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RECENT DEVELOPMENTS IN OPTIMIZATION OF CITRIC ACID FERMENTATION PROCESS-A REVIEW

K. ANAND KISHORE & G. VENKAT REDDY

Department of Chemical Engineering,National Institute of Technology,Warangal-506004, A.P, India

ABSTRACT

Citric acid is produced mainly by submerged fermentation using Aspergillus Niger. The present review reports the study on Optimization of process parameters using one factor at a time (OFAT) method and Response surface methodology (RSM). Regression equations were used to model the fermentation in order to determine optimum fermentation conditions. Maximum citric acid production was obtained at pH: 5.35, temperature:30-32^{0}C, fermentation days of 5.7 days with 221.66 g of substrate/L, 0.479 g of ammonium nitrate/L and 2.33 g of potassium ferrocyanide/L. A maximum citric acid production of 82 g kg^{-1} dry peat moss (DPM) was reached after 72 h with the following optimized nutrient solution, in terms of g kg^{-1} DPM: 967.9 glucose, 15.4 (NH4)2SO4, 43.9 KH2PO4 and 4.0 NaCl. The maximum citric acid production of 354.8 g/kg DPM was resulted from the combination of 19 g phytate/kg DPM, 49 g olive oil/kg DPM and 37 g methanol/kg DPM at 120 h. The optimum experimental condition was found using 7.0 mg/L of Fe^{+3} and 6.5 mg/L of Zn^{+2} in absence of Mn^{+2}.

KEYWORDS: Aspergillus Niger, Citric acid, factorial design, operating variables, Plackett-Burman design and Response Surface methodology.

INTRODUCTION

Citric acid is a weak organic acid and its molecular formula is $C_6H_8O_7$. It is produced commercially by submerged fermentation using Aspergillus niger and has been widely used in the food, beverage, chemical, pharmaceutical and other industries. Citric acid productivity by Aspergillus niger can be improved by the fermentation parameters: Initial sucrose concentration, initial P^H, Additives, Stirrer speed, Incubation period, Fermentation temperature and Nutrient concentration [1]. Sucrose is the most commonly used substrate for producing citric acid by fermentation. A successful process depends on the optimization of the fermentation parameters. Response surface method (RSM) has been the widely used and preferred method for the optimization of fermentation process. The most popular RSM designs is the central composite design (CCD) as it can simultaneously consider several factors at many different levels and corresponding interactions using these factors and a small umber of interactions. It is more efficient than the other methods. Basically, this optimization process involves three major steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model and predicting the response and checking the adequacy of the model [2].

