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Optimization of Chemical Parameters for the Production of Citric acid using Box-Behnken Design

Gopinadh R[1]*, Ayyanna C[2], Ramakrishna Ch[3], Narayana Saibaba KV[1], Ravi Vital Kandisa[1] and Jagadhi R[4]

[1]Central Research Laboratory, Industrial Biotechnology, GITAM Institute of Technology, GITAM University, Visakhapatnam-530045, Andhra Pradesh, India
[2]Department of Chemical Engineering, Andhra University, Visakhapatnam-530003, Andhra Pradesh, India
[3]Director, Directorate of UGC affairs and research activities, GITAM University, Visakhapatnam-530045, Andhra Pradesh, India
[4]Department of Pharmaceutical Sciences, Andhra University, Visakhapatnam-530003, Andhra Pradesh, India

Abstract

Statistical experimental design was employed for the improvement of citric acid production from *Madhuca indica* through submerged fermentation process using mutant fungi *Aspergillus niger* MTCC 282. Efficient production of citric acid by mutant *Aspergillus niger* MTCC 282 grown on a sugar rich byproduct requires the supplementation of the optimized quantities of nutrients. Optimization of chemical parameters for the production of citric acid was done by Box-Behnken design. Box-Behnken design was used to statistically compare different combinations of four different nutrients, namely Ammonium sulphate ($(NH_4)_2SO_4$), Magnesium (Mg^{2+}), Ethylene diamine tetra acetic acid (EDTA) and Potassium phosphate (KH_2PO_4), for the maximum production of citric acid. The optimum nutrient concentrations obtained using the Box-Behnken design were Ammonium sulphate ($(NH_4)_2SO_4$) 0.2138 g/l, Magnesium (Mg^{2+}) 0.1896 g/l, Ethylene diamine tetra acetic acid (EDTA) 0.3968 g/l and Potassium phosphate (KH_2PO_4) 3 g/l. Under the optimum conditions, mutant *Aspergillus niger* MTCC 282 produced the maximum citric acid of 75 g/l of citric acid/ kg substrate at pH 4.

Keywords: *Aspergillus niger*; Box-Behnken design; Submerged fermentation; Mahua flower; Citric acid

Introduction

A variety of fungi is reported to produce organic acids such as citric acid, oxalic acid, succinic acid and malic acid. Among them, citric acid production using the filamentous fungi *Aspergillus niger* is well known and is widely used in food, beverages, chemical and pharmaceutical industries [1]. Citric acid is a biodegradable, environmentally accepted and versatile organic acid produced by fermentation [2]. Presently citric acid from mutant *Aspergillus niger* MTCC 282 is economically produced by using various sources. However, the global demand for citric acid is growing faster than its production, implying more economical processes are required to supplement the present processes [3-6]. Hence in the present study a novel source such as *Madhuca indica* was tested for the production of citric acid.

Mahua (*Madhuca indica*) trees are distributed all over the world. Annual production of dry mahua flowers in India is about 2 million tonnes. The flowers, which are succulent, are eaten raw or cooked. They are used in the preparation of distilled liquors and vinegar. They are also used as feed for livestock. But so far they have not been used for fermentation for the production of citric acid and other metabolites. Carbohydrate source has an important effect on the production of citric acid by *Aspergillus niger* [7]. Flowers of *Madhuca indica* are rich in sugars and contain appreciable amounts of vitamins and calcium [8,9]. *Madhuca indica* is of high nutrient value and has vast potential for good quality of citric acid, which will find extensive export market. It is of low cost, economically and abundantly available substrate.

The most important chemical fermentation parameters influencing the growth of *Aspergillus niger* on a liquid substrate and production of citric acid are: nutrients, substrate composition, nitrogen, phosphorous, potassium and other salts [10,11]. Commercial production of citric acid is performed mainly with *Aspergillus niger*. The fungus *Aspergillus niger* is grown on sugar rich byproducts using submerged fermentation to produce citric acid. To produce maximum amount of citric acid, it is essential to study the influence of physical and chemical environments on citric acid production [11]. Nevertheless, this process requires optimization of nutrients. Therefore, experiments were conducted to find the response of *Aspergillus niger* for the production of citric acid at different combinations of four different nutrients such as Ammonium sulphate ($(NH_4)_2SO_4$), Magnesium (Mg^{2+}), Ethylene Diamine Tetra acetic Acid (EDTA), Potassium phosphate (KH_2PO_4).

