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Optimization of Concentration of Medium Ingredients for Production of Citric Acid using Statistical Methods

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Abstract

The concentration of medium ingredients was optimized by applying statistical methods for production of citric acid by locally isolated *Aspergillus niger* PCSIR-06. Box-bhenken design and response surface methodology were applied for designing experiment and statistical analysis of results. The Box-bhenken design limited the number of experiments to fifty four for studying possible interaction between the six medium ingredients i.e., Sucrose, KH_2PO_4, NH_4NO_3, $MgSO_4$, zinc and iron. The statistical method predicted maximum citric acid yield of 108.69 g/l with medium ingredients concentration Sucrose 149.92 g/l, KH_2PO_4 1.25 g/l, NH_4NO_3, 4.66 g/l, $MgSO_4$ 0.15 g/l, Zn 24.00 mg/l, Fe 0.21 mg/l. The verification run confirmed the predicted yield with the given concentration of medium ingredients. These studies successfully demonstrated the use of Box-Bhenken experimental design for the optimization of nutrient concentration for maximum yield of citric acid.

Key words: Optimize, Medium, Citric acid, Box-bhenken design, Response surface methodology

Introduction

Citric acid (2-hydroxy-1,2,3-propanetricarboxylic acid) is produced by fermentation (Roukas and Kotzekidou 1997; Hang and Woodams, 1998). Citric acid is one of the fermentation products with an estimated annual production of about 1,000,000 tons, the highest level of production worldwide (Soccol *et al.,* 2003). Considerable amount of citric acid is required in several industrial processes (Jianlong, 2000). The food industry consumes about 70% of the total citric acid production, while other industries consume the remaining 30% (Yokoya, 1992; Pandey *et al.,* 2001). Citric acid is used in food, beverage, pharmaceutical, chemical, cosmetic and other industries for applications such as acidulation, antioxidation, flavour enhancement, preservation, plasticizer and as a synergistic agent (Sarangbin *et al.,* 1993; Shankaranand and Lonsane, 1994; Suzuki *et al.*,1996). The filamentous fungus *Aspergillus niger* is the most commonly used microorganism for citric acid production (Roukas 1999).

The excretion of intermediates of the TCA cycle (organic acids; for instance citrate, oxalate or succinate) is a characteristic feature of many anamorphic fungal species, such as *Aspergillus* spp. and *Penicillium* spp. Excretion of organic acids is observed in natural habitats (Gadd, 2004) and during growth on solid/liquid media in the laboratory (Foster, 1949). Commercial production of citric acid is generally by submerged fermentation using the filamentous fungus *Aspergillus niger* (Röhr *et al.,* 1983; Vandenberghe *et al.,* 2000, Roukas 1999).

The demand for citric acid production is increasing faster than its production and hence more economical processes are required. The growth and production of citric acid is greatly affected by medium composition, fermentation parameters and stimulators. The medium components nitrogen, carbon and phosphorus and metal ions play critical role in production of citric acid by *Aspergillus sp.* (Kristiansen and Sinclair, 1978).

In development of industrial processes optimization of conditions has vital importance. One variable at a time (OVAT), a classical optimization methodology is most frequently used. It is time and effort consuming. Moreover, it also lacks the study of interaction between variables (Haaland, 1989). In order to optimize biotechnological processes, the statistical

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approaches provide cost-effective way out as they provide pre planned methods through which interaction between variables can easily be calculated (Haaland, 1989; Gupta *et al.,* 2002). Response surface methodology (RSM) is one of the useful models for studying the effect of several factors influencing the responses by varying them simultaneously and carrying out limited number of experiments. Keeping in view of the above facts, present study was designed to optimize the medium ingredients concentration that play important role in growth of fungus and secretion of metabolic products. In order to study the concentration effects and optimization of concentration of the different medium ingredients on yield of citric acid, Box-Behnken design (1960) and response surface methodology (Deshayes, 1980; Metthews *et al.,* 1981) were applied.

Materials and Methods

Microorganism

Aspergillus niger PCSIR-06 isolated by Rahman *et al.* (2008) was used in present studies.

Organism maintenance

The culture of *Aspergillus niger* was maintained on sterilized potato dextrose agar medium (Potato Extract of 200 g/l, Dextrose 20 g/l and Agar 15 g/l), pH 4.5 and stored at 5°C in the refrigerator. All the culture media, unless other wise stated, were sterilized in autoclave at 15 lbs/inch^2 pressure (121ºC) for 15 min.

Inoculum Preparation

The spores of *A. niger* PCSIR-06 were produced in 250 ml Erlenmeyer flasks containing 40 ml PDA medium. The medium was inoculated with spores from the stock culture and incubated at 28°C for seven days. The spores were recovered by stirring using a solution of Tween 80 0.01%.

The suspension obtained, containing 10^8 spores/ml was used as inoculum.

