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STATISTICAL DESIGN AND OPTIMIZATION OF METAL ION CONCENTRATIONS FOR SUBMERGED FERMENTATION OF CITRIC ACID

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ABSTRACT

The present investigation reports the statistical design and optimization of metal ions involved in the submerged fermentation of citric acid by *Aspergillus Niger* NCIM 705. Citric acid is one of the bulk chemicals synthesized by fermentation. The supply of natural citric acid is limited and hence the demand can be satisfied by fermentation processes. Since recent times, *Aspergillus Niger* is predominantly employed for large scale production of citric acid. The optimization of fermentation conditions has got lot of impact on the economy of the process. The study on interactions of different physical and chemical parameters of fermentation will permit satisfactory modelling and optimization of the process. Copper (Cu^{2+}), zinc (Zn^{2+}) and magnesium (Mg^{2+}) are identified as the important metal ions in the biosynthesis of citric acid using *Aspergillus Niger*. Experiments conducted for investigating the effects of the ions in the form of $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$ and $MgSO_4.5H_2O$ on the yields of citric acid are designed by Central Composite Design (CCD), one of the methods of Response Surface Methodology (RSM). The statistical Design-Expert software was used to generate a regression model to predict the combined effect of ions yield of citric acid. A second-order polynomial equation was fitted to the experimental data to construct the response surface model using multiple regressions. The response of ions was predicted by the polynomial equation. With the citric acid production values computed using polynomial equation, the significance of effect of each ion was analyzed using Analysis of variance (ANOVA). It was observed that copper and zinc ions showed good effect on citric acid production whereas magnesium had no impact. With the citric acid production values predicted by CCD second order equation, three-dimensional response surface curves were obtained using RSM. From the studies, 59.592 mg/L of maximum citric acid was fiund to be produced at the optimum ion concentrations of copper: 1.47 mg/L, zinc: 2.46 mg/L and magnesium : 1.25 mg/L.

Key words: Citric acid, Metal ions, Optimization, Central composite design, Regression analysis and Response surface methodology.

INTRODUCTION

Citric acid is an essential biochemical commodity. It is widely found in nature and exists as an intermediate in the citric acid cycle when carbohydrates are oxidized to carbon dioxide[1]. Citric acid (CA) finds profound applications in food, beverage, pharmaceutical and agriculture sectors and hence, there is a greater emphasis on finding more efficient methods of citric acid production by fermentation. As shown in

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Fig. 1, CA is a 6-carbon containing tricarboxylic acid. Many microorganisms such as fungi and bacteria can produce citric acid.

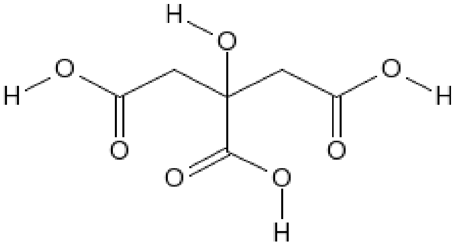

Fig. 1: Structure of citric acid

At present, citric acid is produced commercially using a mutant strain of *Aspergillus Niger* shown in Fig. 2. Citric acid can be produced by extraction from citrus fruits, synthesis and fermentation processes, but fermentation method is predominantly and it accounts for over 90% of the world production. The fermentation process is simple and stable, requires less technical skills, consumes lower energy, less complicated and also requires less sophisticated control systems and easy maintenance[2].

Fig. 2: Aspergillus Niger

The main carbon source used for citric acid production is sugar cane syrup, sugar cane molasses or sugar beet molasses. Beside the use of optimum carbon and nitrogen source concentrations, metal salts and phosphate concentration in the medium were found to influence the yield[3].