The traditional one-factor-at-a-time technique used for optimizing the variables is not only time consuming but also often easily confuses the alternative effects between the components. This method requires a large number of experimental works to determine the optimum levels. The drawbacks of the one factor method can be eliminated by optimizing all the effecting parameters collectively by response surface methodology.

Materials and Methods

Microorganism and preparation of inoculum

The fungus *Aspergillus niger* MTCC 282 was obtained from the Microbial Type Culture Collection (MTCC) Chandigarh, India. The fungus used in this study is a UV-mutant strain. Mutants are produced by mutagenic treatment by the method as described by Hamissa [12]. The organism was maintained on Potato Dextrose Agar (PDA) slants (HIMEDIA, India) at 4°C. The culture was inoculated on PDA slants at 30°C for 10 days. 1 ml of 0.1% Tween 80 (Qualigens, India) solution was added to each slant. Spores were scrapped off, suspended in water at 10 ml. The strain was sub-cultured for every two months interval of time.

***Corresponding author:** Gopinadh R, Central Research Laboratory, Industrial Biotechnology, GITAM Institute of Technology, GITAM University, Visakhapatnam-530045, Andhra Pradesh, India, Tel: 986-626-4077; E-mail: gopinadh@gitam.edu

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Production medium

Madhuca indica was used as carbon source for fermentation. Flowers of *Madhuca indica* were collected from agency area in Visakhapatnam, Andhra Pradesh. These flowers were washed thoroughly under tap water and juice was extracted using mortar and pestle. The juice was collected in a beaker and was filtered using Whatman filter paper 50. The filtrate was collected for citric acid production.

Fermentation conditions for submerged fermentation

The submerged fermentation was performed in 250 ml Erlenmeyer flasks. 100 ml of production medium is supplemented with Ammonium sulphate ($(NH_4)_2SO_4$), Magnesium (Mg^{2+}), Ethylene diamine tetra acetic acid (EDTA), Potassium phosphate (KH_2PO_4) to different flasks. The pH of the medium was adjusted to 4.0 by 1M HCl and autoclaved at 121°C for 15 minutes at 15 psi. After autoclaving, the flasks were inoculated with 1 ml (1.0×10^6 spores/ml) of mutant *Aspergillus niger* MTCC 282 inoculum and were incubated at 30°C for 264 hours on an orbital shaking incubator at a speed of 150 rpm.

Analytical procedures

A known volume of fermentation broth was removed for every 24 hours under aseptic conditions and centrifuged at 6000 rpm. The supernatant was analyzed for the estimation of citric acid and total reducing sugars. Citric acid was estimated by the method as described by Marrier and Boulet [13]. Reducing sugars were determined as described by Miller [14].

Experimental design and optimization

Response Surface Methodology (RSM) is a collection of mathematical and statistical techniques for empirical model building. Response surface methods are employed to find factor settings (operating conditions) that produce the best response [15]. Box-Behnken Design (BBD) method is used to examine the relationship between one or more response variables and a set of quantitative experimental variables or factors [16]. In order to describe the nature of response surface in the experimental region and elucidate the optimal concentrations of the most significant independent variables Box-Behnken design was used. In the present study Box-Behnken design was used to optimize medium constituents and conditions for production of citric acid.

Preliminary investigations indicated that Ammonium sulphate ($(NH_4)_2SO_4$), Magnesium (Mg^{2+}), Ethylene diamine tetra acetic acid (EDTA), Potassium phosphate (KH_2PO_4) are highly significant variables for citric acid production. Thus these variables were chosen for optimization studies and designated as X_1, X_2, X_3 and X_4 respectively. The parameters and their range in coded values used in the experiment were described in Table 1.

