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Optimization of Operating Variables in the Fermentation of Citric acid using Response Surface Methodology

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Abstract. Citric acid is a commercially important product synthesized mainly by submerged aerobic fermentation using *Aspergillus Niger*. The large scale demand of the organic acid requires the development of more effective and efficient production process by optimizing its operating variables. The present work aims at optimizing three of its significant identified process variables: initial sucrose concentration, stirrer speed and initial medium pH and studies their effect on the yield of citric acid. Central Composite Design (CCD) was employed to design the number of experiments to be fifteen different combinations of variables to grow *Aspergillus Niger* at 30^0C for 5 days of fermentation, and to measure the resulting citric acid. The results were used to produce a second order polynomial equation using CCD defining the yield of citric acid as a function of above said variables. The equation obtained was optimized using Response Surface Methodology (RSM). Initial sucrose concentration, stirrer speed and initial medium pH levels were found to have a significant positive effect on citric acid. A maximum citric acid concentration of 59.84 g/l was observed to reach after 5 days of fermentation with the optimized variables of 140 g/l initial sucrose concentration, 240 rpm stirrer speed and 6.0 initial medium p^H.

Keywords: Citric acid, *Aspergillus Niger,* Response surface method.

1. Introduction

Citric acid is one of the few bulk chemicals produced by fermentation, and is the most exploited biochemical product. The supply of natural citric acid is limited and the demand can only be satisfied by biotechnological fermentation processes. Nowadays, *Aspergillus niger* is almost exclusively used for industrial scale production of citric acid [1]. The optimization of fermentation conditions are of primary importance in the development of any fermentation process owing to their impact on the economy and practability of the process [2]. The diversity of combinational interactions of physical and chemical fermentation parameters and production do not permit satisfactory detailed modeling. In the present work, we report the sequential optimization strategy for citric acid production by *A. niger* through statistically designed experiments as an effective tool for fermentation engineering and its validation by Genetic Algorithms. Citric acid is widely used in the food, beverage, chemical, pharmaceutical and other industries [3].

The Yield of Citric acid can be increased by optimizing the operating variables such as Initial sucrose concentration, Initial medium p^H, Stirrer speed, Fermentation time, Fermentation temperature and O_2 flow rate which are identified to be vital in its fermentation [4]. The present study deals with the optimization of three variables as stated in abstract. Citric acid can be made from a number of carbon-containing raw materials such as Beet molasses, Brewery wastes, Carob pod extract, Coconut oil, Corn starch, Date syrup, Glycerol, Olive oil, Apple pomace, Carrot waste, Cassava bagasse, Sucrose etc [5-6]. Sucrose is preferable to glucose as *A. niger* has a potent extracellular mycelium bound invertase that is active at low pH and rapidly hydrolyzes sucrose and hence it is taken as the raw material in the present work. Response Surface

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Methodology is employed the optimization tool [7]. Although many microorganisms can be used to produce citric acid, *A. niger* remains the main industrial producer. Specific strains that are capable of overproducing citric acid have been developed for various types of fermentation processes [8]. The yield of citric acid from these strains often exceeds 70% of the theoretical yield on the carbon source. Citric acid production by fermentation is the most economical and widely used way of obtaining the product [9]. More than 90 % of the citric acid produced in the world is obtained by fermentation, which has its own advantages like operations are simple, need less sophisticated control systems, energy consumption is lower and the frequent power failures do not critically affect the functioning of the plant [10]. The submerged technique is widely used for citric acid production. It is estimated that about 80% of world production is obtained by submerged fermentation. This fermentation process is employed in large scale bases requires more sophisticated technique and rigorous control. On the other hand, it presents several advantages such as higher productivity and yields, lower labor costs, and lower contamination risk [11].

1.1. Response Surface Methodology (RSM)

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. Response Surface Methodology explores the relationships between explanatory variables (input variables) and response variables (output variables) [12].