Optimization of the fermentation conditions for citric acid production by *A. Niger* can be done using empirical or statistical based optimization methods. Statistical optimization is a proven tool for overcoming the limitations of the empirical method. Widely used and preferred method of Statistical optimizations for the optimization of fermentation is the response surface method (RSM). It is a more efficient technique since it can provide statistical data with a relatively small number of experiments. Moreover, it is a valuable tool for measuring interactions among factors and for the prediction of optimal fermentation conditions. Another strong point of using statistical optimization is that no complex calculations are required to analyze the resulting data[4].

Comprehensive review of previous works on citric acid production opined that the role of physical and chemical variables is very much significant in the enhancement of citric acid yield for the given amount of substrate. The reports on effective optimization of fermentation conditions: Initial sucrose concentration, initial pH, stirrer speed, incubation period, fermentation temperature, O_2 flow rate, additives, metal ions involved in the production of citric acid have been scarce. The present study addresses the gaps and reports the work carried out on statistical design of experiments and optimization of metal ion concentrations by RSM for enhancing the yield of CA.

Response surface methodology

RSM is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions and has successfully been used in the optimization of bioprocesses. In statistics, Response Surface Methodology explores the relationships between input variables and one or more response variables (output variables). It is a scientific approach for determining optimum conditions which combines special experimental designs with Taylor first and second order equations. The RSM process determines the surface of the Taylor expansion curve which describes the response (yield, impurity level, etc.). RSM involves different designs like Box-Wilson Design, Central Composite Design and Factorial Design[2].

Central composite design

CCD is used for building a second order (quadratic) model for the response variable without using a complete three-level factorial experiment. It is efficient than the other methods. 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. CCD's are designed to estimate the coefficients of a quadratic model[1]. All point descriptions will be in terms of coded values of the factors. A CCD has three groups of design points: (a) Two-level factorial design points- for the two factor case there are four design points: (-1, -1) (+1, -1) (-1, +1) (+1, +1), (b) Axial points- for a two factor problem, the star points are: (-Alpha, 0) (+Alpha, 0) (0, -Alpha) (0, +Alpha) and (c) Centre points- are points with all levels set to coded level 0, the midpoint of each factor range: (0, 0)[5].

Previous work

Optimization of the medium components, including ethanol, methanol, phytate, olive oil and surfactant was carried out using ‘one-factor-at-a-time’ and central composite design (CCD) methods. Based on the results of ‘one-factor-at-a-time’ optimization, phytate, olive oil and methanol were the selected additives to test the effect on citric acid production using CCD. Maximum citric acid production in optimized condition by CCD represented about a 2.7-fold increase compared to that obtained from control before optimization[6].

Studied citric acid (CA) production by *Aspergillus Niger* ATCC9642 from whey with different concentrations of sucrose, glucose, fructose, galactose riboflavin, tricalcium phosphate and methanol in surface culture process. It was found that whey with 15% (w/v) sucrose with or without 1% methanol was the most favourable medium producing the highest amount (106.5 g/L) of citric acid. Lower CA was produced from whey with other concentrations of sugars and other additives used. Highest biomass of *A. Niger* was produced with the addition of riboflavin. In general, extension of the fermentation (upto 20 days) resulted in an increase in CA and biomass, and decrease in both residual sucrose and pH values[7].

Optimization of medium constituents including yeast extract, moisture content of the substrate, KH_2PO_4 and Na_2HPO_4 was carried out using Plackett–Burman design and these variables were subsequently optimized using a central composite design (CCD). Maximum citric acid production in optimized condition by using CCD is greater than that obtained by using Plackett-Burman Design[8].

Used statistical designs to enhance the production of citric acid in submerged culture. For screening of fermentation medium composition significantly influencing citric acid production, the two-level Plackett–Burman design was used. Response surface methodology (RSM) was adopted to acquire the best process conditions. The five level central composite designs was used for outlining the optimum values of the fermentation factors initial pH, aeration rate and temperature on citric acid production. A near optimum medium formulation was obtained using this method with increased citric acid yield by five-folds[9].