Coding of the variables was done according to the following equation (1)

$$x_i = (X_i - (\overline{X_i}) / \Delta X_i \qquad i = 1, 2, 3 \ldots k, \qquad (1)$$

Where

X_i = dimensionless value of an independent variable

$\overline{X_i}$ = real value of an independent variable

ΔX_i = Real value of an independent variable at centre point

= Step change

To construct the response surface model, the following second-order polynomial equation was fitted to the data using multiple regressions.

$$Y = \beta_o + \sum_{i=1}^{k}\beta_i x_i + \sum_{i=1}^{k}\beta_{ii}X_i^2 + \sum_{i<}\sum_{j}\beta_{ij}X_iX_j + \varepsilon \qquad (2)$$

Where

Y = response, X = factors,

β_k = regression coefficients and

ε = error term with a normal distribution, mean of 0, and standard deviation of σ.

The values of the coefficients as well as the optimum concentrations were calculated using Matlab. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2.

Results and Discussion

Effects of nutrient levels on citric acid production

Box- Behnken design was used to optimize medium constituents for the production of citric acid. In order to approach the optimum response region of citric acid yield, significant independent variables were further explored, each at three levels according to Box-Behnken. Table 2 represents the design matrix of the coded variables together with the experimental results of citric acid yield. All cultures were grown in 100 ml aliquots in triplicates and the averages of the observations were used. According to the applied design, 30 combinations were executed and their observations were fitted to the following second order polynomial model.

$$Y=-2.1695+159.439X_1-244.357X_2+28.6463X_3+39.972X_4-42.2621X_1^2-137.19X_2^2-20.9154X_3^2-4.43352X_4^2-1.45125X_1X_2+23.5856X_1X_3-64.986X_1X_4-37.2275X_2X_3+110.296X_2X_4-10.0856X_3X_4$$

The goodness of fit of equation was determined by computing predicted citric acid production values and comparing them to those measured. At the model level, the correlation measures for the estimation of the regression equation are the multiple correlation factor R and the determination coefficient R^2. From Table 3, R^2 for citric acid production was 0.997. This value indicates a high degree of correlation between the experimental and predicted values. It is also a measure of fit to the model, which implies a good agreement between the observed and predicted response [7,17,18]. The significance and adequacy of the second-order equation was determined by using analysis of variance (ANOVA). The result of the ANOVA is shown in Table 4. Ammonium sulphate, Magnesium, EDTA, KH_2PO_4 had shown significant impact on citric acid production by *Aspergillus niger* MTCC 282 at 72 hours of fermentation.

Response surface curves

The 3D response surface plots are the graphical representation of the regression equation used to determine the optimum values of

S.No	Variable	Parameter	Coded and actual Level		
			-1	0	1
1	X_1	Ammonium sulphate (g/l)	0.2	0.4	0.6
2	X_2	Magnesium (g/l)	0.05	0.15	0.25
3	X_3	EDTA (g/l)	0.2	0.4	0.6
4	X_4	KH_2PO_4 (g/l)	2	2.5	3

Table 1: Coded Values used in Box-Behnken design to optimize the chemical parameters for citric acid production.

