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Optimization of citric acid production by *Aspergillus niger* NRRL 567 grown in a column bioreactor

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Abstract−Citric acid production using *Aspergillus niger* NRRL 567 grown on peat moss has been optimized in a column bioreactor using a statistically based method. A 2^3 full factorial design with eight fermentation conditions was applied to evaluate significance on citric acid production and their interactions between variables, where the three independent variables evaluated were aeration rate, bed depth and temperature. Aeration rate and fermentation temperature were identified to be significant variables. Citric acid production markedly increases with aeration rate and fermentation temperature; however, the bed depth of solid substrate showed an insignificant effect on citric acid production. The optimum fermentation condition for citric acid production in a column bioreactor consisted of aeration rate of 0.84 vvm, bed depth of 22 cm and fermentation temperature of 32 °C. Under a given condition, a maximum citric acid production of 120.6 g/*l* was predicted and matched well with the experimental value of 123.9 g/kg.

Key words: *Aspergillus niger*, Citric Acid, Solid Substrate Fermentation, Optimization, Peat Moss

INTRODUCTION

Solid substrate fermentation (SSF) involves the growth of microorganisms on moist solid substrates in the absence of free flowing water [1,2]. In general, the solid substrate acts as physical support, provides nutrients and holds water meeting the requirements of the growth of microorganism [3-5]. As SSF provides growth conditions simulating natural habitats, it is presently gaining interest for the manufacturing of bulk chemicals and highly purified enzymes at a lower cost than submerged fermentation. Currently, SSF has been widely used for food fermentation, enzyme production by the Koji process, mushroom production, mould-ripening of cheese and partial composting of agricultural residues [5-8].

The decrease of operating cost is the one of attractive aspects of SSF when SSF utilizes agro-industrial byproducts as a solid substrate. The potential use of agro-industrial byproducts with SSF for citric acid production has been intensively studied over the past five decades [9,10]. The white rot fungus *A. niger* can be grown on sugar-rich agro-industrial byproducts using SSF to produce organic acids for various purposes, including bioremediation. Weak organic acids such as citric, tartaric, oxalic and formic acids have been used as chelating agents for the bioremediation of soils and sediments contaminated with heavy metals [11]. The organic acids produced by *A. niger* have carboxyl groups which tend to donate protons (H^+), resulting in negatively charged carboxyl groups capable of competing against soil particles for heavy metal adsorption. A prerequisite for the development of such an *in-site* bioremediation technique, using the fungus *A. niger*, is the optimization of a semi-continuous production technique using sugar-rich organic byproducts.

The objective of the present study was, therefore, to evaluate citric acid production by *A. niger* when grown using a semi-continuous system consisting of a column bioreactor holding the sugar-rich byproduct periodically supplemented with nutrient solution. This column bioreactor also requires the flushing of its solid substrate to recover the citric acid produced for the bioremediation of heavy metals from contaminated soils. The sugar-rich byproduct was simulated by using peat moss wetted with a nutrient solution. The physical fermentation parameters optimized were: aeration rate, bed depth of solid substrate and fermentation temperature. The present study applied a 2^3 FFD to identify the optimum fermentation condition for citric acid production and to evaluate the interactions between the variables.

EXPERIMENT

1. Microorganism

Aspergillus niger NRRL 567 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and was stored at −76 °C, in tubes containing glycerol (30% v/v). *A. niger* spores were produced on a potato dextrose agar (Sigma, St. Louis, MO, USA) plates kept at 30 °C. After ten days of incubation on potato dextrose agar plates, 10 ml of 0.1% Tween 80 (Sigma, St. Louis, MO, USA) was added to each plate to harvest the spores. Diluted spore suspensions of 4.0×10^6 spores/ml were counted with a hemocytometer and prepared as inoculum as described by Kim et al. [12].