Suggested that among the various fungal strains screened for citric acid production, *Aspergillus niger* is known to produce considerable amounts of citric acid and other organic acids when cultivated in carbohydrate-rich medium in solid substrate fermentation (SSF). The effects of various nutrients, fermentation parameters and of initial level of potential stimulators were evaluated with respect to citric acid production by *A. niger* grown on damp peat moss. As compared to submerged and semi-continuous fermentation, batch type SSF could take higher levels of initial glucose and produce the high concentration of citric acid within a shorter period of time[10].

Suggested that Response surface methodology (RSM) is widely used and preferred method for the optimization of fermentation medium. Another strong point of using RSM for optimization is that no complex calculations are required to analyze the resulting data. One-at-a-time strategies are more complex when a large number of factors need to be optimized and they will give limited information on the interactive effects. These strategies identify the input variables that can have a significant effect on the response and they can reduce the number of experiments to be carried out later when using RSM[11].

Studied submerged fungal fermentation of citric acid. Numerous studies attempting to increase citric acid production through the search for either newer strains or more efficient processes have been extensively reviewed. *Aspetgillus niger* remains the organism of choice for the production of citric acid, which involves a number of very specific environmental conditions including initial pH, dissolved oxygen tension, the concentrations and types of carbon, nitrogen and phosphorus in addition to various trace elements[12].

Suggested that the possible optimum level of moisture content, incubation temperature, and initial pH 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 pH 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[13].

EXPERIMENTAL

Materials and methods

Materials

Glass fermentor, *Aspergillus niger*, sucrose, autoclave, NaOH and phenolphthalein indicator, $(NH_4)_2SO_4$, KH_2PO_4, NaCl and potato dextrose agar medium comprising dextrose : 20 (g/L), yeast extract: 0.1 (g/L) and agar-agar : 20 (g/L).

Experimentation

A 1.2 Liter capacity fermentor equipped with standard control and instrumentation as shown in Fig.3 was used for the citric acid fermentation. The fermentor was thoroughly cleaned with water and sterilized in an autoclave for 20 minutes. The sterilized fermentor was placed in the main assembly and tube connections were given for water and air. Then the sterilized medium containing vegetative inoculums was transferred to the fermentor from the conical flask after 24 hours of incubation. The power was switched on. The experiments designed were conducted as per the standard procedure. Samples were collected from the fermentor and analyzed for citric acid production.

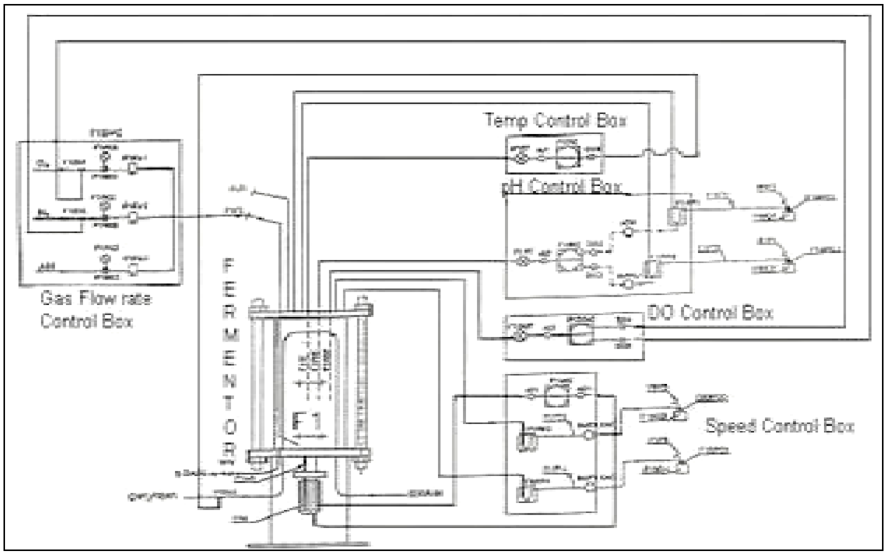

Fig. 3: Experimental setup

Estimation of citric acid

Citric acid was determined titrimatically by using 0.1 N NaOH and phenolphthalein indicator. The amount of citric acid produced is determined in terms of normality using the material balance equation ($N_1V_1 = N_2V_2$) and was later converted into g/L.