Run No	X_1	X_2	X_3	X_4	Experimental Yield (g/l)	Predicted Yield (g/l)
1	0	0	0	0	63.2092	63.17875
2	0	0	-1	-1	60.2092	59.95509
3	-1	1	0	0	62.8258	63.14466
4	-1	-1	0	0	68.3970	68.13571
5	0	0	0	0	63.2092	63.17875
6	0	-1	-1	0	64.2680	64.64021
7	1	0	1	0	54.2260	54.18126
8	1	0	-1	0	56.1991	56.07438
9	0	-1	0	-1	66.2092	66.57774
10	0	0	1	1	60.2792	60.49529
11	1	0	0	-1	59.1991	59.19458
12	0	1	-1	0	61.2092	61.08021
13	1	0	0	1	50.2092	50.51754
14	0	0	0	0	63.2092	63.17875
15	0	-1	1	0	62.1900	62.34935
16	0	0	-1	1	66.2092	66.29237
17	0	-1	0	1	60.1500	59.8683
18	-1	0	1	0	63.2092	63.34207
19	0	1	1	0	56.1530	55.81115
20	0	0	0	0	63.0260	63.17875
21	0	1	0	1	66.2092	65.8488
22	-1	0	0	-1	57.5230	57.24503
23	1	-1	0	0	57.5030	57.14611
24	0	1	0	-1	50.2092	50.49904
25	-1	0	0	1	74.5275	74.56239
26	-1	0	-1	0	68.9560	69.00888
27	1	1	0	0	51.8157	52.03896
28	0	0	0	0	63.2092	63.17875
29	0	0	1	-1	58.3135	58.19225
30	0	0	0	0	63.2092	63.17875

Table 2: Experimental and Predicted yield of citric acid values.

Term	Coef	SE Coef	T	P
Constant	63.1787	0.12676	498.418	0.000
X_1	-5.5239	0.08963	-61.629	0.00
X_2	-2.5246	0.08963	-28.166	0.000
X_3	-1.8900	0.08963	-21.086	0.000
X_4	2.1601	0.08963	24.100	0.000
$X_1{}^* X_1$	-1.6905	0.11857	-14.257	0.000
$X_2{}^* X_2$	-1.3719	0.11857	-11.570	0.000
$X_3{}^* X_3$	-0.8366	0.11857	-7.056	0.000
$X_4{}^* X_4$	-1.1084	0.11857	-9.348	0.000
$X_1{}^* X_2$	-0.0290	0.15525	-0.187	0.854
$X_1{}^* X_3$	0.9434	0.15525	6.077	0.000
$X_1{}^* X_4$	-6.4986	0.15525	-41.860	0.000
$X_2{}^* X_3$	-0.7445	0.15525	-4.796	0.000
$X_2{}^* X_4$	5.5148	0.15525	35.523	0.000
$X_3{}^* X_4$	-1.0086	0.15525	-6.497	0.000

Table 3: Regression analysis for citric acid production.

S.No	Source	DF	Seq SS	Adj SS	Adj MS	F	P
1	Regression	14	875.213	875.213	62.515	648.46	0.000
2	Linear	4	541.497	541.497	135.374	1404.21	0.000
3	Square	4	33.287	33.287	8.322	86.32	0.000
4	Interaction	6	300.429	300.429	50.072	519.38	0.000
5	Residual Error	15	1.446	1.446	0.096		
6	Lack-of-Fit	10	1.418	1.418	0.142	25.35	0.001
7	Pure Error	5	0.028	0.028	0.006		
8	Total	29	876.659				

Table 4: Analysis of Variance (ANOVA) for citric acid production.

the variables within the ranges considered [19]. The 3D plots for the interactions cyclically between two variables among three the variables are presented in Figures 1-6. The main target of response surface is to hunt efficiently for the optimum values of the variables such that the response is maximized [19]. Each contour curve represents an infinitive number of combinations of the two test variables while the other variable maintained at zero level (center). The maximum predicted value is identified by the surface confined in the smallest ellipse in the contour diagram. Elliptical contours are obtained when there is a perfect interaction between the independent variables [20].

Three-dimensional response surface curves show the interaction between the two variables, while fixing that of the others. Figure 1 shows the interactive effect of KH_2PO_4 and EDTA on citric acid production at 72 hours of fermentation. In the plot, an elliptical response surface in the entire region was found from the second order quadratic equation for the citric acid production. The results showed that the citric acid production was considerably affected by varying the concentration of KH_2PO_4 and EDTA. The maximum production was obtained at the point of intersection of the major and minor axes of the ellipse [21-24].

