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Analysis of Physicochemical Variables and Their Interactive Effect on Uricase Production from *Alcaligenes faecalis*

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Abstract: Uricase catalyzes the conversion of uric acid into allantoin and hydrogen peroxide by oxidation reaction. It has therapeutic potential in treating gout, hyperuricemia and analysis of uric acid in biological fluids. The interactive effect of various physico-chemical variables on uricase production from isolated *Alcaligenes faecalis* strain has been studied through response surface methodology. The role of most significant factors were evaluated and medium pH (7.0), inducer (uric acid) concentration (3 g/L), agitation rate (150 rpm) and incubation time (45 h) were found to play critical role in the production of enzyme. The production of uricase by *A. faecalis* increased up to 2.5-fold after applying the RSM.

Key words: Uricase, *Alcaligenesfaecalis*, Response surface methodology, Hyperuricemia.

Introduction

Uricase is useful for enzymatic determination of uric acid in clinical analysis of biological fluids and used as a protein drug to reduce uric acid accumulation [12]. In humans, uricase gene has been inactivated during the course of evolution [8], biological reason for this loss has not been explained with valid theory. Due to absence of uricase gene in humans, the plasma concentration of uric acid is rather high [11] and some time its abnormal rise in blood can lead to development of disease known as gout. In some cases, hyperuricemia resulted into increased insulin absorption resistance in tissues of patient with type 2 diabetes and increased nephropathy progression [7, 18]. Gout prevalence has been increased in direct association with age, lifestyle and dietary factors, such as heavy consumption of beer and liquor as well as protein rich diets [4, 22]. Allopurinol and febuxostat are anti-hyperuricemia drugs that competitively inhibit xanthine oxidase.

Allopurinol has been used as first-line drug for treating gout, but refractory gout occurs when patients suffer from hypersensitivity or non-responsiveness to allopurinol, intolerance to allopurinol toxicity [15,19,22]. Polyphenols[10,14,25], coumarins [9], ellagic acid, valoneic acid dilactone [26] have been reported to be potent plant-based xanthine oxidase inhibitor.

The main hindrance for using plant based medicine for treatment of gout is extraction of these compounds from their crude extract [20]. The enzyme uricase that convert insoluble uric acid into soluble allantoin has been proved to be a better alternate for treating gout. Several microorganisms has been reported to produce uricase such as *Aspergillus niger* [1], *Alcaligenes faecalis* [3], *Bacillus* sp. TB-90 [27], *Mucorhiemalis* [28], *Giomastrics gueg* [5], *Pseudomonas aeruginosa* [24].

The present study focuses on achieving the maximum uricase production from novel isolate *Alcaligenes faecalis*. The interactive effect of various physicochemical parameters and their combinations on uricase production has been studied through applying response surface methodology.

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Material and methods

Chemicals

The chemicals used in experiment, were of analytical grade and were purchased from Hi-Media, and MP Biochemicals, India.

Microorganism and culture conditions

The *Alcaligenes faecalis* used in the study was isolated from soil samples of the bird sanctuary. The isolation was carried out on nutrient agar medium (pH 7.0) containing 3 g/L uric acid. Initially, culture medium (pH 6.5) containing (g/L): Dextrose-10.0, Yeast extract- 2.0, NaCl -5.0, Uric acid- 3.0 was used for uricase production at 30°C (150 rpm) in a temperature controlled orbital shaker.

Uricase assay

The enzymatic assay for detecting uricase activity was carried out by the method as described by Mahler *et al*., [21]. Reaction mixture consists of 3 mL of 20 mM boric acid buffer of pH 8.5, 75 µL of 3.57 mM uric acid solution and 25 µL of enzyme and incubated at 30°C for 20 minutes. The decrease in the uric acid concentration in test solution due to the uricase action was measured with the aid of a UV-visible spectrophotometer at 293 nm. One unit of enzyme activity was defined as the amount of uricase required to convert 1 µmol of uric acid into allantoin per minute at 30°C and at pH 8.5, considering milli molar extinction co-efficient of uric acid at 293 nm as 12.6 $mM^{-1}cm^{-1}$.

Screening of the medium components on the basis of Plackett-Burman design

Screening of the most important physicochemical components affecting the production of uricase by *A. faecalis* was performed by Plackett-Burman design. A total of seven components were selected for this study viz., dextrose, yeast extract, NaCl, inducer concentration, pH, Temperature, agitation rate, inoculum volume, production time, respectively. A set of 12 combinations was generated by the software, each being represented at two levels, high (+1) and low (-1), in which the uricase activity was determined individually. A pareto graph showing the positive effect of these constituents was also prepared. Design Expert (Version 10.0) was used for Plackett-Burman design and regression analysis. The effect of each variable was calculated using the following equation:

$$E = (\Sigma M^{+} - \Sigma M^{-})/N \quad \text{Eq. 1}$$

where 'E' is the effect of the tested variable and M^{+} and M^{-} are responses (uricase activity) of experiments at which the parameter was at its higher and lower level, respectively, and N is the number of experiment carried out.