LITERATURE REVIEW

Carried out the optimization of process parameters such as moisture content, incubation temperature and initial pH (fixed) for the improvement of citric acid production from oil palm empty fruit bunches using traditional one-factor-at-a-time (OFAT) method and response surface methodology (RSM). The possible optimum levels of moisture content, incubation temperature, and initial p^H were found from the OFAT study to be 70%, 30 - 32^{o}C and 5.5 - 8 respectively. The optimum moisture content of 70.3% (v/w) and incubation temperature of 33.1°C with initial p^H of 6.5 gave the maximum production of citric acid (369.16 g/kg of dry EFB). The analysis of variance (ANOVA) of the statistical optimization using central composite design showed that moisture content ($p<0.001$) and incubation temperature ($p<0.0001$) as well as the interaction of these two parameters were highly significant for the citric acid production [3]. Suggested that palmyra jaggery is a suitable substrate for increasing the yield of citric acid using A.niger MTCC 281 by submerged fermentation. The quantitative effects of P^H, temperature, time of fermentation, sugar concentration, nitrogen concentration and potassium ferrocyanide on citric acid production were investigated using a statistical experimental design. Regression equations were used to model the fermentation in order to determine optimum fermentation conditions. After optimizing media components and conditions of fermentation, maximum citric acid production was obtained at p^H 5.35, 29.76^0 C, 5.7 days of fermentation with 221.66 g of substrate/l, 0.479 g of ammonium nitrate/l and 2.33 g of potassium ferrocyanide/l [4]. Investigated the effect of potassium ferrocyanide ($K_4Fe(CN)_6$) concentration on citric acid production and the prevention of solvent toxicity using corn oil. Citric acid concentration increased with increasing $K_4Fe(CN)_6$ concentration. Solvent toxicity was reduced using corn oil in the extractive citric fermentation. After optimization with response surface method, Citric acid production by extractive fermentation was increased approximately 40% [5]. Optimized the initial glucose, nitrogen (N), phosphorus (P) and NaCl levels of a nutrient solution. Seventeen different combinations of nutrients were tested to grow A. niger at 30^{0}C for 48 and 72 h, and to measure the resulting citric acid production. With the central composite design method (CCD), the results were used to produce a second order equation defining citric acid production as a function of initial glucose, N, P and NaCl levels. A peak citric acid production of 82 g/ kg dry peat moss (DPM) was reached after 72 h with the following optimized nutrient solution in terms of g/kg DPM: 967.9 glucose, 15.4 $(NH_4)_2SO_4$, 43.9 KH_2PO_4 and 4.0 NaCl [6]. Presented the effect of Fe^{+3}, Zn^{+2}, and Mn^{+2} on citric acid production by A. niger NRRL 2001. The culture medium composition was glucose (120 g/L) KH_2PO_4 (1.0 g/L); K_2HPO_4 (1.0 g/L), $MgSO_4.7H_2O$ (0.5 g/L), $(NH_4)_2SO_4$ (3.0 g/L). The ions Fe^{+3}, Zn^{+2} and Mn^{+2} had their concentrations changed according to an experimental design. The experiments were carried out in an orbital shaker at 200 rpm and 30°C. The strain produced an extracellular polysaccharide that was also quantified. No oxalic acid formation was observed using this experimental condition. Metal contents were not significant for the production of the polysaccharide. The highest production rate (2.95 g/L/ day) was reached after 10 days of fermentation. After this period, the productivity decreased slightly. The conversion into citric acid increased continuously, yielding 45.8% in 20 days of fermentation [7]. Suggested that the optimization of the medium, including ethanol, methanol, phytate, olive oil and surfactant was carried out using "one-factor-at-a-time" and central composite design (CCD) methods. Optimization using OFAT was performed and the supplement of ethanol and methanol between 15 and 30g/kg dry peat moss (DPM) enhanced citric acid production while higher levels than 30 g/kg DPM had an inhibitory effect on citric acid production at 48 and 72h of incubation. Based on the results of OFAT optimization, phytate, olive oil and methanol were the selected additives to test the effect on citric acid production using CCD [8]. The three variables were identified to have significant effects on citric acid production and the maximum citric acid production of 354.8 g/kg DPM was resulted from the combination of 19 g phytate/kg DPM, 49 g olive oil/kg DPM and 37 g methanol/kg DPM at 120h. Maximum citric acid production in optimized condition by CCD represented about a 2.7 fold increase compared to that obtained from control before optimization [9].

MATERIALS AND METHODS

Culture Preparation

A pure culture of Aspergillus niger a type of fungus was obtained from national chemical laboratory, Pune (India). This strain of bacterium is reported to capable of producing citric acid utilizing sucrose as a raw material. The culture was preserved in refrigerator by periodic sub culture on potato dextrose agar.

Sub Culture Preparation

The microorganism is subcultured once in a month. This can be done by using two different media namely potato dextrose agar medium or Asthana hakas liquid medium which will not contain any agar component. Throughout the experiment potato dextrose agar medium was prepared and used.

Potato Dextrose Agar Medium

The hundred grams of peeled potatoes were cut into small pieces and suspended in 1000 ml of distilled water and steamed for 30 min. remove the extract by filtration using maslein cloth make the final volume to 1000 ml. Add nutrient agar along with growth medium. To each of test tubes 15 ml of this potato dextrose agar in the test tubes, colonies of fungi is introduced on it, and is incubated at 30^0 C for 7 days and then it is stored at 4^0 C in a refrigerator.

Asthana Hakas Medium

Take the medium (glucose, potassium nitrate, KH_2PO_4, $MgSO_4.7H_2O$ and distilled water) in a conical flask. Sterilize the conical flask at 15 psia and 121^0 C for 30 minutes. After the sterilization allow it for cooling then transfer the conical flask along with the asthana hakas medium to the laminar flow chamber and expose the conical flask to UV light for 20 minutes. Then colonies of fungi are introduced in it and are incubated at 30^0 C for 7 days and then it is stored at 4^0 C in a refrigerator.

Estimation Methods

Citric acid was estimated gravimetrically using pyridine-acetic anhydride method as reported by Marrier and Boulet (1958). Citric acid was determined titrimetrically by using 0.1 N NaOH and

Phenolphthalein indicator. Amount of citric acid determined in normality using material balance equation $N_1V_1 = N_2V_2$.

RESULTS AND DISCUSSIONS

Optimization of media constituents

RSM is a sequential procedure with an initial objective of leading the experimenter rapidly and efficiently to the general vicinity of the optimum. Statistical inference techniques can be used to assess the importance of individual factors. Table.1 is a complete three factor two level factorial (2^3) experimental design (CCD) for citric acid fermentation. The three factors are substrate concentration, potassium ferrocyanide and ammonium nitrate.