The interactive effects of KH_2PO_4 and Mg^{2+} on citric acid production at 72 hours of fermentation with fixed level of EDTA and Ammonium sulphate are shown in Figure 2. KH_2PO_4 concentration was found to affect citric acid production nonlinearly at all concentrations of Mg^{2+}. The inhibition by citric acid was due to chelation of Mg^{2+} which is required to chelate the co-substrate ATP, and is most probably irrelevant under physiological conditions where Mg^{2+} is present in excess. Increase in the KH_2PO_4 concentration from 2 to 3 g/l leads to an increase in citric acid production [25,26].

Figure 3 shows the elliptical response surface plot of citric acid production as a function of EDTA and Mg^{2+} at 72 hours of fermentation. The predicted citric acid production decreased at the lower values of ranges for both EDTA and Mg^{2+} concentrations [27]. Initial EDTA concentration strongly affected citric acid production by mutant *Aspergillus niger* MTCC 282. Decrease in the citric acid production is due to the chelation of Mg^{2+} ions. Optimum yields were obtained when Mg^{2+} was in low concentration. EDTA and Mg^{2+} affected the yield of citric acid other than their effect on the growth of mycelium. Their optimum concentrations for maximum citric acid production were interdependent. The maximum production of citric acid was predicted at the EDTA and Mg^{2+} concentration level of about 0.4 g/l and 0.15 g/l [28-30].

Figure 4 represents the surface response curve of citric acid production as a function of Ammonium sulphate $(NH_4)_2SO_4$ and KH_2PO_4. Citric acid production increased with increased concentrations of KH_2PO_4, and decreased concentrations of Ammonium sulphate. The maximum production of citric acid was predicted at the Ammonium

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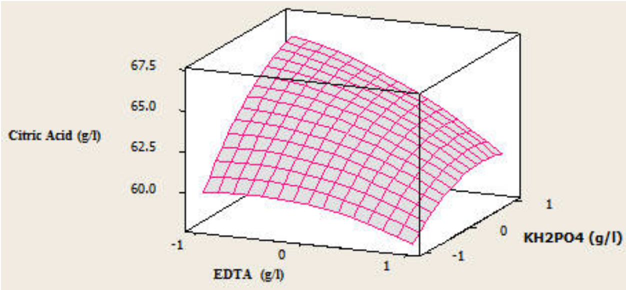

Figure 1: Response surface curve for citric acid production representing the interaction between KH_2PO_4 and EDTA at 72 hours.

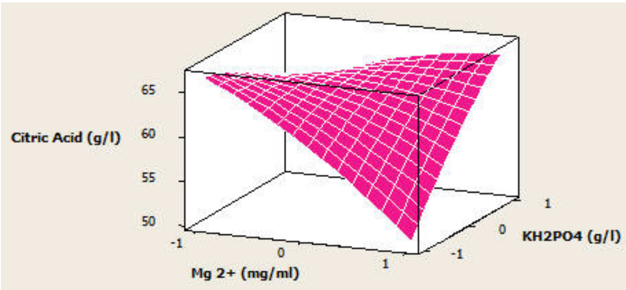

Figure 2: Response surface curve for citric acid production representing the interaction between KH_2PO_4 and Mg^{2+} at 72 hours.

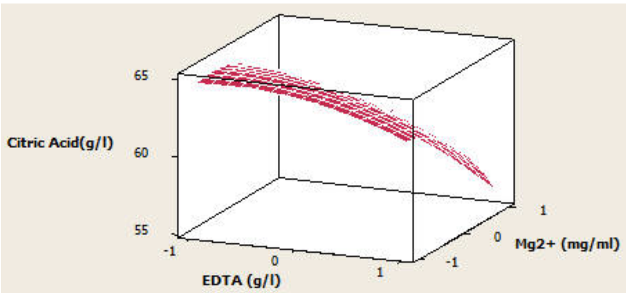

Figure 3: Response surface curve for citric acid production representing the interaction between Mg^{2+} and EDTA at 72 hours.