Fermentation technique

Citric acid production experiments were carried out using sterilized medium containing glucose, NH_4NO_3, KH_2PO_4 and $MgSO_4$ as per model design in TUNAIR™ Cell growth Shake Flask System, Shelton Scientific-IBI, working volume 500 ml, shaking speed 200 rpm and filter size 0.22 micron nitrocellulose. 5% v/v inoculum was used for inoculating the medium through out the studies. The whole system was incubated at 30±1ºC for 168 hrs.

Analytical methods

After the completion of each experiment the fermented broth was centrifuged at 10,000 rpm for 10 minutes and the supernatant was used for analysis.

Estimation of Citric Acid

Citric acid was estimated by using pyridine- acetic anhydride method as reported by Marrier and Boulet (1956). One ml of the diluted culture filtrate along with 1.30 ml of pyridine was added in the test tube and swirled briskly. Then 5.70 ml of acetic anhydride was added in the test tube. The test tube was placed in a water bath at 32°C for 30 min. The absorbance was measured by double beam spectrophotometer HITACHI U 2000 at 405 nm and citric acid contents of the sample were estimated by comparison with reference to the standard. The percentage of citric acid was determined.

Experimental design and optimization by RSM

Response surface methodology is a group of experimental techniques to evaluate relationship between cluster of controlled experimental factors and measured responses according to one or more selected criteria. The variables selected were Sucrose, KH_2PO_4, NH_4NO_3, $MgSO_4$, Zn and Fe. Box-Behnken design (Box and Behnken, 1960) was applied to describe the nature of the response surface in the experimental region and to identify the optimum medium components concentration for maximum yield of citric acid.

The actual and coded values along with units for different variables are shown in Table I.

Table II presents the design matrix for medium components sucrose, KH_2PO_4, NH_4NO_3, $MgSO_4$, Zn and Fe consisting of 54 trials to study the yield of citric acid. Each variable was studied on three levels, coded -1, 0 and +1 for low, middle and high values.

In order to predict the optimal point, a second order polynomial function was fitted to correlate the relationship between the independent variable and the response citric acid yield. For six factors, the equation was

Table I: The coded and actual values of the factors in Box-Behnken design

Factors	Name of factors	Units	Low coded	Low actual	Centre coded	Centre actual	High coded	High actual
A	Sucrose	gl^{-1}	-1	50.00	0	100.00	1	150.00
B	KH_2PO_4	gl^{-1}	-1	1.25	0	3.13	1	5.00
C	NH_4NO_3	gl^{-1}	-1	1.25	0	3.13	1	5.00
D	$MgSO_4$	gl^{-1}	-1	0.125	0	0.31	1	0.375
E	Zn	mgl^{-1}	-1	6.00	0	15.00	1	24.00
F	Fe	mgl^{-1}	-1	0.20	0	0.50	1	0.80

$$Y = \beta_0+\beta_1A+\beta_2B+\beta_3C+\beta_4D+\beta_5E+\beta_6F+\beta_{12}AB+\beta_{13}AC+\beta_{14}AD+\beta_{15}AE+\beta_{16}AF+\beta_{23}BC+\beta_{24}BD+\beta_{25}BE+\beta_{26}BF+\beta_{34}CD+\beta_{35}CE+\beta_{36}CF+\beta_{45}DE+\beta_{46}DF+\beta_{56}EF+\beta_{11}A^2+\beta_{22}B^2+\beta_{33}C^2+\beta_{44}D^2+\beta_{55}E^2+\beta_{66}F^2$$

Where Y is the measured response Citric Acid yield, β_o is the model constant, β_1, β_2,.... β_6 are linear coefficient, β_{11}, β_{22}, β_{33}, β_{44}, β_{55}, β_{66} are quadratic coefficient, β_{12}, β_{13},.... β_{46} are cross product coefficient and A,B,C …… F are coded independent variables.

Table II: Box-Behnken design for six factors influencing citric acid yield

Std order	Design Id	Run order	Point type	Factors					
				A	B	C	D	E	F
1	10	17	IBFact	0	1	-1	0	-1	0
2	23	21	IBFact	0	0	-1	1	0	1
3	3	33	IBFact	-1	1	0	-1	0	0
4	31	28	IBFact	-1	0	0	1	1	0
5	7	6	IBFact	-1	1	0	1	0	0
6	25	11	IBFact	-1	0	0	-1	-1	0
7	29	43	IBFact	-1	0	0	-1	1	0
8	26	7	IBFact	1	0	0	-1	-1	0
9	48	4	IBFact	1	0	1	0	0	1
10	41	12	IBFact	-1	0	-1	0	0	-1
11	8	40	IBFact	1	1	0	1	0	0
12	15	44	IBFact	0	-1	1	0	1	0
13	28	26	IBFact	1	0	0	1	-1	0
14	38	10	IBFact	0	1	0	0	-1	1
15	18	19	IBFact	0	0	1	-1	0	-1
16	35	39	IBFact	0	-1	0	0	1	-1
17	16	31	IBFact	0	1	1	0	1	0
18	36	15	IBFact	0	1	0	0	1	-1
19	42	37	IBFact	1	0	-1	0	0	-1
20	37	14	IBFact	0	-	0	0	-1	1
21	27	16	IBFact	-	0	0	1	-1	0
22	43	23	IBFact	-	0	1	0	0	-1
23	21	35	IBFact	0	0	-1	-1	0	1
24	13	51	IBFact	0	-	-1	0	1	0
25	30	32	IBFact	1	0	0	-1	1	0
26	20	2	IBFact	0	0	1	1	0	-1
27	2	38	IBFact	1	-1	0	-1	0	0
28	5	54	IBFact	-1	-1	0	1	0	0
29	47	29	IBFact	-1	0	1	0	0	1
30	1	48	IBFact	-1	-1	0	-1	0	0

Table II: To be contd.