RESULTS AND DISCUSSION

The effects of metal ions: Cu^{2+}, Zn^{2+} and Mg^{2+} on the fermentation of citric acid are studied experimentally and the ions are optimized using response surface methodology. The range of concentrations of ions under study is shown in Table 1.

Table 1: Selected range of ionic concentrations

Variable	Parameter	Units	Low	High
x_1	$CuSO_4.5H_2O$	mg/L	0.5	4.5
x_2	$ZnSO_4.7H_2O$	mg/L	1.8	5.4
x_3	$MgSO_4.5H_2O$	mg/L	0.1	2.5

Using central composite design, fifteen different combinations of experiments ($2^k + 2k + 1$, where ‘k’ is no. of variables) were designed for three ionic concentrations[13]. The fermentation was carried out at the experimental levels shown in Table 2 as suggested by CCD.

Table 2: Experimental levels

Run No.	x_1	x_2	x_3	Yield of citric acid (Y) g/L
1	0.9	2.4	1.3	24.579
2	0.5	5.4	2.5	29.805
3	4.5	5.4	0.1	32.118
4	4.5	5.4	2.5	34.051
5	1.0	3.6	1.3	37.52
6	2.5	3.6	0.9	43.502
7	0.5	1.8	0.1	10.598
8	0.5	1.8	2.5	12.763
9	1.4	1.8	2.5	21.152
10	2.5	3.6	1.3	36.19
11	2.5	2.4	1.3	29.018
12	0.9	1.8	2.5	15.729
13	0.5	5.4	0.1	21.941
14	1.5	3.6	1.3	31.257
15	4.5	1.8	0.1	37.621
16	2.5	3.6	0.9	53.502

Statistical analysis

The statistical design expert software was used to generate a regression model in order to predict the combined effect of copper, zinc and magnesium ions on the yield of citric acid. The CCD produced the following second-order polynomial equation (1) for predicting the yield of citric acid (Y) as a function of x_1, x_2 and x_3.The response of tested variables was predicted by the equation.

$$\begin{aligned} Y = {} & 8.09419 + 17.44664\, x_1 + 15.74456\, x_2 - 0.94147\, x_3 + 1.55956\, x_1 x_2 \\ & - 1.41097\, x_1 x_3 + 0.35492\, x_2 x_3 - 3.26010\, x_1^2 \\ & + 2.26608\, x_2^2 + 2.62804\, x_3^2 \end{aligned} \quad \ldots(1)$$

The goodness of fit of the equation was determined by computing predicted citric acid production values and correlating them with those measured. R^2 value for the citric acid production was found to be 0.9529. With the citric acid production values computed using polynomial equation, the significance of the effect of each copper, zinc and magnesium ions was analyzed using ANOVA (Analysis of variance) as shown in Table 3.

Effects of variables

Copper and zinc ions observed to show good effect on citric acid production whereas magnesium had no impact as copper and zinc would be used initially to produce energy and build up enzymes for fungal growth to reach its maximum. Magnesium is most likely required in very limited quantities and therefore had no impact. The coefficients of the second-order regression (Eq. 1) measured the effect of the metal ions on the response (Y). The coefficients for copper and zinc were positive indicating that higher levels of such nutrients result in higher citric acid production at 8 days of fermentation. The coefficient for magnesium was negative, indicating that it had a negative effect on citric acid production.