1.2. Central Composite Design

CCD is used for building a second order (quadratic) model for the response variable without needing to use 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 [13]. A CCD has three groups of design points: (a) Two-level factorial or fractional factorial design points (b) Axial points and (c) Center points

1.3. Literature review

M.Y.Lu, I. S. Maddox J. D. Brooks' Experiments showed that kumara and taro were excellent substrates for citric acid production by solid-substrate fermentation using *Aspergillus niger*. Conversely, potato was a poor substrate, although it supported profuse fungal growth. A kinetic analysis of citrate production from kumara showed an overall reactor productivity of 0.48 g citrate/kg wet weight kumara per hour, with a yield of 0.54 on a weight basis (g citrate produced/g starch used). Maximum citrate production rates were observed after 2 to 3 days of fermentation, while the fungal growth rate was still high. The optimum moisture content of the kumara for citrate production was 65% (w/w) or above, while metal ions were shown not to be inhibitory to the process. [14]

P. Ambati and C. Ayyanna suggested that palmyra jaggery is a suitable substrate for increasing the yield of citric acid using *Aspergillus 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. 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 [15].

2. Materials and Methods

2.1. Materials

Glass fermentor, *Aspergillus Niger*, Sucrose, Autoclave, NaOH and phenolphthalein indicator, (NH4)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) .

2.2. Experimental Set-up

A 1.2 liter capacity fermentor made of glass equipped with standard control and instrumentation was used for the citric acid fermentation. The fermentor equipped with a flat blade impeller with three blades. Two 500 ml bottles were provided to the fermentor for the addition of acid and base, one silicon tube was provided for the addition of sterilized silicon oil to control foaming. The fermentor has arrangements for measuring pH and temperature by digital pH controller and digital temperature sensor. Cooling water supply was provided to maintain the temperature in the fermentor at the desired level. There are provisions for supplying Air, N_2 and O_2 at desired flow rates. The experimental set up is shown in Fig.1.

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. The experimental conditions maintained were: Fermentation temperature 30^0C and air flow rate 1-lpm. Using central composite design different combinations of variables were obtained and fermentation was carried out. Samples were collected from the fermentor and analyzed for citric acid production.

2.3. Estimation methods

Citric acid was determined titrimatically by using 0.1 *N* NaOH and phenolphthalein indicator. The end point is pink color. Amount of citric acid determined in Normality using the material balance equation ($N_1V_1 = N_2V_2$) and was later converted into g/l.

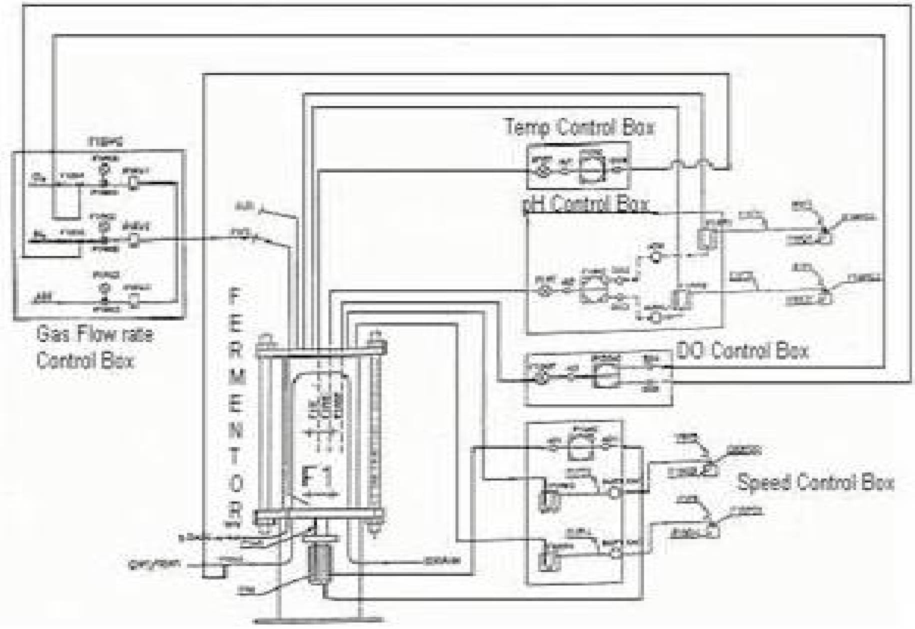

Fig. 1: Experimental setup

3. Results and Discussion

In the present study, the effects of the variables initial sucrose concentration, stirrer speed and medium pH on citric acid production using *Aspergillus Niger* were studied and were optimized using Central composite design and compared with genetic algorithms. Before that using central composite design a different combinations of variables were obtained. The maximum and minimum values are denoted (+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 package. The variable range selected for the production and optimization of Citric acid is as shown in table. 1.