2. Solid Substrate

Sphagnum peat moss (Schultz company, Mississauga, Ontario, Canada) was used as the solid substrate. It was dried at 60 °C for 48 h and screened to remove all particles larger than 2.0 mm, before

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Table 1. Physico-chemical characteristics of the sphagnum peat moss

	Units	Value
Bulk density	kg/m^3	276.4
Moisture content	%	52.7
Carbon	% DW	53.1
Nitrogen	% DW	2.1
Phosphorus	% DW	0.01
Potassium	% DW	0.15
Ash	% DW	2.8
Volatile solids	% DW	97.1
NH_4-N absorption capacity	mg/kg	27.5
pH	-	4.38
Coarse particle (>2.00 mm)	% DW	14.3
Medium particle (1.00-2.00 mm)	% DW	18.4
Fine particle (0.5-1.00 mm)	% DW	20.5
Very fine particle (<0.5 mm)	% DW	46.8

Note: All analysis are reported on a dry weight basis; the carbon content was calculated from {(100%−ash (%))/183}; DW=dry weight [23].

being rewetted and inoculated with *A. niger* NRRL 567. The peat moss was characterized for density, total carbon, total nitrogen, total phosphorus, pH and NH_4-N absorption (Table 1). Dry peat moss samples of 40 and 80 g were placed in 0.5 L autoclave bags (Sigma Chemical Co, USA) and wetted with a nutrient solution. The nutrient solution provided the following glucose and salt levels per kg DPM: 250 g glucose, 15.4 g $(NH_4)_2SO_4$, 43.9 g KH_2PO_4 and 4.0 g NaCl. The moisture content of the dry peat moss (DPM) was further raised to 80% by adding an additional amount of distilled water. Each wetted peat moss sample was supplemented with stimulators, namely olive oil (Bertolli, Laval, Qc, Canada) and phytate (40%wt, Aldrich, St. Louis, MO, USA), on the basis of 28.9 and 19.6 g/kg DPM, respectively, as described by Barrington and Kim [13]. Once supplemented with nutrients and stimulators, the wet peat moss samples were autoclaved for 20 minutes at 121 °C. And then, all samples received a third stimulator, methanol (99+% wt, Sigma Chemical Co, USA), at a concentration of 40.9 g/kg DPM, and 1.0 ml of inoculum containing 4×10^6 spores/ml for every 20 g DPM. The initial pH of the peat moss was determined by thoroughly mixing the content of each inoculation bag, removing 5 g of wet sample, soaking it in distilled water at a ratio of 1 : 10 and measuring its pH with a pH meter (Sartorius PB20, Canada).

3. Column Bioreactor

The semi-continuous fermentation was performed in a column bioreactor with the following dimensions: internal diameter 7 cm, height 23 cm and internal volume 0.89 L. The cylindrical bioreactor consisted of a 3 mm thickness Plexiglas with acrylic top and bottom. Non-sterile air was saturated with water by passing through humidifiers and supplied from the bottom. Outlets on the top and bottom allowed for air exhaustion and water drainage, respectively. After inoculation, the bioreactor was placed in an incubator for 12 days under non-sterile conditions.

4. Fermentation

Each column could hold up to 300 g of wet peat moss. The two bed depths tested corresponded to 22±0.5 cm (270 g of wet peat moss) and 11±0.5 cm (135 g of wet peat moss). The effect of temperature was tested by incubating the column bioreactors for 12 days in a closed chamber maintained at 22 and 32 °C. The aeration rates tested were 0 and 0.84 vvm (volumes of air per minute per working volume of bioreactor). Under non-sterile condition, the peat moss was fed and washed at four-day intervals as follows. The peat moss contained in each column bioreactor was soaked twice for one hour, using non-sterile de-ionized water. After each soaking with 100 ml of de-ionized water, the excess water was drained and collected to determine its citric acid and glucose content. Once soaked and drained twice, each column bioreactor was placed in an incubator for 24 h to remove excess free flowing water. After completion of all washing processes, the 135 and 270 g wet samples each received 20 ml and 40 ml of concentrated glucose solutions (500 g/L), respectively. On day 12, the entire content of each column bioreactor was harvested for glucose and citric acid determination, by soaking in distilled water for 60 min at 150 rpm at room temperature for citric acid and glucose extraction [14].