Std order	Design Id	Run order	Point type	Factors					
				A	B	C	D	E	F
31	14	42	IBFact	0	1	-1	0	1	0
32	33	34	IBFact	0	-1	0	0	-1	-1
33	39	8	IBFact	0	-1	0	0	1	1
34	46	41	IBFact	1	0	-1	0	0	1
35	12	20	IBFact	0	1	1	0	-1	0
36	32	53	IBFact	1	0	0	1	1	0
37	6	27	IBFact	1	-1	0	1	0	0
38	22	52	IBFact	0	0	1	-1	0	1
39	17	49	IBFact	0	0	-1	-1	0	-1
40	4	22	IBFact	1	1	0	-1	0	0
41	44	1	IBFact	1	0	1	0	0	-1
42	19	3	IBFact	0	0	-1	1	0	-1
43	24	46	IBFact	0	0	1	1	0	1
44	9	30	IBFact	0	-1	-1	0	-1	0
45	11	5	IBFact	0	-1	1	0	-1	0
46	45	9	IBFact	-1	0	-1	0	0	1
47	34	25	IBFact	0	1	0	0	-1	-1
48	40	45	IBFact	0	1	0	0	1	1
49	0	24	Centre	0	0	0	0	0	0
50	0	36	Centre	0	0	0	0	0	0
51	0	13	Centre	0	0	0	0	0	0
52	0	50	Centre	0	0	0	0	0	0
53	0	18	Centre	0	0	0	0	0	0
54	0	47	Centre	0	0	0	0	0	0

Software Design-Expert (V.7.1.6, Statease, Minneapolis, 2008) was used for regression analysis of experimental data and graphical analysis. The quality of fit of the polynomial model equation was expressed by a coefficient of determination R^2. The optimal concentrations of critical variables were obtained by analyzing contour plots. The statistical analysis of the model was represented in the form of Analysis of Variance (ANOVA). All the experiments were performed in triplicate and the mean values were subjected for analysis.

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Results and Discussions

Model diagnostics

Residual analysis (Fig. 1) was used to examine the adequacy of the presented model. Fig. 1a checks the lurking variables that may have influenced the response during the experiment. The plot showed a random scatter. Trends indicate a time related variable lurking in the background. Blocking and randomization provide insurance against trends ruining the analysis. Fig. 1b represents the difference between the actual and predicted response. The residual differences were large enough when compared with the actual and predicted one. The difference was due to the presence of noise during the experimentation. Box-Cox plot provides a guide line for selecting the correct power law transformation. The lowest point on Box Cox plot represents the value of lambda (λ) that results in the minimum residual sum of squares in the transformed model. Here the value of Lambda is 'λ =1' which means no transformation is required (Fig. 1c).

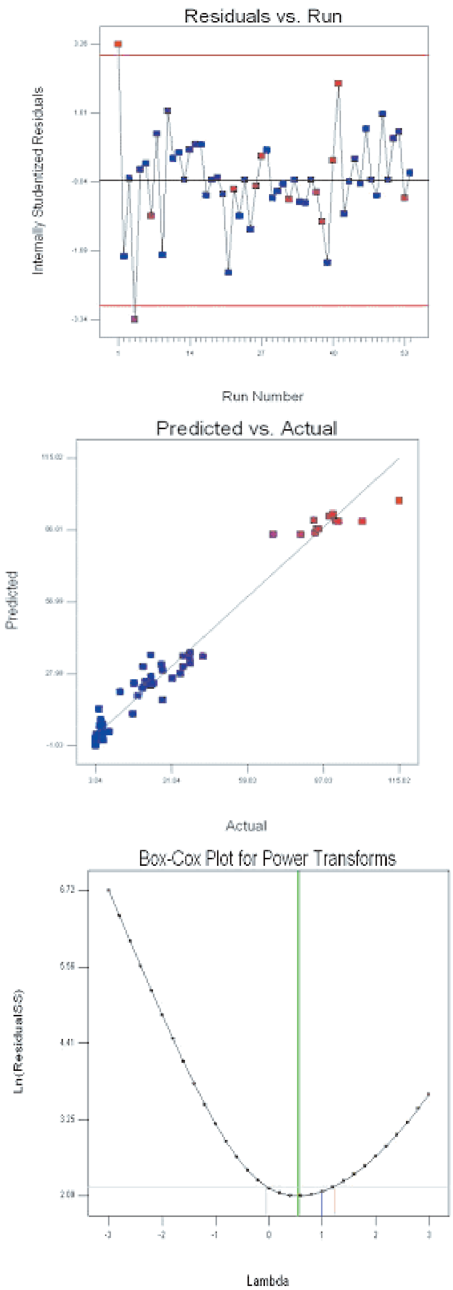

Fig. 1: Residual diagnostic of model for citric acid yield (a) Residual versus run order (b) Perdicted versus actual (c) Box-Cox Plot for power transformation Lambda Current=1, Best=0.56 Low C.I. = - 0.05 High C.I.=1.23