Table.1

Variable	Parameter	Units	Low	High
x_1	Initial sucrose concentration	g/l	80	200
x_2	Stirrer speed	rpm	170	310
x_3	Medium pH	-	5	7

Using central composite design a total of 21 different combinations of variables were tested to grow *Aspergillus Niger* at 30^0C and to measure the resulting citric acid production.

3.1. Statistical Analysis

The statistical software package Design-Expert ® 8.0, was used to generate a regression model in order to predict the effect of combined parameters on responses such as citric acid production and yield. To construct the response surface model, a second-order polynomial equation was fitted to the data using multiple regressions. The response of tested variables can be predicted by the quadratic polynomial equation. The CCD produced the following second-order polynomial equation for predicting the yield of citric acid (Y) as a function of three variables [16].

$$Y = -231.03984 + 1.698\,x_1 + 0.67425\,x_2 + 20.32186\,x_3 - 2.01231\times10^{-3}x_1x_2 - 0.22202\,x_1x_3 - 0.058782\,x_2x_3 + 3.52061\times10^{-4}x_1^2 + 4.50494\,10^{-4}x_2^2 + 1.93079\,x_3^2 \qquad (1)$$

where x_1 = initial sucrose concentration, x_2 = stirrer speed and x_3 = initial medium pH.

The goodness of fit of the equation was determined by computing predicted citric acid production values and correlating them with those measured. At 5 days of fermentation, R^2 value for the citric acid production was 0.97.

With the citric acid production values computed using equation (1), the significance of each parameter was analyzed using ANOVA table. For citric acid production at 30^0 C, initial sucrose concentration, stirrer speed and initial medium pH were observed to exert a significant effect. Thus all these three variables have significant impact on citric acid production by Aspergillus *Niger* at 5 days of incubation.

3.2. 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.

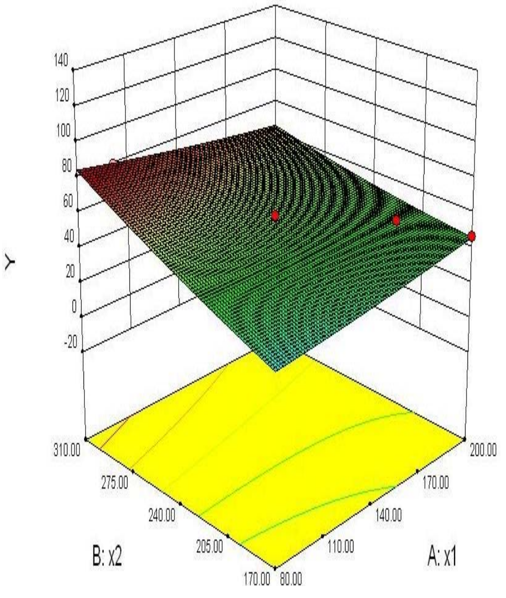

Fig. 2: Response surface curve for citric acid production as a function of initial sucrose concentration and stirrer speed while initial medium pH was fixed at 6.0.