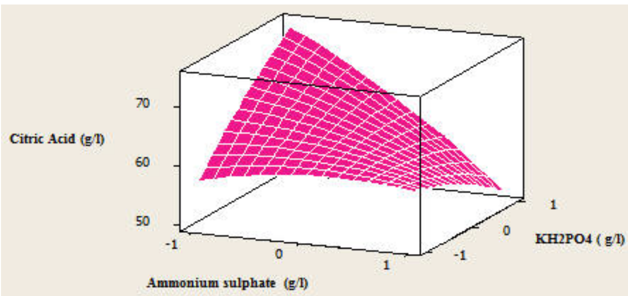

Figure 4: Response surface curve for citric acid production representing the interaction between KH_2PO_4 and Ammonium sulphate at 72 hours.

sulphate $(NH_4)_2SO_4$ 0.2 g/l and KH_2PO_4 3 g/l. High amounts of KH_2PO_4 concentrations favored good yield of citric acid [31,32].

The interactive effect of EDTA and Ammonium sulphate on citric acid production is plotted in Figure 5. Citric acid production in submerged fermentation had a significant effect at low concentration of Ammonium sulphate and high concentration of EDTA. The maximum production of citric acid was predicted at the Ammonium sulphate $(NH_4)_2SO_4$ 0.2 g/l and EDTA 0.4 g/l. The data obtained thus indicate that low ammonium sulphate concentrations are essential for good yield in high concentrations of EDTA [33].

Figure 6 is the response surface plot for variation in the citric acid production, as a function of Mg^{2+} and Ammonium sulphate at 72 hours of fermentation. The production of citric acid was affected by the concentration of Mg^{2+} and Ammonium sulphate [34,35]. At Low concentration of Ammonium sulphate, medium concentration of Mg^{2+} had significant effect on increasing citric acid production at submerged fermentation. The maximum production of citric acid was predicted at the Mg^{2+} and Ammonium sulphate 0.15 g/l and 0.2 g/l.

Figure 7 represents contour plots of citric acid production. Contour plot shows how each response relates to two continuous design variables, while holding the other variables in the model at specified levels.

Figure 8 represents response optimizer plot for citric acid production. Response optimizer plot helps to identify the combination of input variable settings that jointly optimize a single response or a set of responses. Joint optimization satisfies the requirements for all the responses in the set, which is measured by the composite desirability. It provides optimal solution for the input variable combinations [36,37]. Using the response optimizer, the maximum response for citric acid production was 75 g/l with a desirability of 1. The analysis shows that maximum production of citric acid was produced at the following concentrations: Ammonium sulphate $((NH_4)_2SO_4)$ 0.2138 g/l, Magnesium (Mg^{2+}) 0.1896 g/l, Ethylene diamine tetra acetic acid (EDTA) 0.3968 g/l, Potassium phosphate (KH_2PO_4) 3 g/l.

Conclusions

The optimization work in this study has given optimal nutrient concentrations and potent organism for citric acid production from

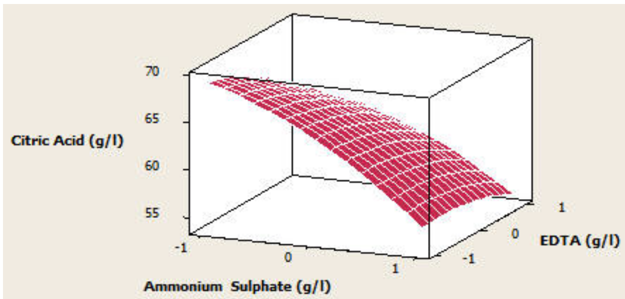

Figure 5: Response surface curve for citric acid production representing the interaction between EDTA and Ammonium sulphate at 72 hours.

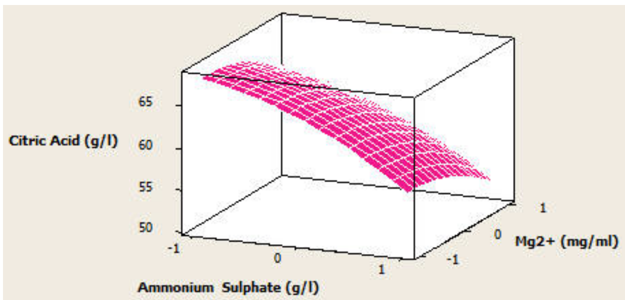

Figure 6: Response surface curve for citric acid production representing the interaction between Mg^{2+} and Ammonium sulphate at 72 hours.