5. Citric Acid and Glucose Determination

For each washing session, the washing effluent was collected and centrifuged at 11,000×g for 10 minute. After pyridine and acetic anhydride were added to develop color, citric acid was determined by spectrophotometry at 420 nm [15]. Glucose was analyzed by the 3,5-denitrosalicylic acid (DNSA) method as described by Miller [16]. After citric acid and glucose concentrations were measured at each sampling session, citric acid concentration was calculated based on the dry weight of peat moss. The citric acid yield and glucose consumption were calculated as follows:

$$\text{Citric acid yield (\%)} = 100\times[\text{produced citric acid (g/}l\text{)/utilized glucose (g/}l\text{)}] \quad (1)$$

$$\text{Glucose consumption (\%)} = 100\times[\text{utilized glucose (g/}l\text{)/initial glucose (g/}l\text{)}] \quad (2)$$

6. Full Factorial Design (FFD)

A 2^3 FFD was applied to determine the effect of fermentation variables on citric acid production by *A. niger* NRRL567 grown in column bioreactor. The 2^3 FFD procedure requires two levels of each variable, where each high and low levels are coded values of +1 and −1 (Table 2). Thus, eight combinations of fermentation conditions were tested and their input variables are listed in Table 3.

The combinations of eight conditions applied to produce a model expressed by using the following equation:

Table 2. Coded values used in 2^3 FFD to optimize the fermentation variables for citric acid production in column bioreactor

	Variables	Unit	Coded and actual level	
			−1	1
X_1	Aeration rate	vvm	0	0.84
X_2	Bed depth	cm	11	22
X_3	Temperature	°C	22	32

vvm (min^{-1}): volume of aeration (ml)/working volume of bioreactor (ml)/minute (min).

$$Y=\beta_0+\beta_1\chi_1+\beta_2\chi_2+\beta_3\chi_3+\beta_{12}\chi_1\chi_2+\beta_{23}\chi_2\chi_3+\beta_{13}\chi_1\chi_3+\beta_{123}\chi_1\chi_2\chi_3 \quad (3)$$

where

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

The significance and adequacy of the model Eq. (3) was measured by analysis of variance (ANOVA), defined as "a method for estimating the amount of variation within all treatment and comparing it to the variables between treatments" [17]. The F-test was applied to evaluate the statistical significance of the model. The F value represents "the ratio of the mean square due to regression to the mean square due to error" [18]. High F values imply that models are significant and can accurately predict the experimental results. The importance of the F values was interpreted as a level of provability (P), where a level under 0.05 implies a level of confidence of over 95%. The coefficients of all linear terms of the model equation provided a measure of the effect of the level of the independent variable on the response [19].

RESULTS AND DISCUSSION

1. Optimization Using 2^3 FFD

The experimental levels of citric acid concentration and yield obtained from the eight runs are listed in Table 3. Citric acid production ranged from 30 to 124 g/kg DPM, and the yield ranged from 4.4 to 17.8%. The highest levels of citric acid and yield were obtained with an aeration rate of 0.84 vvm, peat moss bed depth of 22 cm and temperature of 32 °C. Citric acid production level and yields measured at 32 °C were four times as high as that measured at 22 °C, implying temperature had significant positive effect on citric acid production. As an aeration rate of 0.84 vvm increased citric acid production and yield by a factor of 3 compared to the run with no aeration, the aeration rate was the variable having a significant effect. Also, the bed depth of peat moss was the variable which had an effect, as doubling its bed depth increased citric acid production and yield, but the effect of bed depth on responses was not significant compared to other variables.

The experimental levels were used to generate model equations for predicting citric acid production and yield, by computing the significant linear, squared and interaction coefficients. Neglecting the terms, χ_2 and $\chi_1\chi_2\chi_3$, which were insignificant, the result of 2^3 FFD produced the following equation that describes the effects of the variables on the responses. The equation shows that citric acid production ($Y_{CA\ total}$) and yield ($Y_{CA\ yield}$) are a function of the coded levels of the three tested input variables. The model equations were used to predict those optimal fermentation conditions for best citric acid production:

$$Y_{CA\ total}=54.47+16.01\chi_1+21.46\chi_3+6.75\chi_1\chi_2+17.38\chi_1\chi_3+4.54\chi_2\chi_3 \quad (4)$$

$$Y_{CA\ yield}=9.41+1.82\chi_1+3.37\chi_3+0.45\chi_1\chi_2+2.60\chi_1\chi_3+0.81\chi_2\chi_3 \quad (5)$$

