+1.26\chi_1\chi_3+0.25\chi_2\chi_3 \quad \ldots(3)$$

$$CA_{240h}=7.89+3.95\chi_1+0.12\chi_2-0.76\chi_3-0.39\chi_1^2-0.23\chi_2^2-1.41\chi_3^2+0.41\chi_1\chi_2-0.17\chi_1\chi_3-0.17\chi_2\chi_3 \quad \ldots(4)$$

The R^2 of these equations (3) and (4), comparing the predicted to the measured values, shows a high degree of correlation, as it ranges between 0.97 and 0.94. Accordingly, the value of R^2 shows that only around 3% and 6% of the total variations were not explained by the second-order polynomial equations (Tables 2,3). The *F* value, Fisher variance ratio, is a stastically valid measure of how well the factors describe the variation in

Table 1. Central composite design (CCD) for the screening medium

Variables	Components	Unit	Coded and actual level				
			-1.68	-1	0	+1	+1.68
X_1	Glucose	g/L	6.36	20.0	40.0	60.0	73.6
X_2	$(NH_4)_2SO_4$	g/L	0.16	0.5	1.0	1.5	1.84
X_3	KH_2PO_4	g/L	1.59	5.0	10.0	15.0	18.4

Table 2. Experimental-design contain of experimental and predicted concentration of citric acid at 196 and 240 hr

RunNo	Glucose(g/L)	$(NH_4)_2SO_4$(g/L)	KH_2PO_4(g/L)	196 hr		240 hr	
				Observed	Predicted	Observed	Predicted
1	20.0	0.5	5.0	9.03	8.69	1.21	2.61
2	60.0	0.5	5.0	10.67	10.44	9.46	10.04
3	20.0	1.5	5.0	7.60	7.21	1.74	2.38
4	60.0	1.5	5.0	12.70	11.67	11.68	11.44
5	20.0	0.5	15.0	2.76	2.96	1.51	1.79
6	60.0	1.5	15.0	10.21	9.77	9.12	8.52
7	20.0	1.5	15.0	3.09	2.50	1.40	0.87
8	6.36	1.0	15.0	12.51	12.01	10.59	9.23
9	73.6	1.0	10.0	2.96	3.23	1.19	0.15
10	40.0	1.0	10.0	11.79	12.70	12.45	13.43
11	40.0	0.16	10.0	8.64	8.72	8.01	7.04
12	40.0	1.84	10.0	8.28	9.37	6.53	7.44
13	40.0	1.0	1.59	8.78	9.56	6.57	5.18
14	40.0	1.0	18.4	4.64	5.03	1.30	2.63
15	40.0	1.0	10.0	6.92	7.18	8.21	7.89
16	40.0	1.0	10.0	6.96	7.18	8.04	7.89
17	40.0	1.0	10.0	7.85	7.18	7.42	7.89

R^2 (coefficient of determination) = 0.9652 (196 hr) and 0.9393 (240 hr)

Table 3. ANOVA of the second-order model for citric acid production

	196 hr			240 hr		
	Sum of Squares	*F* value	*P* level	Sum of Squares	*F* value	*P* level
Model	155.85	21.56	0.0003*	253.47	14.43	0.0017*
X1	108.22	134.75	< 0.0001*	220.83	94.43	< 0.0001*
X_2	0.51	0.63	0.4534	0.51	0.22	0.6536
X_3	24.77	30.84	0.0009*	6.39	2.73	0.1423
X_1^2	0.87	1.08	0.3333	1.97	0.84	0.3895
X_2^2	4.91	6.12	0.0426*	0.75	0.32	0.5887
X_3^2	0.020	0.024	0.8804	23.31	9.97	0.0160*
X_1X_2	3.68	4.58	0.0695	0.64	0.28	0.6159
X_1X_3	12.78	15.91	0.0053*	0.71	0.30	0.5981
X_2X_3	0.51	0.63	0.4518	0.71	0.30	0.5981

X_1 = Glucose; X_2 = $(NH_4)_2SO_4$; X_3 = KH_2PO_4
*Significant at the 95% level

Table 4. Optimization of citric acid production

	Glucose. g/L	$(NH_4)_2SO_4$ g/L	KH_2PO_4 g/L	Predicted Citric acid production g/L	
	X_1	X_2	X_3	196 hr	240 hr
1	60.0	1.50	8.08	11.74	12.0
2	60.0	1.50	8.19	11.74	12.0
3	60.0	1.50	8.42	11.75	11.98
4	60.0	1.50	8.13	11.74	12.0
5	60.0	1.50	7.45	11.72	12.0

the mean of data [1]. The ANOVA indicated that two second order regression models were higly significant, as an evident from *F*-test with a very low *p*-values. Similarly, glucose (X_1) and KH_2PO_4 (X_3) had a significant effect on citric acid production at 196 and 240 hr while $(NH_4)_2SO_4$ (X_2) had no significant effects within a tested level. Proven accurate in predict citric acid production, equations (3) and (4) were used to analyze the interactive effects of the three factors. The results of equations including their linear, quadratic and cross product terms, were applied to plot a 3-dimentional curve predicting citric acid production at 196 and 240 hr[26,27].