Central composite design to study lack of fit and ANOVA of designed statistical model

Central composite design (CCD) was employed to optimize the fermentation conditions which have shown positive effect on uricase production viz., inducer concentration, pH, production time or incubation time and agitation rate, keeping rest of the production conditions and medium components same. The CCD contained a total of 30 experimental runs. The statistical model was validated through experiments under shake flask conditions for the production of uricase as experiments predicted by design expert.

Result and discussion

Since, manual optimization of process parameters has many drawbacks associated with it, as it does not explain the interactive effect of different variables used in experiment. So it affects interpretation of experimental results [2,6]. Fisher developed the basic theory of experimental design to get over this difficulty [23], which proves the transcendence of study of more than one factor at a time over only one factor at a time [16]. The Response Surface Methodology (RSM) measures the interaction between the response(s) and the independent variables [13] and defines the effect of the independent variables either alone or in combination. This method is an important tool to find out the optimum physicochemical conditions necessary for the scale up of the process and to reduce the number and cost of experiments [17]. In present work, RSM has been applied to optimize the process parameter and their interactive effect on production of uricase by *Alcaligenes faecalis*.

Screening of important physicochemical conditions using Plackett–Burman design

The effect of seven independent variables (dextrose, yeast extract, NaCl, inducer concentration, pH, Temperature, agitation rate, inoculum volume and production time) on the production of uricase enzyme was observed. The study was carried out using Plackett-Burman design which gives 12 different combinations of physicochemical conditions selected for the experiment.

Table 1 shows the seven selected independent variables and their interactive effect on the production of uricase enzyme. Result obtained by performing Plackett-Burman design was further used to construct a Pareto chart (Fig. 1) to find out the variables which has positive effect on uricase production. It was found that inducer concentration, pH, production time and agitation rate has positive effect on uricase production.

CCD to study lack of fit and ANOVA of designed statistical model

CCD was employed to optimize the fermentation conditions, namely, inducer concentration, pH, production time and agitation rate, keeping rest of the production conditions and medium components same (Table 2). Second-order polynomial equation was derived to explain the dependence of uricase production on medium components. The results of CCD were fitted into second order polynomial equation for the prediction of response on the bases of coded value:

Responses = R1= +28.40 +3.64A +4.34B + 1.92C +0.99D +3.14AB +1.34AC +0.92BD + 0.19 CD-6.84A^2 -4.97B–6.79C^2 -3.39D^2 Eq.2

The analysis of variance of the quadratic regression model suggested that the model was significant. Three dimensional (3-D) graphs were generated for regression analysis of CCD design, using pair wise combination for uricase production. These 3-D response surface plots describe the combined effect of each independent variable upon the uricase acivity.

The CCD contained a total of 30 experimental runs. The maximum production of uricase obtained experimentally through response surface methodology was 31.9 IU.

Aanalysis of variance (ANOVA) analysis of CCD results was carried out and four process

Table 1. Screening of important factors using Plackett-Burman design

Run	Dextrose (g/L)	Y.E (g/L)	NaCl (g/L)	Inducer (g/L)	pH	Temperature (ºC)	Agitation (rpm)	Inoculum volume (%, v/v)	Production time (h)	Response (IU)
1	1	1	1	5	5	45	200	1	72	6.5
2	1	1	5	0	9	45	100	6	72	0.0
3	10	10	5	0	5	25	200	1	72	0.0
4	10	10	1	5	9	45	100	1	18	1.2
5	1	10	5	0	9	45	200	1	18	0.0
6	10	10	1	0	5	45	100	6	72	0.0
7	1	10	1	5	9	25	200	6	72	12.5
8	10	1	5	5	9	25	100	1	72	9.5
9	10	1	5	5	5	45	200	6	18	1.8
10	10	1	1	0	9	25	200	6	18	0.0
11	1	10	5	5	5	25	100	6	18	0.7
12	1	1	1	0	5	25	100	1	18	0.03