Response analysis of citric acid yield

The model obtained by statistical package "Design expert" describing the response as function of variable was as follows:

Citric acid yield = 22.89 + 41.92 A - 1.46 B - 2.61 C - 2.25 D - 1.59 E + 1.21 F - 0.47 AB + 0.24 AC + 1.107 AD + 2.15 AE - 2.9 AF - 2.70 BC + 0.91 BD - 1.01 BE + 0.48 BF - 5.08 CE - 4.05 CE - 4.11 CF + 0.01 DE - 0.45 DF - 4.54 EF + 21.73 A^2+ 3.11 B^2 + 0.43 C^2 - 1.80 D^2 + 0.84 E^2 + 1.36 F^2

The results of 2nd order response surface model fitted in the form of analysis of variance (ANOVA) are depicted in Table IV. The Model F-value of 27.21 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob>F" less than 0.0500 indicate model terms are significant.

In this case A , CF and A^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. As there are many insignificant model terms, model reduction could improve the model. The "Pred R-Squared" of 0.7866 is in reasonable agreement with the "Adj R-Squared" of 0.9303. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Here the ratio of 16.785 indicates an adequate signal. This indicates a better precision and reliability of the experiment.

As stated earlier that p-values of "prob. > F" less than 0.0500 indicate significant model terms. Here in this particular case A, CF and A^2 are significant model terms. The "lack of fit value" of 82.77 is significant relative to pure error. A reduced model obtained describing the response as function of the most significant variables is as follows:

Citric Acid Yield = 22.89 + 41.91A - 4.11CF + 21.73A^2

The relationship of controlled experimental factors and measured responses on the basis of different selected criteria are shown in Fig. 2-6.

The effect of combination of sucrose with other factors i.e., KH_2PO_4, NH_4NO_3, $MgSO_4$, Zn and Fe, in the form of response surface and contour plots are presented in Fig. 2.