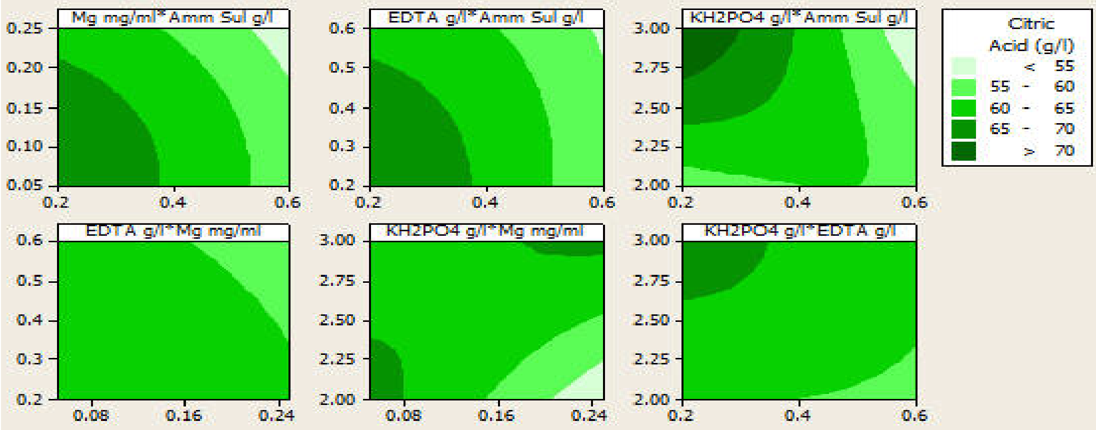

Figure 7: Response contour plots of citric acid.

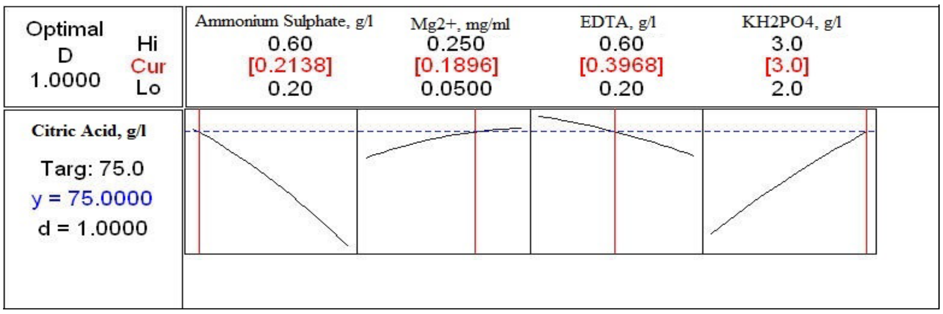

Figure 7: Response optimizer for citric acid production.

Maduca indica. The statistical based optimization using the Box-Behnken design method was proved to be useful for optimizing the nutrient compositions for citric acid production by mutant *Aspergillus niger* MTCC 282 grown on potato dextrose agar medium. For citric acid production, Ammonium sulphate ($(NH_4)_2SO_4$), had a positive effect as it enhanced cell growth resulting in high production of citric acid, The second-order polynomial model obtained was able to identify citric acid production at specific levels of nutrients within the tested range. The optimum values obtained using the Box-Behnken design were Ammonium sulphate ($(NH_4)_2SO_4$) 0.2138 g/l, Magnesium (Mg^{2+}) 0.1896 g/l, Ethylene diamine tetra acetic acid (EDTA) 0.3968 g/l, and Potassium phosphate (KH_2PO_4) 3 g/l. Using the optimal nutrient concentrations, mutant *Aspergillus niger* MTCC 282 produced the maximum citric acid level of 75 g/l of citric acid/ kg substrate at pH 4.

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