Table.1

Variable	Parameter	Level				
		-1.68	-1	0	1	1.68
Equation 1						
X_1	Substrate, g/l	120.2	150	200	250	270.7
X_2	Potassium ferrocyanide, g/l	1.7	2	2.5	3	3.2
X_3	Ammonium nitrate, g/l	0.34	0.4	0.5	0.6	0.65
Equation 2 &3						
X_1	p^H	3.405	4	5	6	6.95
X_2	Temperature ^0C	22.025	25	30	35	37.975
X_3	Fermentation time, days	1.81	3	5	7	8.19

Utilization of sucrose or pure sugars as a carbon source in the fermentation media for large scale production of citric acid is rather un-economical and a cheaper carbohydrate source should be used. Palmyra jiggery was used as a carbon source by the submerged fermentation process to optimize the substrate concentration level for the highest production of citric acid. Using CCD method, a total of 20 experiments with different combinations of substrate concentrations, ferrocyanide and nitrogen sources were performed. The response was observed at 5 days of fermentation at the maximum citric acid production. Results were analyzed using ANOVA. Regression equation or optimization of medium constituents shows that citric acid production Y_1 (g/l) is the function of the substrate concentration X_1 (g/l), potassium ferrocyanide X_2 (g/l) and ammonium nitrate X_3 (g/l).

$$\hat{Y}_1 = -934.986 + 3.1525x_1 + 218.3233x_2 + 1693.95x_3 - 0.004867x_1^2 - 36.2440x_2^2 - 1.663.63x_3^2 - 0.2807x_1x_2 + 26.2x_2x_3 - 0.71x_1x_3. \quad (1)$$

The predicted optimum level of X_1, X_2 and X_3 were obtained by applying regression analysis to the above equation using MATLAB software. In the optimization studies, the optimum levels of media constituents for higher citric acid production of 74.8 g/L can be attained at 221.36g/L of substrate concentration, 2.33 g of ferrocyanide/L and 0.479 g of nitrogen source/L.

Effect of Potassium Ferrocyanide on Citric Acid Production

Ferrocyanide could be responsible for increasing citric acid production by inhibiting isocitric dehydrogenase, blocking the citric acid cycle at that point and controlling the rate of cell growth during fermentation. Ferrocyanide stimulates the activity of some citric acid condensing enzymes by suppressing the toxic effect of some ions during citric acid fermentation of molasses. Therefore, the effect on production was examined with media containing 0.02, 0.04, 0.06, 0.08% (w/v) of $K_4Fe(CN)_6$. Addition of 0.06% (w/v) potassium ferrocyanide gave the maximum citric acid concentration (Fig. 1).

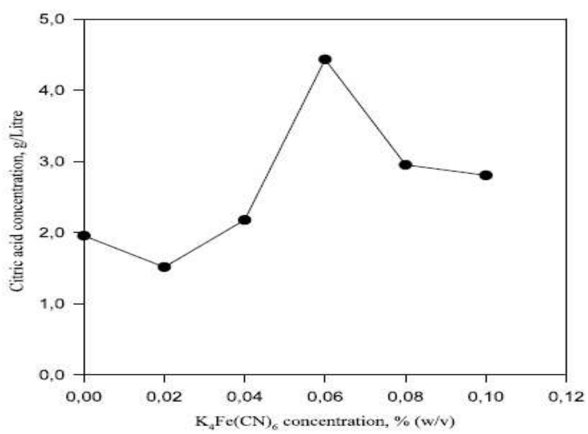

Fig. 1. The effect of $K_4Fe(CN)_6$ concentration on citric acid production. $T = 30$ °C, $N = 100$ rpm, $t = 4$ days, $Q_v = 3$ vvm.

Modeling the Effect of Nutrient Level on Citric Acid Production

Each of the 17 nutrient solutions prescribed by CCD resulted in a different level of citric acid accumulation. These levels provided the data to produce the CCD second-order polynomial equations for predicting citric acid production (Y) as a function of coded value of variables at both 48 and 72 h

$$
\begin{aligned}
Y_{48\mathrm{h}} = {} & 15.87 + 3.41\chi_1 - 5.20\chi_2 + 0.77\chi_3 - 0.68\chi_4 \\
& - 3.42\chi_1^2 + 0.077\chi_2^2 - 0.12\chi_3 - 1.02\chi_4^2 \\
& + 1.15\chi_1\chi_2 - 1.10\chi_1\chi_3 - 0.096\chi_1\chi_4 \\
& - 0.18\chi_2\chi_3 + 5.01\chi_2\chi_3 + 0.40\chi_3\chi_4
\end{aligned} \tag{2}
$$