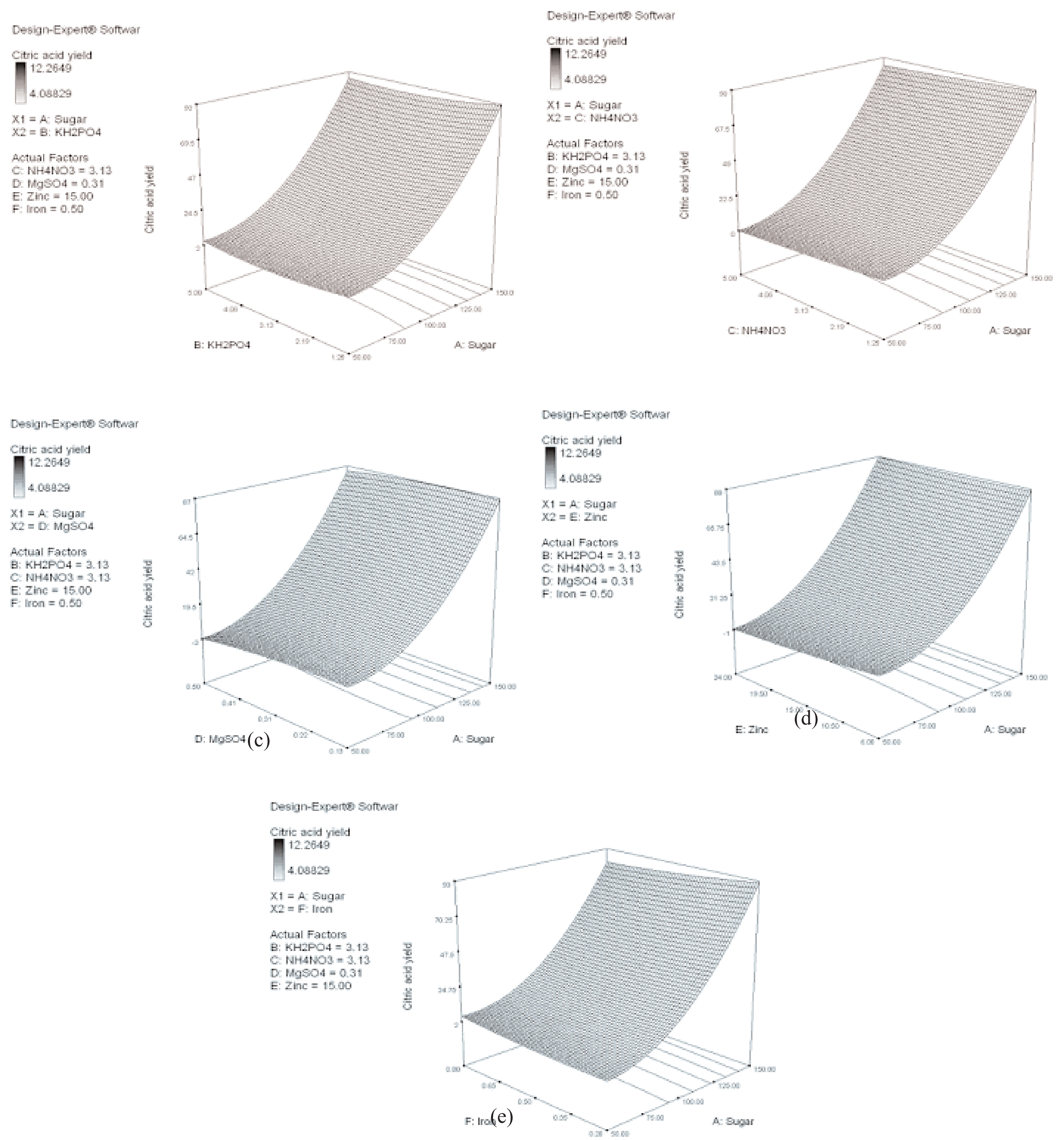

Fig. 2: **Response surface and contour plot showing effect of sucrose in combination with other nutrient factors on citric acid yied by *Aspergillus niger* PCSIR-06**
a) Sucrose + KH_2PO_4 b) Sucrose + NH_4NO_3 c) Sucrose + $MgSO_4$,
d) Sucrose + Zn e) Sucrose + Fe

The variation in concentration of all the individual ingredients with sucrose while keeping the rest of the factors at central level showed similar trends and revealed that sucrose is a major factor controlling the yield of citric acid in this case. The excessive concentration of carbon source is required for citric acid overflow during fermentation (Kristian and Sinclair, 1978).

The data regarding the variation in concentration in combinations of KH_2PO_4 with other individual nutrients while keeping the remaining nutrients at central points is presented in Fig. 3. The data revealed that maximum citric acid yield achieved was about 30g/l in combinations of KH_2PO_4 at maximum level with minimum level of NH_4NO_3 while rest of the ingredients kept at central points. These results confirmed the findings of Kristiansen and Sinclair (1979) that nitrogen must be limited to attain highest yields of citric acid. An appropriate balance of nitrogen and phosphate is important for the accumulation of citric acid in batch culture (Shu and Johnson, 1948). The other combinations showed mixed effect on yield of citric acid.

The response surface and contour plot of the effects of NH_4NO_3 concentration in combination with other nutrients i.e, $MgSO_4$, Zn and Fe revealed that citric acid yield was possible in the range of $\simeq$ 10.0 to $\simeq$ 30 g/l (Fig. 4). Fig. 4 also unzip the fact that NH_4NO_3 a nitrogen source is required at maximum level with minimum levels of metals (Fe, Zn) and $MgSO_4$ for maximum yield of citric acid and vice versa. In defined media nitrogen is supplied as ammonium sulfate or ammonium nitrate. The advantage of ammonium salts is that they decrease pH as they are consumed, which is a prerequisite of citric acid production (Papagianni *et al.,* 2005).

The data presented in Fig. 5 regarding variation in combination of $MgSO_4$ with Zn and Fe showed more or less the same pattern of citric acid yield. The combination of $MgSO_4$ $\simeq$ 0.41g/l , Zn $\simeq$ 15.0 mg/l and Fe $\simeq$ 0.5 mg/l while keeping rest of ingredients at central level gave maximum yield of citric acid.

The studies on optimization of Zn and iron keeping other nutrients at central point (Sucrose, NH_4NO_3, KH_2PO_4,