Table 3. Experiment and predicted values of citric acid production and yield

Run no	X_1	X_2	X_3	Citric acid production (g/kg DPM)		Citric acid yield (%)	
				Experimental	Predicted	Experimental	Predicted
1	0	11	22	42.4	45.7	8.7	8.1
2	0.84	11	22	33.5	29.4	6.1	5.6
3	0	22	22	22.4	23.1	4.9	5.6
4	0.84	22	22	29.8	33.9	4.4	4.9
5	0	11	32	48.8	44.8	8.5	8.0
6	0.84	11	32	114.7	98.0	16.6	15.9
7	0	22	32	36.3	40.3	8.3	8.7
8	0.84	22	32	123.9	120.6	17.8	18.5

R^2=0.988 (citric acid production) and 0.986 (citric acid yield), X_1=aeration rate; X_2=bed depth of solid substrate; X_3=fermentation temperature.

Table 4. ANOVA table: influences of fermentation variables on citric acid production and yield

	Citric acid production and yield					
	Citric acid (g/kg DPM)			Citric acid yield (%)		
	Sum of Squares	F value	P level	Sum of Squares	F value	P level
Model	11002.07	506.81	0.0340*	180.72	626.84	0.0306*
X_1	2890.28	798.85	0.0225*	26.43	549.98	0.0271*
X_2	90.32	24.96	0.1258	2.44	50.82	0.0887**
X_3	4786.33	1322.90	0.0175*	90.99	1893.65	0.0146*
X_1X_2	180.50	49.89	0.0895**	1.60	33.34	0.1092
X_1X_3	3003.13	830.04	0.0221*	53.98	1123.33	0.0190*
X_1X_3	51.51	14.24	0.1649	5.28	109.91	0.0605**

*Significant at the 95% level
**Significant at the 90% level

where the coefficients χ_1, χ_2 and χ_3 correspond to the coded value of fermentation parameters.

The goodness of fit of the regression equation was checked by determining its coefficient of determination (R^2), where the R^2 of models was found to be 0.988 and 0.986 for citric acid production and yield, respectively. These values indicate that 98.8 and 98.6% of the variability in the response can be explained by equations, which indicates good agreement between the predicted responses by models and the experimental data. F values of 506.8 and 626.8 for citric acid production and yield were obtained in ANOVA (Table 4). For the citric acid production and its yield, χ_1 (aeration rate) and χ_3 (temperature) indicated that these two variables have significant positive effects on Reponses ($P<0.05$) within the tested range.

The effects of the independent variables on citric acid production and yield were studied by using response surface curves which were generated by regression models. The interaction between aeration rate and bed depth was plotted at a fixed fermentation temperature of 27 °C. Fig. 1A shows citric acid production significantly increased with the aeration rate. At a bed depth of 22 cm, citric acid production increased noticeably with increased aeration rate. However, at constant aeration rate, citric acid production did not changed significantly with a depth of bed. As compared to a bed depth of 22 cm, a bed depth of 11 cm demonstrated less aeration effect. Under optimum levels, a maximum citric acid production of 77.2 g/*l* was predicted. A similar response surface curve was obtained for citric acid yield (Fig. 1B), which also maximized at 11.7%, but varied little between bed depth. Thus, thick beds of solid substrates require a forced aeration to support high citric acid production. The effect of aeration rate has been intensively studied by many researchers who reported that the growth of *A. niger* for citric acid is aerobic, that forced aeration is indispensable for the over-production of citric acid [20].