$$
\begin{aligned}
Y_{72\mathrm{h}} = {} & 29.47 + 18.90\chi_1 - 11.58\chi_2 + 5.89\chi_3 + 0.17\chi_4 \\
& + 2.04\chi_1^2 - 1.01\chi_2^2 - 2.92\chi_3^2 + 0.058\chi_4^2 \\
& - 3.52_1\chi_2 + 1.37\chi_1\chi_3 + 3.96\chi_1\chi_4 \\
& + 0.11\chi_2\chi_3 + 11.78\chi_2\chi_4 - 0.86\chi_3\chi_4
\end{aligned} \tag{3}
$$

The goodness of fit of the equations was determined by computing predicted citric acid production values and correlating them with those measured. At 48 and 72 h of fermentation, R_2 values for citric acid production were 0.99 and 0.97, respectively. With the citric acid production values computed using Eqns. (2) and (3), the significance of the effect of each parameter (glucose, $(NH_4)_2SO_4$, KH_2PO_4 and NaCl) was analyzed using ANOVA.

Response Surface Curves

Three-dimensional response surface curves were obtained using citric acid production values predicted by the CCD second order equations. For each curve, two nutrients were varied while the others were fixed.

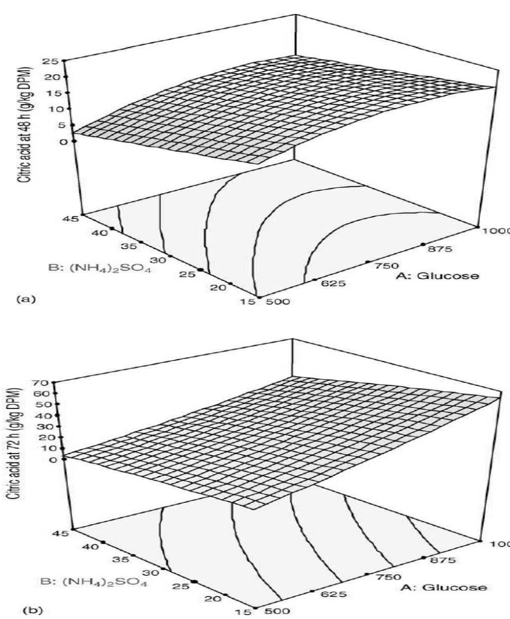

Fig.2. Response Surface Curve for Citric Acid Production as a Function of Initial Glucose and $(NH_4)_2SO_4$ (G Kg$^-$1 DPM) at 48 H (A) and 72 H (B), While the other Variables are Fixed at 30 G KH_2PO_4 and 8 G Nacl Kg$^-$1 DPM.

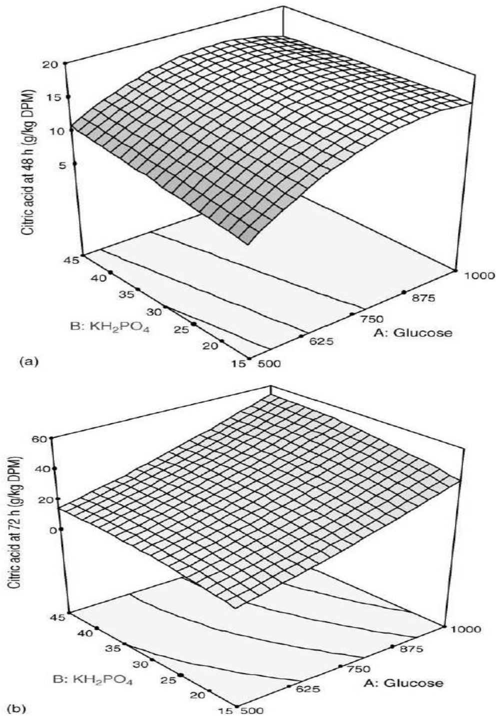

Fig.3. Response Surface Curve for Citric Acid Production as a Function of Initial Glucose and KH_2PO_4 (G Kg^{-}1 DPM) at 48 H (A) and 72 H (B), While the Other Variables are Fixed at 30 G $(NH_4)_2SO_4$ and 8 G Nacl Kg^{-}1 DPM.