The predicted interaction between glucose and $(NH_4)_2SO_4$ on citric acid production is presented in Figs. 1a and 1b, respectively. Both plots show an increase in citric acid production with increasing initial glucose concentration within all ranges of $(NH_4)_2SO_4$ tested. Increasing the initial $(NH_4)_2SO_4$ concentration did not significantly increased citric acid production. Thus, maximum citric acid production was predicted using the highest levels of supplemented glucose and but not necessarily the high level of $(NH_4)_2SO_4$. Similar observations for citric acid production were reported by Favela-Torres *et al*.[6]. It was found that optimal levels of sugar supplementation ranged between 10 to 15% (w/v) and that citric acid production increased with glucose levels.

The interactions between glucose and KH_2PO_4, on citric acid production at a constant $(NH_4)_2SO_4$ level of 1.0 g/L, after 196 and 240 hr, are shown in Figs. 2a and 2b, respectively. Citric acid production significantly increased with glucose levels but not with KH_2PO_4 level both at 196 and 240 hr. The interaction between $(NH_4)_2SO_4$ and KH_2PO_4 at a constant glucose level of 40 g/L, is illustrated in Figs. 3a and 3b at 196 and 240 hr, respectively. Citric acid production was found to decrease with increasing levels of supplemented KH_2PO_4 at 196 hr of fermentation while a maximum

citric acid production was obtained with 9 g/L of KH_2PO_4 at 240 hr. However, citric acid production was not significantly affected by levels of $(NH_4)_2SO_4$. The second order regression model predicted the citric acid production, which was considerably affected by varying the concentration of glucose. Thus, higher citric acid levels are achievable with higher level of glucose. This result shows that cheese whey is deficient in readily available sugar for citric acid production and whey-based medium can produced high level of citric acid only with high glucose supplementation.

Cube graphs were used to predict the simultaneous interaction between all three nutrient supplements at 196 and 240 hr of fermentation (Figs. 4a and 4b). Each cube has eight corners representing eight different experimental conditions. The plus (+) and minus (-) signs represent the coded levels (-1 and +1) for each nutrient[28]. The cube's upper right side gives a maximum citric acid production when using the highest concentration of glucose (A+) along with

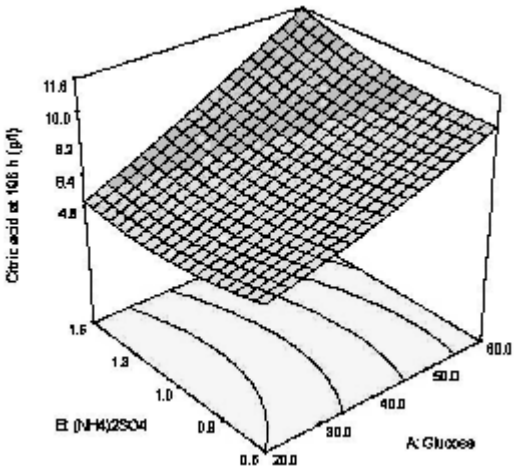

Fig. 1(a). Predicted citric acid production as a function of glucose and $(NH_4)_2SO_4$ with constant levels of KH_2PO_4 (10 g/L) and whey at196 hr

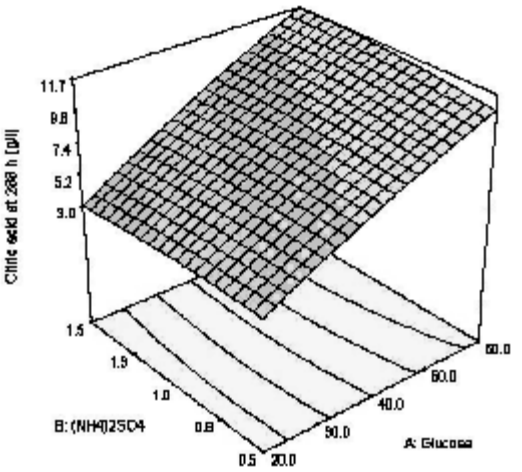

Fig. 1(b). Predicted citric acid production as a function of glucose and $(NH_4)_2SO_4$ with constant levels of KH2PO4 (10 g/L) and whey at 240 hr