The interactive effect of aeration rate and fermentation temperature on citric acid production and yield are plotted in Figs. 2A and

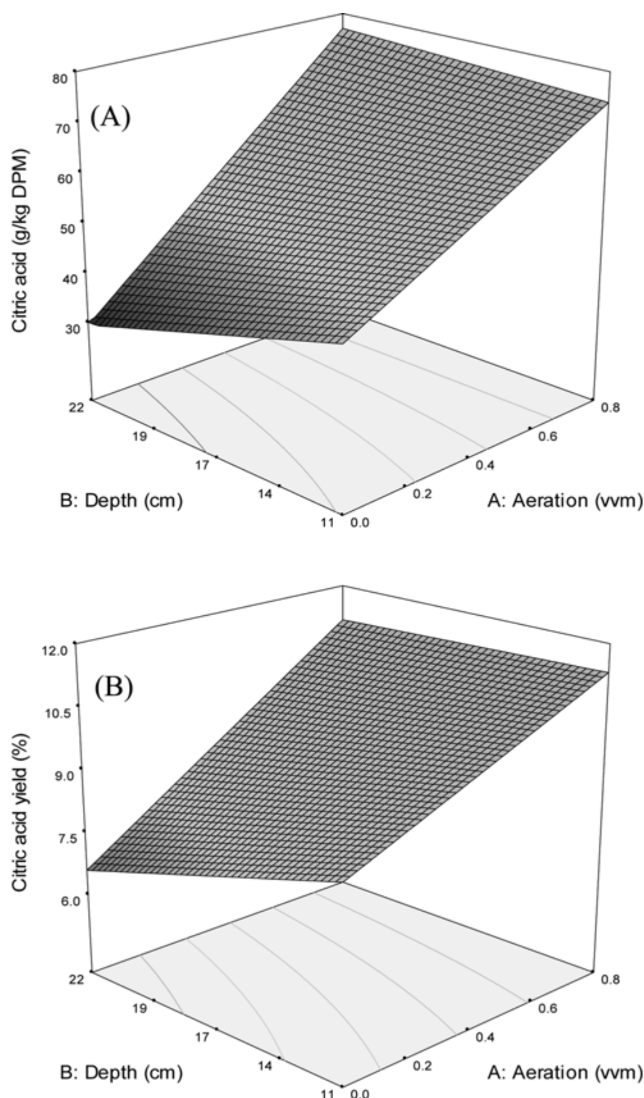

Fig. 1. Citric acid production from *A. niger* NRRL 567 (A) and production yield (B) as a function of aeration rate and bed depth of solid substrate at a constant fermentation temperature (27 °C).