At 48 and 72 h of fermentation, citric acid production increased with increasing levels of glucose and decreased with increasing levels of $(NH_4)_2SO_4$ (Fig. 2a and b). A maximum citric acid concentration of 65 g kg^{-}1 DPM was achieved with initial levels of 1000 g glucose kg^{-}1 DPM and 15 g $(NH_4)_2SO_4$ kg^{-}1 DPM. Similar low levels of nutrients, such as N, were found to limit the growth of A. niger in continuous fermentation, which then fostered higher citric acid production and yield. The interactive effects of glucose and KH_2PO_4 on citric acid production at 48 and 72 h with fixed levels of $(NH_4)_2SO_4$ and NaCl is illustrated in Fig. 3a and b.

Evaluation of the Most Significant Medium Constituents Affecting Citric Acid Production

Beet molasses, a by-product of beet sugar industry, contains trace elements such as, sodium, calcium, zinc and copper that may induce the production of citric acid by A. niger. The positive effect of corn steep liquor, a by-product of corn wet milling, could be due to the presence of phytate in corn steep liquor.

Optimization of Fermentation Conditions by the Central Composite Design

In order to optimize the fermentation parameters including initial pH, aeration rate and fermentation temperature as independent variables for maximum citric acid yield. Independent variables were investigated each at five levels according to the central composite design.

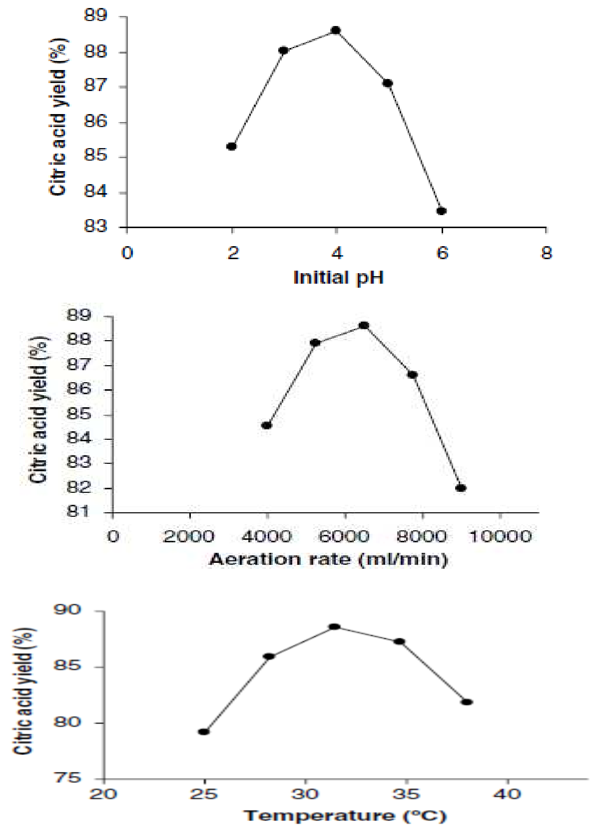

Fig.4 The Relation Between the Response (Citric Acid Yield %) and Different Independent Variables Showing the Predicted Optimal Values Based on the Central Composite Experimental Results.

Estimated Effects of Metal Ions

According to the results, Fe^{+3} had no significant effect on citric acid production but was significant on citric acid yield. Zn^{+2} and Mn^{+2} had significant effects on citric acid production and yield at the considered confidence interval. The effect of manganese on both responses was strongly negative. The interaction between zinc and manganese was also strongly negative on citric acid production and yield responses. On the other hand, manganese had significant effect on oxalic acid production but was not significant on oxalic acid yield. Iron had a small negative effect on oxalic acid production and yield.

CONCLUSIONS

For high productivity of citric acid, optimization of process variables is required. The present review includes the study on nutrients: initial glucose, nitrogen, phosphorous, NaCl levels and the additives phytate, olive oil and methanol were tested for the production of citric acid. Process parameters such as moisture content, incubation temperature and initial pH (fixed) for the improvement of citric acid production from oil palm empty fruit bunches was carried out. The quantitative effects of PH, temperature, time of fermentation, sugar concentration, nitrogen concentration and potassium ferrocyanide on citric acid production were investigated.Using CCD, citric acid production was found to be highly and positively influenced by the initial glucose level while negatively influenced by the initial N level. Phosphorous and NaCl supplementation had a limited positive and an insignificant effect on citric acid production, respectively. The negative effect exerted by N was likely due to its impact on fungal growth, which activates the TCA and the consumption of citric acid for the production of energy and cell constituents. Plackett–Burman design offers good and fast screening procedure and mathematically computes the significance of large number of factors in one experiment, which is time saving and maintain convincing information on each component. Though, it is not of first priority in the screening program to examine the interaction between these large numbers of variables. Aspergillus niger NRRL 2001 was not strongly affected by Fe+3.

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