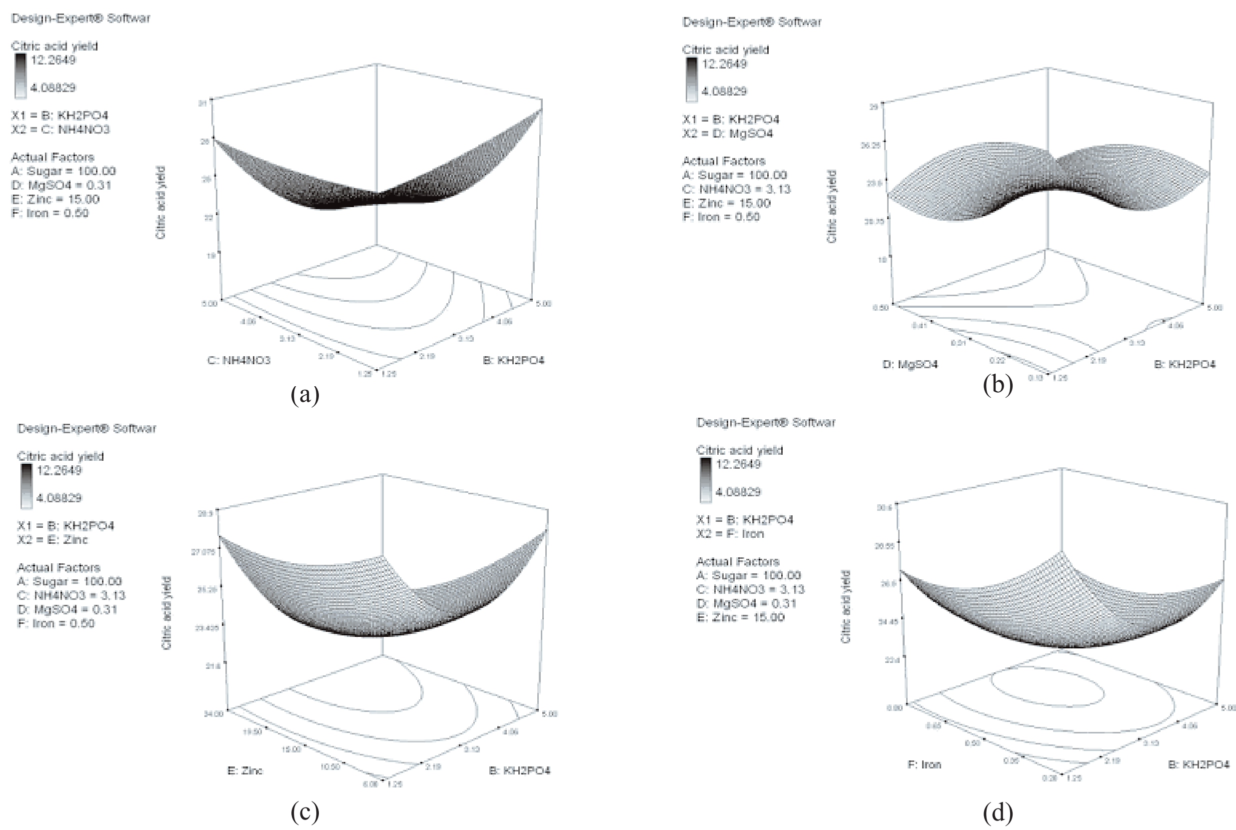

Fig. 3: Response surface and contour plot showing effect of sucrose in combination with other nutrient factors on citric acid yield by *Aspergillus niger* PCSIR-06
a) KH_2PO_4 + NH_4NO_3 b) KH_2PO_4 + $MgSO_4$, c) Sucrose + Zn d) Sucrose + Fe