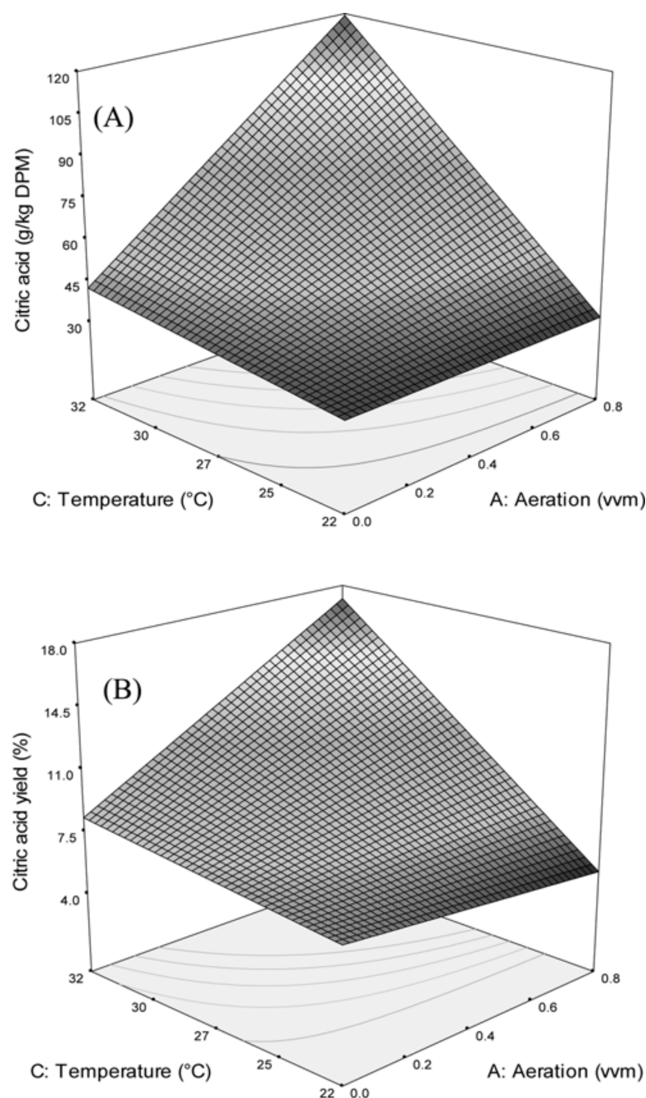

Fig. 2. Citric acid production from *A. niger* NRRL 567 (A) and production yield (B) as a function of aeration rate and fermentation temperature at a constant bed depth of solid substrate (16.5 cm).

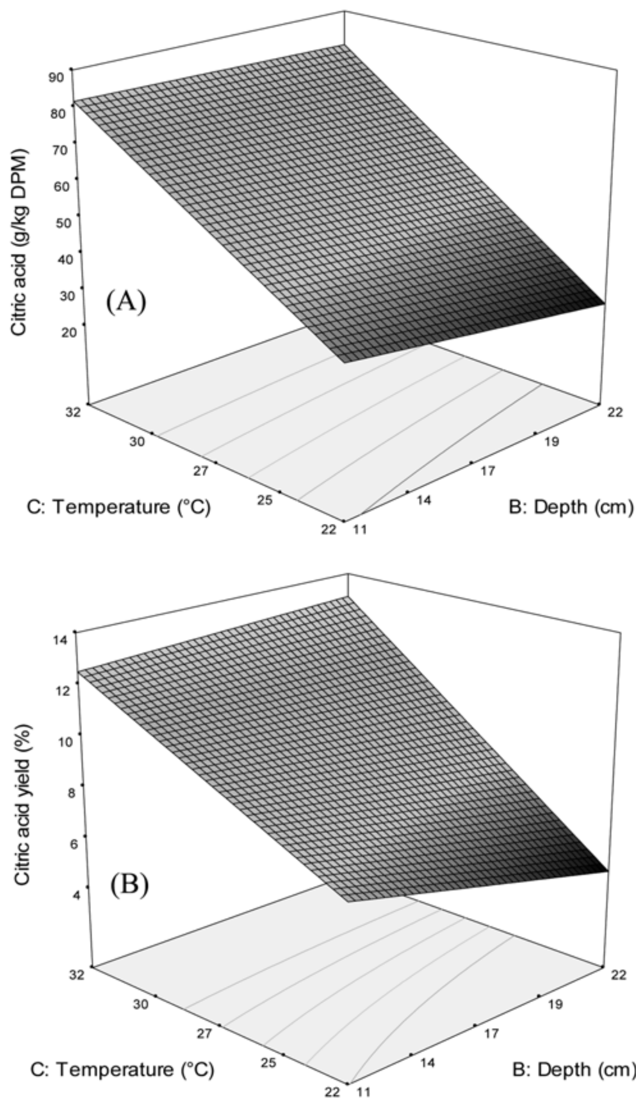

Fig. 3. Citric acid production from *A. niger* NRRL 567 (A) and production yield (B) as a function of bed depth of solid substrate and temperature at a constant level of aeration rate (0.42 vvm).

