OPTIMIZATION OF INCUBATION TIME, FERMENTATION TEMPERATURE & O$_2$ FLOW RATE IN CITRIC ACID FERMENTATION USING RESPONSE SURFACE METHODOLOGY (RSM)

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International Journal of Advanced Engineering Technology E-ISSN 0976-3945

ABSTRACT
Citric acid is an important commercial product and it is produced mainly by submerged aerobic fermentation. The large scale use of this organic acid requires development of effective and efficient production process. The yield of Citric acid can be enhanced if the main operating variables: Initial sucrose concentration, Initial pH, Stirrer speed, Incubation time, Fermentation temperature and O$_2$ flow rate are properly optimized. The present investigation involves batch fermentation of Citric acid from sucrose with Aspergillus Niger NCIM 705 and optimization of three variables: Incubation time, Fermentation temperature and O$_2$ flow rate out of the said variables. Response Surface Methodology (RSM) has been employed to optimize the variables and to study their effects on the yields of citric acid. For the three optimizable variables, fifteen different combinations of experiments were designed using Central Composite Design (CCD). The experiments were conducted accordingly and the concentrations of resulting citric acid were measured. The experimental results obtained were used to produce a second order equation with the yield of citric acid as a function of Incubation time, Fermentation temperature and O$_2$ flow rate. Different levels of the three variables were found to have a significant positive effect on citric acid production. A maximum concentration of 52.49 g/l for citric acid was found at Initial sucrose concentration of 155g/l, Initial medium pH of 6.0 and stirrer speed of 240 rpm with the RSM optimized variables: 5 days of fermentation time, 30°C of fermentation temperature and 1.0 lpm of O$_2$ flow rate.

KEYWORDS Citric acid, Aspergillus Niger, Fermentation variables, Optimization, Central composite design, Response surface methodology.

INTRODUCTION:
Citric acid is one of the bulk chemicals produced from a wide range of carbon-containing raw materials such as molasses, Corn starch, Date syrup, Sucrose etc. Present work uses sucrose as raw material as A. niger has got potent mycelium bound invertase that is active at low pH and rapidly hydrolyzes sucrose. Citric acid is widely used in the food, beverage, pharmaceutical, cosmetic and other industries [1, 5].

Citric acid productivity can be increased by optimizing its fermentation variables: Initial sucrose concentration, Initial pH, Stirrer speed, Incubation time, Fermentation temperature, Oxygen flow rate, Additives and Nutrients’ concentration. Response Surface Methodology is employed for optimizing the title variables [1, 7].

Response Surface Methodology:
RSM is a collection of statistical techniques for designing experiments, evaluating the effects of factors and searching for the optimum conditions and it has successfully been used in the optimization of bioprocesses. RSM explores the relationships between explanatory variables (input variables) and response variables (output variables). CCD is one of the methods of RSM. A CCD is used for building a second order (quadratic) model for the response variable without using a complete three-level factorial experiment. 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. A CCD has three groups of design points: (a) Two-level factorial or fractional factorial design points (b) Axial points and (c) Center points [10, 2].

a. Factorial Points: The two-level factorial part of the design consists of all possible combinations of the +1 and -1 levels of the factors. For the two factor case there are four design points: (-1, -1) (+1, -1) (-1, +1) (+1, +1)

b. Axial Points: The star points have all of the factors set to 0, the midpoint, except one factor, which has the value +/- Alpha. For a two factor problem, the star points are: (-Alpha, 0) (+Alpha, 0) (0, -Alpha) (0, +Alpha).

c. Center Points: Center points, as implied by the name are points with all levels set to coded level 0-the midpoint of each factor range: (0, 0). Design descriptions and analyses for designed experiments are best done with coded factors.

PREVIOUS WORK:
Y. D. Hang et al. considered Corncobs as a substrate for citric acid production by Aspergillus Niger. Methanol had a significant effect on fungal production of citric acid from corncobs. Of the four cultures examined, A. niger NRRL 2001 was found to produce the highest amount of citric acid (250 g/kg dry matter of corncobs) after 72 h of growth at 30°C in the presence of 3% methanol. The yield of citric acid was over 50% based on the amount of sugar consumed [13]. Luciana P. et al. evaluated three different agro-industrial wastes, sugar cane bagasse, coffee husk and cassava bagasse for their efficiency in production of
citric acid by a culture of *Aspergillus Niger*. Cassava bagasse best supported the mould's growth, giving the highest yield of citric acid among the tested substrates. Results showed the fungal strain had good adaptation to the substrate (cassava bagasse) and increased the protein content (23 g/kg) in the fermented matter. Citric acid production reached a maximum (88-g/kg dry matter) when fermentation was carried out with cassava bagasse having initial moisture of 62% at 26°C for 120 h [4].