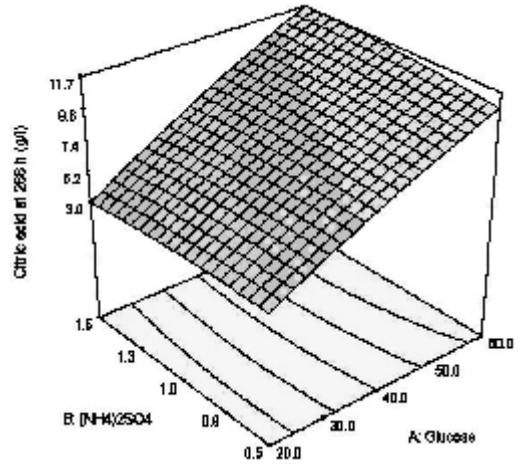

Fig. 2(a). Predicted citric acid production as a function of glucose and KH_2PO_4 with constant levels of $(NH_4)_2SO_4$ (1 g/L) and whey at196 hr

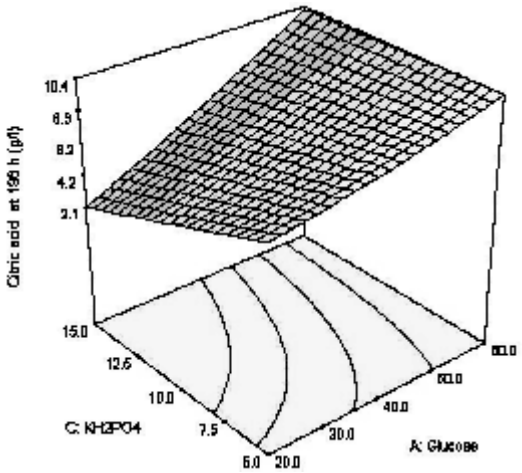

Fig. 2(b). Predicted citric acid production as a function of glucose and KH_2PO_4 with constant levels of $(NH_4)_2SO_4$ (1 g/L) and whey at240 hr

the highest concentration of $(NH_4)_2SO_4$ (B+), both after 196 and 240 hr of fermentation.

Optimum nutrient composition

Optimum levels of nutrient supplementation were predicted using second order regression models. All combinations give similar results, the following solution was selected as the most economical combination producing 12.0 g/L of citric acid: glucose at 60 g/L, $(NH_4)_2SO_4$ at 1.5 g/L and KH_2PO_4 at 8.1 g/L (Table 4). Under this nutrient level, the validation flask test showed that citric acid of 12.4 g/L was produced at 240 hr (Fig. 5). The measured citric acid production agreed with that predicted by the second-order equations (3) and (4), of 12.0 g/L at 240 hr. This production level represents 2 folds increase, as compared to the whey medium with 60 g/L glucose producing 6.1 g/L of citric acid.

Citric acid production obtained with the supplemented whey-based medium was compared to that obtained with the Czapek-Dox medium [29]. This conventional medium for submerged

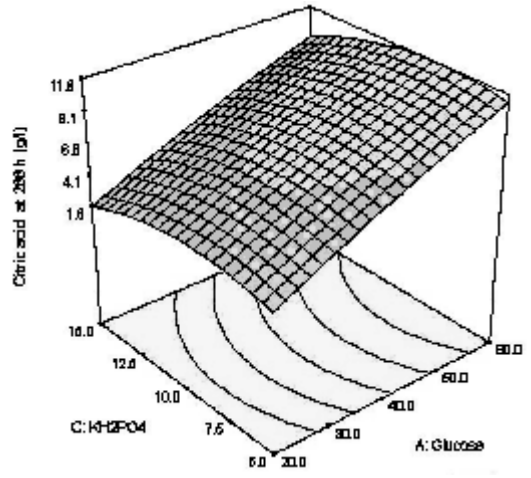

Fig. 3(a). Predicted citric acid production as a function of $(NH_4)_2SO_4$ and KH_2PO_4 with constant levels of glucose (40 g/L) and whey at 196 hr

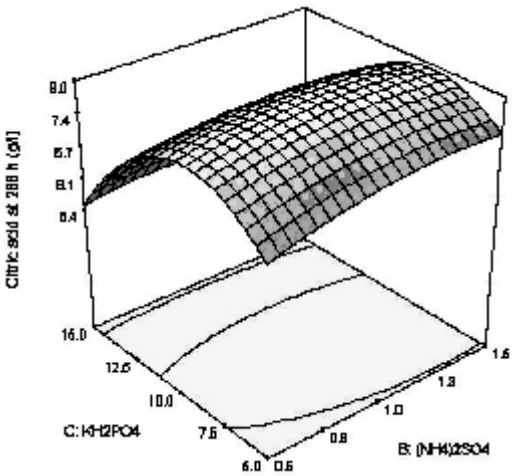

Fig. 3(b). Predicted citric acid production as a function of $(NH_4)_2SO_4$ and KH_2PO_4 with constant levels of glucose (40 g/L) and whey at 240 hr