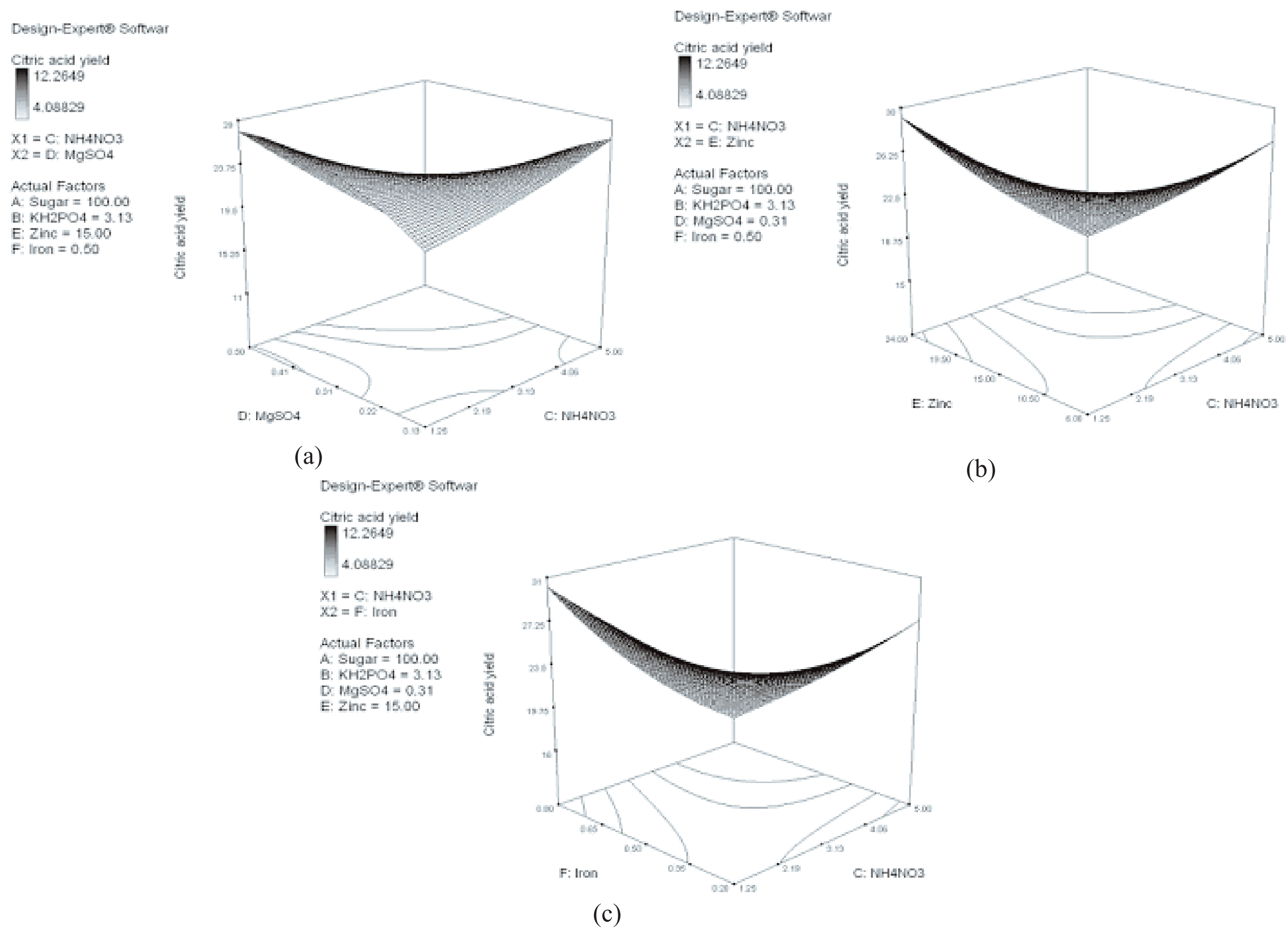

Fig. 4: Response surface and contour plot showing effect of NH_4NO_3 in combination with other nutrient factors on citric acid yield by *Aspergillus niger* PCSIR-06
a) NH_4NO_3 + $MgSO_4$, b) NH_4NO_3 + Zn c) NH_4NO_3 + Fe

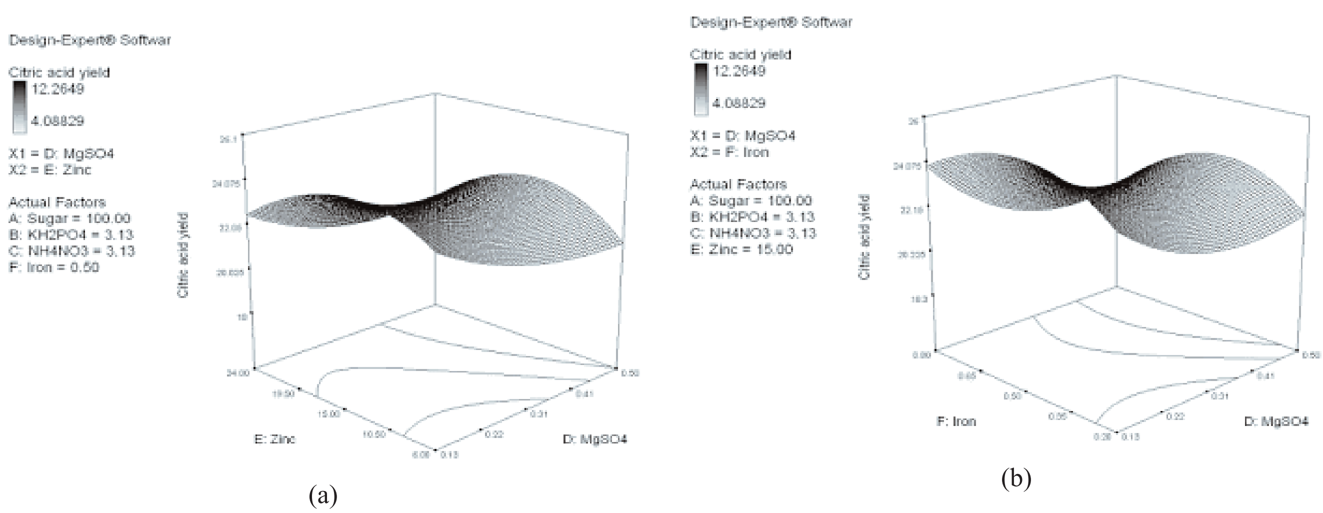

Fig. 5: Response surface and contour plot showing effect of $MgSO_4$ in combination with other nutrient factors on citric acid yield by *Aspergillus niger* PCSIR-06
a) $MgSO_4$,+Zn b) $MgSO_4$ + Fe

Table III: Analysis of variance (ANOVA) for the quadratic model for citric acid yield