Table 3: ANOVA table

Source	Sum of squares	F value	P value
Model	2764.776	22.4711	< 0.0001
A-x_1	396.9787	29.0385	0.0357
B-x_2	216.416	15.83056	0.0265
C-x_3	93.39617	6.831815	0.0026
AB	130.8621	9.572403	0.0114
AC	46.89751	3.430495	0.0937
BC	2.76504	0.202259	0.6625
A^2	94.01295	6.876931	0.0255
B^2	14.19884	1.038628	0.3322
C^2	5.179351	0.378863	0.5520

Response surface curves

Three-dimensional response surface curves were obtained with RSM using citric acid production values predicted by CCD second order equation. For each curve,the concentration of two metal ions were varied while the other was fixed.

At 8 days of fermentation, citric acid production was found to increase with increasing levels of $CuSO_4.5H_2O$ till 1.47 mg/L and then a slight decrease was observed above 1.47 mg/L. When zinc concentration was increased from 1.8 to 2.5 mg/L, citric acid concentration was increased for all initial copper concentrations. A maximum citric acid production of 56.78 g/L was achieved at 1.47 mg/L of copper and 2.46 mg/L of zinc as shown in Fig. 4.

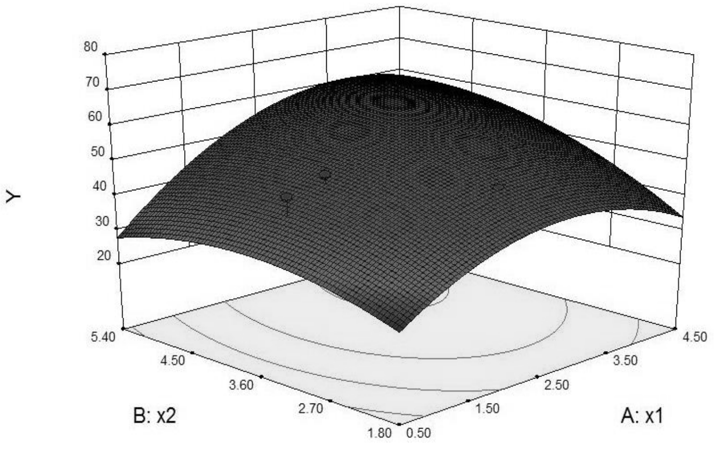

Fig. 4: Response surface curve for citric acid production as a function of copper and zinc concentrations at 8 days of fermentation, while magnesium concentration was fixed at 1.25 mg\L

At 8 days of fermentation, citric acid production was observed to increase with increasing levels of copper and decreased with increasing levels of magnesium. When magnesium was increased from 0.2 to 2.5 mg/L, citric acid concentration was dropped for all initial copper concentration. A maximum citric acid production of 56.78 g/L was achieved at 1.47 mg/L of copper and 1.25 mg/L of magnesium as shown in Fig. 5.

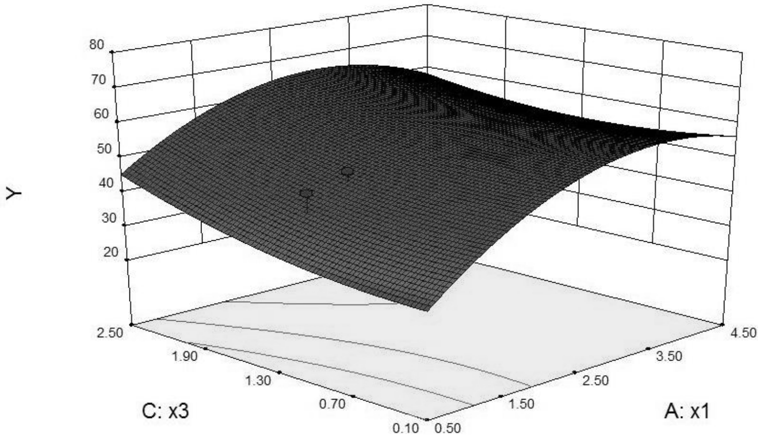

Fig. 5: Response surface curve for citric acid production as a function of copper and magnesium concentrations at 8 days of fermentation, while zinc concentration was fixed at 2.46 mg\L