Nehad Z. Adham observed that Natural oils with high unsaturated fatty acids content when added at concentrations of 2% and 4% (v/v) to beet molasses (BM) medium caused a considerable increase in citric acid yield from *Aspergillus niger*. The fermentation capacities were also examined for production of citric acid using BM-oil media under different fermentation conditions. Maximum citric acid yield was achieved in surface culture in the presence of 4% olive oil after 12 days incubation [9].

Murad A. El-Holi et al. studied on 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 was studied. 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 riboflavins. In general, extension of the fermentation (up to 20 days) resulted in an increase in CA and biomass, and decrease in both residual sucrose and pH values [8]. Jin-Woo Kim studied the effects of various nutrients (glucose, (NH4)2SO 4, KH2PO4 and NaCl), fermentation parameters (moisture content, temperature, inoculum density, composition of solid substrate and particle size) and of initial level of potential stimulators (ethanol, methanol, phytate and surfactant) were evaluated with respect to citric acid production by A. niger grown on damp peat moss. Three variables including aeration, thickness of solid substrate bed and incubation temperature were optimized using a 23 full factorial design (FFD). Under optimum, the total citric acid production and yield were 120.6 g/kg DPM and 18.5% respectively. A third experiment (study 3) compared the production of citric acid by *A. niger* in submerged fermentation using cheese whey, as opposed to batch and semi-continuous fermentation using peat moss. Various fermentation conditions such as nutrients (glucose, (NH4)2SO 4 and KH2PO4), stimulators (methanol, olive oil and phytate) and fermentation parameters (pH, fermentation time and inoculum density) were optimized using a central composite design (CCD). Citric acid production improved citric acid production by a factor of 13.3 when compared to the production of citric acid by *A. niger* NRRL 567 using whey-based medium (50 g/l) alone [3].

**MATERIALS AND METHODS:**

**Experimentation:**

A 1.2 liter capacity fermentor made of glass, *Aspergillus Niger*, Sucrose, Autoclave, NaOH, phenolphthalein indicator and Potato dextrose agar medium shown in table. 1 is used for the fermentation of Citric acid.

Fermentor equipped with standard control and instrumentation as shown in Fig.1 was used for the citric acid fermentation. 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. Thus the system was ready for the process. The power was switched on. Using central composite design (2\(^k\)+2\(k+1\), where ‘k’ is no. of variables) 15 different combinations of experiments were designed and fermentation was carried out. Samples were collected from the fermentor and analyzed for citric acid production.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose</td>
<td>20</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>0.1</td>
</tr>
<tr>
<td>Agar-Agar</td>
<td>20</td>
</tr>
</tbody>
</table>

**Estimation of Citric Acid**

Citric acid was determined titrimatically by using 0.1 N NaOH and phenolphthalein indicator. The end point was pink color. Amount of citric acid was determined
in Normality using the material balance \((N_1V_1 = N_2V_2)\) equation and was later converted into g/l. Where \(N_1\) = concentration of NaOH, \(N_2\) = concentration of citric acid, \(V_1\) = volume of NaOH run down in ml, \(V_2\) = volume of culture broth used in ml [6].

RESULTS AND DISCUSSION

Using CCD different combinations of experiments were designed. The maximum and minimum values are denoted as (+1) and (-1) respectively. The center point is the average of maximum and minimum values. The alpha values (-1.68 and +1.68) are default values taken in the design expert software. The effects of Incubation time, Fermentation temperature and \(O_2\) flow rate on citric acid production were experimentally studied and were optimized using RSM. The range of values selected for the variables under study are as shown in table 2.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Parameter</th>
<th>Units</th>
<th>Low</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>(x_1)</td>
<td>Incubation Time</td>
<td>days</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>(x_2)</td>
<td>Fermentation Temperature</td>
<td>°C</td>
<td>28</td>
<td>32</td>
</tr>
<tr>
<td>(x_3)</td>
<td>(O_2) flow rate</td>
<td>lpm</td>
<td>0.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Statistical Analysis

The statistical software Design-Expert® 8.0 was used to generate a regression model for predicting the effect of combined parameters (Incubation time, Fermentation temperature and \(O_2\) flow rate) on the responses (citric acid yield). To construct the response surface model, a second-order polynomial equation was fitted to the experimental data obtained using multiple regressions. The response of tested variables can be predicted by the quadratic polynomial equation [12].