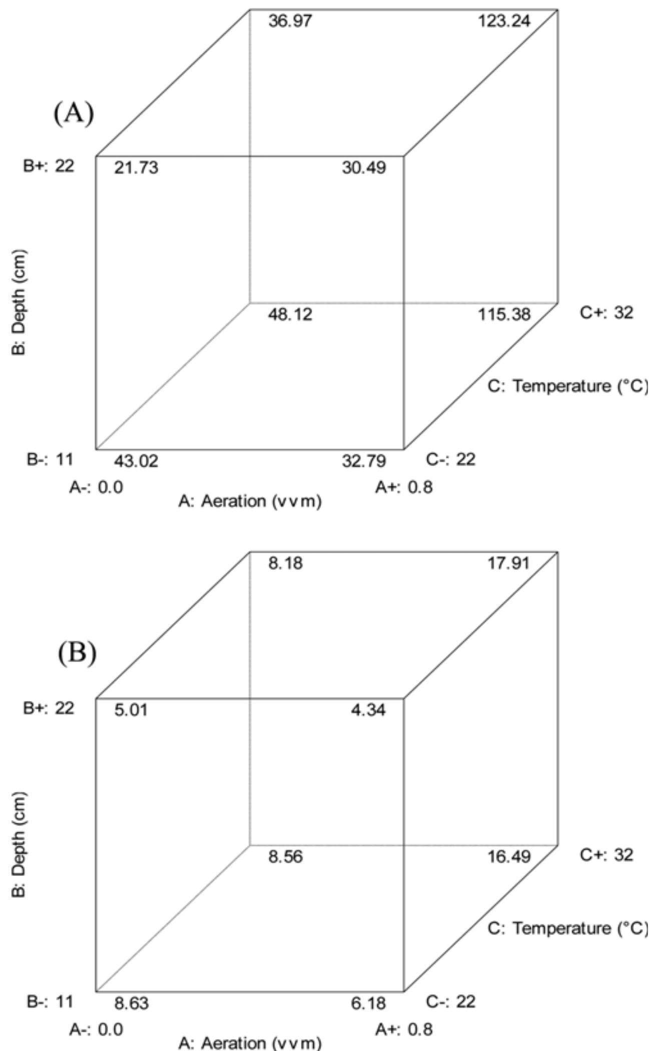

Fig. 4. Cube plot showing the interactive effect of aeration rate, bed depth and fermentation temperature on citric acid production (A) and production yield (B). The cube corner values are the level of citric acid production and yield predicted using the polynomial Eqs. (4) and (5).

2B. At a high fermentation temperature, aeration rate had a significant effect on citric acid production, while aeration rate showed no significant effect in citric acid production at a fermentation temperature of 22 ºC. Thus, the highest citric acid production was obtained at 32 ºC with forced aeration. As the cylindrical column bioreactor has a relatively small surface area compared to its bed volume, surface aeration leads to insufficient oxygen supply within the center of the solid substrate. However, at low fermentation temperature, cell growth and citric acid production are mainly limited by temperature, and surface aeration suffices in supplying oxygen to *A. niger.*

Figs. 3A and 3B plot the interactive effect of fermentation temperature and bed depth on citric acid production and yield, respectively. The two plots show the dominant effect of the fermentation temperature on citric acid production and yield, while the depth of solid substrate shows an insignificant effect. The highest citric acid production was achieved at a combination of fermentation temperature of 32 ºC and a bed depth of 22 cm.

Cube plots were developed (Figs. 4A and 4B) to evaluate the combined effect of the three tested variables simultaneously. A cube plot has eight corners representing eight experimental conditions. The plus (+) and minus (–) signs represent the coded level (–1 and +1) of each variable [17]. The maximum citric acid production and yield were predicted at a high level of aeration rate (A+), a thick solid substrate (C+) and a high fermentation temperature (C+). The cube plot indicated two dominant surfaces, the back side with a high temperature and the right side high aeration rate, both increasing citric acid production and yield. However, citric acid production and yield were not significantly different between the top and the bottom sides. Thus, the combined influence of temperature and aeration rate had a dominant effect on citric acid production and yield.

CONCLUSIONS

The statistically based optimization procedure using an FFD proved

to be an effective method in optimizing fermentation conditions. In the experiment using the column bioreactor, citric acid production and yield were 124 g/kg DPM and 18%. The low productivity of citric acid in the column bioreactor may result from a low surface aeration, a channeling of nutrient solution, a temperature gradient, a variation of moisture content and a restricted gas exchange. However, *in-situ* soil flushing using fungal organic acids, especially citric acid, represents a biodegradable approach and effective remediation technique compared to using chemical chelators [21,22]. As the semi-continuous fermentation of citric acid in solid substrate provides citric acid production and the flushing of its solid substrate to recover the citric acid for the bioremediation of heavy in the non-sterile operating condition without contamination, it still has the potential for using *in-situ* remediation system for heavy metal contaminated soil. Thus, to achieve this purpose, an appropriate bioreactor type and a future optimization of fermentation condition are required.

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