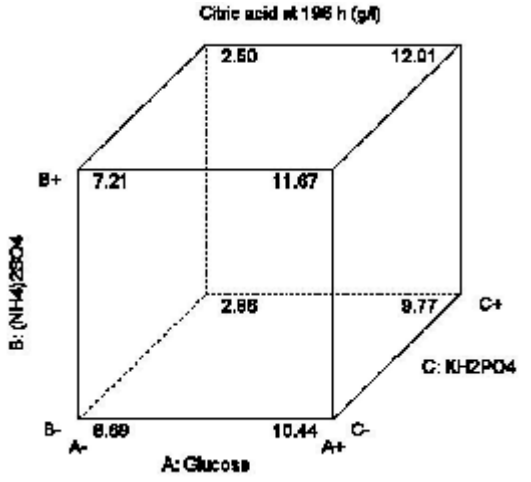

Fig. 4(a). Cube plot of CCD for 196 hr of fermentation. The cube corner values are the levels of citric acid production predicted using the second-order polynomial equation (3) applied to a whey medium

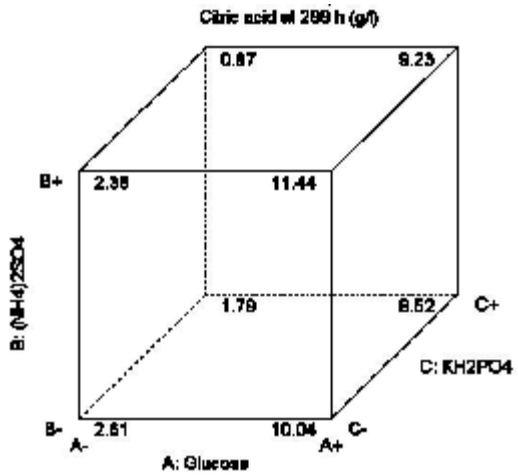

Fig. 4(b). Cube plot of CCD for 240 hr of fermentation. The cube corner values are the levels of citric acid production predicted using the second-order polynomial equation (4) applied to a whey medium

fermentation produced a maximum citric acid production of 8.7 g/L, after 196 hr, with 60 g/L of supplemented glucose. The whey-based medium produced 27% more citric acid at 196 hr fermentation, as compared to the Czapek-Dox medium.

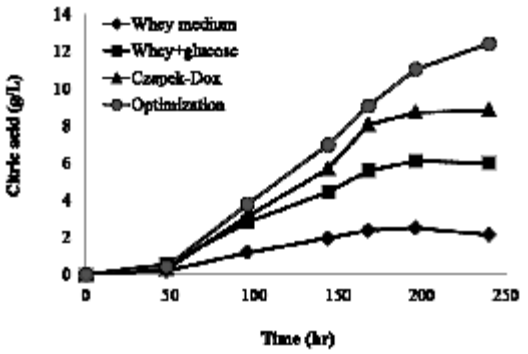

Fig. 5. Citric acid production in 250 ml flask using a control medium of a whey medium with 60 g/L glucose; a whey medium supplemented with 59.6 g/L glucose, 1.5 g/L $(NH_4)_2SO_4$ and 7.6 g/L KH_2PO_4; and a Czapek-Dox medium containing 60 g/L glucose and other nutrient. (♦: whey medium; ■: whey medium+glucose; ▲: Czapek-Dox medium; ●: optimized medium).

CONCLUSIONS

The statistically-based optimization procedure, CCD, was a valid and efficient tool in optimizing the supplementation of a whey-based medium for citric acid production by *A. niger*. This optimization method leads to a 2 fold increase in citric acid production, compared to the whey-based medium with 60 g/L glucose supplementation. For citric acid production by *A. niger*, glucose and KH_2PO_4 supplementation was found to have a highly significant effect, whereas, $(NH_4)_2SO_4$ was found to have no significant effect at 196 and 240 hr. Nutrient supplementation of the whey-based medium increased citric acid production; the final level was increased from the citric acid production obtained with a Czapek-Dox medium. To further improve citric acid production using a whey-based medium, additional testing is required to optimize other fermentation conditions such as pH, inoculation density, temperature and stimulators such as methanol, ethanol, fatty acid and phytate.

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