Table 4: Optimum ionic levels

x_1 mg/L	**x_2 mg/L**	**x_3 mg/L**	**Y g/L**
1	3.6	1.3	42.0621
0.9	2.4	1.3	35.7411
4.5	5.4	0.1	56.7877
1.47	**2.46**	**1.25**	**59.5920**
2.5	3.6	0.9	55.7515
0.5	5.4	2.5	40.066
0.5	1.8	0.1	22.1413
1.4	1.8	2.5	45.5999
0.5	1.8	2.5	36.1209
4.5	1.8	0.1	37.3903
0.5	5.4	0.1	23.0199
1.5	3.6	1.3	48.6013
4.5	5.4	2.5	54.2274
0.9	1.8	2.5	40.9858
2.5	2.4	1.3	48.9777

Fig. 6 represents citric acid production as a function of zinc and magnesium at fixed level of copper concentrations after 8 days of fermentation. Citric acid production was maximized with zinc at 2.46 mg/L and magnesium at 1.25 mg/L, while not significantly affected by variation of magnesium. Maximum citric acid production was achieved with the highest levels of copper and magnesium, as an increase in magnesium resulted in a slight increase in citric acid production only for copper levels exceeding 1.5 mg/L. Thus, copper strongly affected citric acid production by *Aspergillus Niger*, while magnesium had insignificant effects.

Citric acid production using the optimized ionic solution

Numerical optimization was carried out to determine the optimum composition of the ionic solution, within the range of coded levels from -1 to 1 at 8 days of fermentation. The optimum ionic levels found out are as shown in Table 4. Although the numerical optimization process suggested 20 possible optimal media, the fourth solution was found to be the most economical, producing the most citric acid of 59.592 mg/L for the copper concentration of 1.47 mg/L, zinc 2.46 mg/L and magnesium 1.25 mg/L.

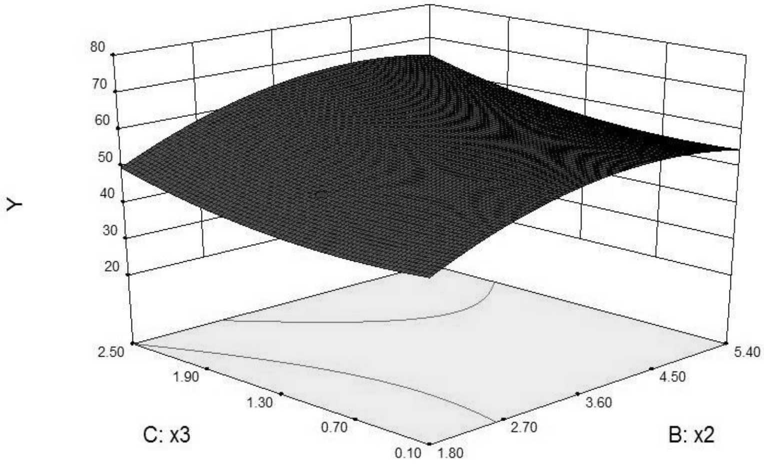

Fig 6: Response surface curve for citric acid production as a function of zinc and magnesium concentrations at 8 days of fermentation, while copper concentration was fixed at 1.47 mg\L

CONCLUSION

For high productivity of citric acid and economic operation of the plant, optimization of process variables is required. In the present study, metal ion concentrations of copper (Cu^{2+}), zinc (Zn^{2+}) and magnesium (Mg^{2+}) are tested for the production of citric acid. From the optimization studies, maximum citric acid concentration of 59.592 mg/L was observed at the optimum concentrations of copper : 1.47 mg/L, zinc : 2.46 mg/L and magnesium : 1.25 mg/L.

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