The effect of combined parameters on citric acid production

The second-order polynomial equation for predicting the yield of citric acid \((Y)\) as a function of the three variables generated by CCD is:

\[
y = -1463.44 + 1.99043x_1 + 98.2544x_2 + 1.24709x_3 + 0.35126x_{12} + 0.029100x_{13} - 0.053142x_2 - 1.16985x_3 - 1.63925x_2 - 2.87616x_3
\]

(1)

Where \(x_1\) = Incubation time, \(x_2\) = Fermentation temperature and \(x_3\) = \(O_2\) flow rate.

The goodness of fit of the equation was determined by computing predicted citric acid yields and correlating them with those measured. At 6.0 medium \(pH\), \(R^2\) value for the citric acid production was 0.90. With the citric acid yields computed using equation (1), the significance of each parameter was analyzed using ANOVA table. For citric acid production at medium \(pH\) of 6.0, Incubation time, Fermentation temperature and \(O_2\) flow rate were observed to exert a significant effect. Thus all the three variables have significant impact on citric acid production.

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 variables were varied while the other variable was fixed.

Fig. 2 Response surface curve for citric acid production as a function of Incubation time and Fermentation temperature while \(O_2\) flow rate was fixed at 1.0 lpm.

Fig. 3 Response surface curve for citric acid production as a function of Incubation time and \(O_2\) flow rate, while Fermentation temperature was fixed at 30°C.

In the figs. 2 & 3, at initial medium \(pH\) of 6.0, citric acid production was increased with increasing levels of incubation time and as well as with increasing levels of fermentation temperature. A slight decrease in citric acid production was observed for \(O_2\) flow rate above 1.0 lpm. When \(O_2\) flow rate was increased from 1.0 –
2.5 lpm, citric acid production dropped for levels of incubation period. A maximum citric acid concentration of 52.49 g/l was achieved with incubation time of 5 days and fermentation temperature of 30°C.

![Fig:4 Response surface curve for citric acid production as a function of Fermentation temperature and O\textsubscript{2} flow rate, while the other variable Incubation time was fixed at 5 days.]

In the fig. 4, the interactive effects of Fermentation temperature and O\textsubscript{2} flow rate on citric acid production at a medium pH of 6.0 with fixed levels of Incubation time was shown. Yield of citric acid was increased with the increase in Fermentation temperature from 28°C to 30°C and also with the increase in O\textsubscript{2} flow rate from 0.5 lpm – 1.0 lpm for all levels of Incubation time. It was found that citric acid production was decreased with the the increase in Fermentation temperature above 30°C, this is because of accumulation of by-products such as oxalic acid above 30°C. The optimum values obtained using RSM are shown in table.3.

**Table.3: The optimum values obtained using RSM**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Units</th>
<th>Optimum value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation period</td>
<td>days</td>
<td>5</td>
</tr>
<tr>
<td>Fermentation temperature</td>
<td>°C</td>
<td>30</td>
</tr>
<tr>
<td>O\textsubscript{2} flow rate</td>
<td>lpm</td>
<td>1.0</td>
</tr>
<tr>
<td>Citric acid yield</td>
<td>g/l</td>
<td>52.49</td>
</tr>
</tbody>
</table>

**CONCLUSIONS**

Based on the results of the study, the conclusions can be made as: For high productivity of citric acid, optimization of its process variables is required. In the study, Incubation time, Fermentation temperature and O\textsubscript{2} flow rate were tested for the production of citric acid and found that maximum yield of citric acid (52.49 g/l) was obtained at the RSM optimized values of 5 days of Incubation time, 30°C of Fermentation temperature and 1.0 lpm of O\textsubscript{2} flow rate.

**ACKNOWLEDGMENTS**

I am very much thankful to Prof. G.Venkat Reddy (Supervisor) for his constant support and encouragement. I heart fully thank the Department for providing me the experimental facilities.

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