Source	Sum of squares	df	Mean square	F - Value	p-value Prob > F
Model	49118.78	27	1819.214226	27.21075	< 0.0001
A-Sugar	26253.95	1	26253.95101	392.6913	< 0.0001
B-KH_2PO_4	39.11773	1	39.117734	0.5851	0.4512
C-NH_4NO_3	113.335	1	113.3349525	1.695198	0.2043
D-$MgSO_4$	78.56772	1	78.56771502	1.17517	0.2883
E-Zinc	42.21523	1	42.21522973	0.631431	0.4340
F-Iron	25.1164	1	25.11640356	0.375677	0.5453
AB	1.907194	1	1.907194475	0.028527	0.8672
AC	0.503739	1	0.503738735	0.007535	0.9315
AD	20.09994	1	20.09994222	0.300643	0.5882
AE	39.76439	1	39.76439379	0.594773	0.4475
AF	75.45466	1	75.45466419	1.128607	0.2978
BC	72.35162	1	72.35162453	1.082194	0.3078
BD	7.634949	1	7.634949267	0.114199	0.7381
BE	18.06053	1	18.0605292	0.270139	0.6076
BF	2.152658	1	2.152658044	0.032198	0.8590
CD	235.1543	1	235.154306	3.517302	0.0720
CE	145.6931	1	145.6931008	2.179193	0.1519
CF	288.0419	1	288.0418513	4.308363	0.0480
DE	0.000522	1	0.000522421	7.81E-06	0.9978
DF	1.87795	1	1.877949785	0.028089	0.8682
EF	187.5719	1	187.5718981	2.805591	0.1059
A^2	4805.703	1	4805.703251	71.88092	< 0.0001
B^2	82.69619	1	82.69618826	1.236922	0.2762
C^2	1.469401	1	1.469400545	0.021978	0.8833
D^2	25.23687	1	25.23687393	0.377479	0.5443
E^2	5.399127	1	5.399127091	0.080757	0.7785
F^2	14.22931	1	14.22931036	0.212834	0.6484
Residual	1738.268	26	66.85645369		
Lack of Fit	1738.268	21	82.77465695		
Pure Error	0	5	0		
Cor Total	50857.05	53			
Std. Dev.	8.17658		R-Squared	0.965821	
Mean	35.66168		Adj R-Squared	0.930326	
C.V. %	22.92819		Pred R-Squared	0.786623	
PRESS	10851.73		Adeq Precision	16.78523	

$MgSO_4$) (Fig. 6) revealed that maximum yield of citric acid could be achieved by keeping one of the both metals at minimum and other at maximum level in the medium. The trace metal have important role in accumulation of citric acid (Shu and Johnson (1948). They synergistically influence on yield of citric acid (Kristiansen and Sinclair, 1979).

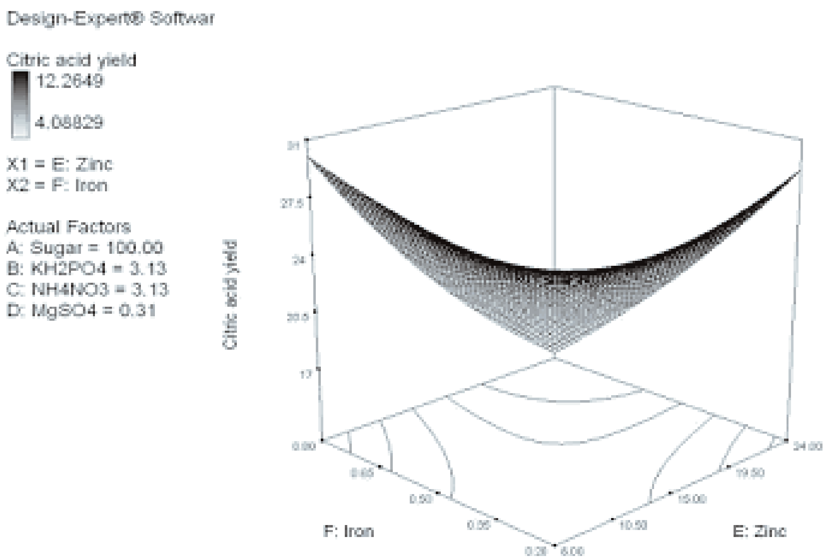

Fig. 6: Response surface and contour plot showing effect of Fe and Zn on citric acid yield by *Aspergillus niger* PCSIR-06

On the basis of above described relationships among the experimental factors, the Model predicted optimized concentrations of medium ingredients i.e., Sucrose 149.92 g/l, KH_2PO_4 1.25 g/l, NH_4NO_3, 4.66 g/l, $MgSO_4$ 0.15 g/l, Zn 24.00 mg/l, Fe 0.21 mg/l for maximum citric acid yield i.e., 108.69 g/l.

Verification

A verification run was conducted in triplicate to confirm the predicted optimized concentrations of the medium ingredients using *Aspergillus niger* PCSIR 06 with requisite conditions. The mean citric acid yield of 102.18± 2.38 g/l was obtained with the optimized concentrations of medium ingredients which is quite close to predicted yield of 108.69 ± 2.92 %. The application of response surface methodology to optimize the medium composition for various processes has also been reported by many workers (Abdel-Fateh and Olama, 2002; Chang *et al.,* 2002; El-Helow *et al.,* 2000; Francis *et al.,* 2003).

Conclusion

It can be safely concluded from the aforsaid study that the application of Box-Bhenken design and response surface analysis to optimize the different factors for maximal production is an efficient method that evaluates the interactive effect of each factor. Further it converts the bioprocess factors correlation into a mathmetical model that predicts where the optimum is likely to be located.

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