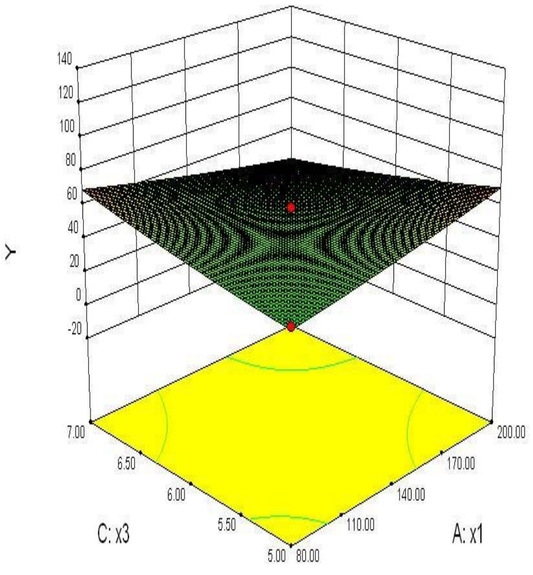

Fig. 3: Respopnse surface curve for citric acid production as a function of initial sucrose concentration and initiral medium pH at 5 dyas of fermentation at 240 rpm.

In the figs. 2 & 3, at 5 days of fermentation, citric acid production was increased with increasing levels of initial sucrose concentration and also with increasing levels of stirrer speed. A slight decrease in citric acid production was observed for stirrer speed above 290 rpm. When initial medium pH was increased from 6 to 7, citric acid concentration dropped for all initial sucrose concentration levels. A maximum citric acid concentration of 59.84 g/l was achieved with values of 140 g/l initial sucrose concentration and 240 rpm stirrer speed.

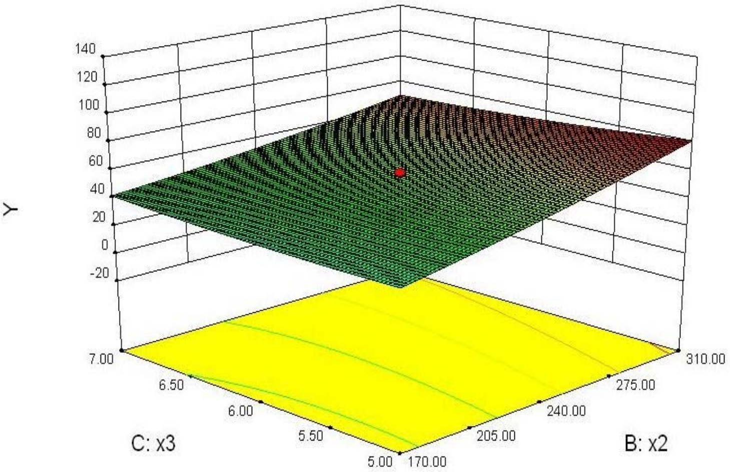

Fig. 4: Response surface curve for citric acid production as afunction of stirrer speed and initial medium pH, while the other variable initial sucrose concentration was fixed at 140 g/l.

In the fig. 4, the interactive effects of stirrer speed and initial medium composition on citric acid production at 5 days of fermentation with fixed levels of initial sucrose concentration was shown. A marginal increase in citric acid production was only found when initialmedium pH was varied from 5.0 to 6.2 was decreased with the medium pH above 6.2. This marginal increase was found for all levels of initial sucrose concentration. The optimum values were found are shown in table. 2.

Table.2

Variable	**Units**	**Optimum value**
Initial Sucrose Concentration	g/l	140
Stirrer speed	rpm	240
Initial medium pH	-	6.0
Citric acid yield	g/l	59.84

4. Conclusions

Based on the results the conclusions can be made as for high productivity of citric acid, optimization of process variables is required. In the present study process variables namely initial sucrose concentration, stirrer speed and initial medium pH were tested for the production of citric acid. Maximum (+1) and minimum (-1) levels of variables initial sucrose concentration (80 – 200 g/l), stirrer speed (170 – 310 rpm) and initial medium pH (5.0 – 7.0) were obtained. Using central composite design 15 combinations of variables were obtained and for each combination the experiment was carried out for the production of citric acid. A second order polynomial equation was developed and the same was optimized using CCD to determine the optimum composition of the variables, within the range of coded levels from +1 to -1 at 5 days of fermentation. Citric acid production was found to be highly and positively influenced by all these three variables initial sucrose concentration, stirrer and initial medium p^H. Maximum yield of citric acid (59.84 g/l) was obtained at 140 g/l initial sucrose concentration, stirrer speed and initial medium p^H.

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