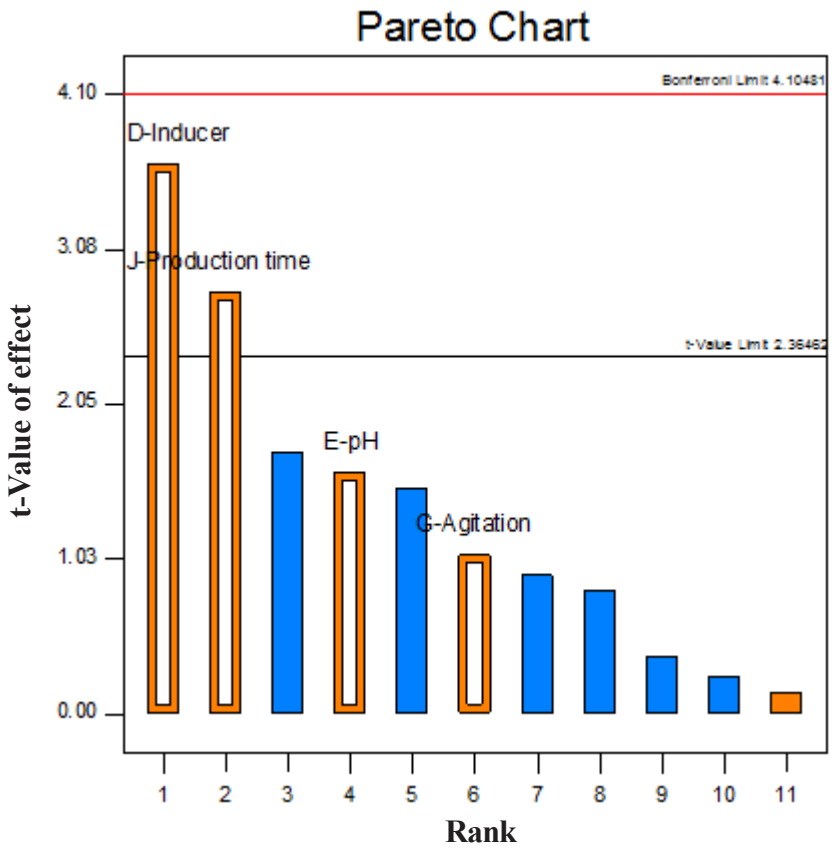

Fig. 1. A pareto chart showing the positive and negative effects of selected variables

Table 2. Central Composite experimental design by using factors having positive effect on uricase activity

Run	Inducer (g/L)	Production time (h)	pH	Agitation (rpm)	Response (IU)
1	2.5	45	7	150	30.1
2	5.0	15	9	200	1.8
3	2.5	45	7	150	29.8
4	0.0	72	5	100	0.0
5	2.5	45	11	150	12.3
6	2.5	45	7	150	29.5
7	5.0	18	5	100	1.7
8	0.0	18	5	100	0.0
9	0.0	18	9	100	0.0
10	2.5	45	7	250	21.9
11	5.0	72	9	100	15.2
12	-2.5	45	7	150	0.0
13	5.0	72	9	200	24.3
14	5.0	72	5	200	11.9
15	0.0	18	9	200	0.0
16	2.5	45	7	150	29.8

table 2. (continued).

Run	Inducer (g/L)	Production time (h)	pH	Agitation (rpm)	Response (IU)
17	2.5	45	7	150	31.9
18	0.0	72	5	200	0.0
19	2.5	45	7	150	20.9
20	2.5	99	7	150	26.9
21	5.0	18	5	200	1.5
22	0.0	72	9	100	0.0
23	5.0	72	5	100	5.7
24	2.5	45	7	50	17.6
25	7.5	45	7	150	16.9
26	5.0	18	9	100	1.4
27	2.5	-9	7	150	0.0
28	0.0	72	9	200	0.0
29	0.0	18	5	200	0.0
30	2.5	45	3	150	0.0

order were suggested by Design expert 10.0. Cubic and Quardratic process order proved best and processed for further analysis due to low standard deviation (2.69) and high R-squared value (0.779) respectively (Table 3).

Three dimensional (3D) graphs were generated for regression analysis of CCD design of pair wise combination of four factors for uricase production. These 3D response surface plots described the effects of the independent variables and combined effects of each independent variable upon the uricase activity.

These 3-D response surface plots describe the effects of the independent variables and combined effect of each independent variable upon the response i.e., uricase activity (Fig. 2a-f). The 3-D response surface plots were constructed by plotting the response i.e., uricase activity on the Z-axis against any two independent variables, while maintaining other variables at their optimal levels. As shown in Fig. 2(a) curvature in the response surface indicates lower and higher values of both, inducer (uric acid) and production time or production time (h) did not result in higher response. The increase in the uric acid concentration from 1 to 3 g/L and prolonged production time from 18 to 45 h led to increase in uricase activity. However, further increase in both the components consequently decreases the enzyme activity (Fig. 2a). More or less similar type of proûles were observed for effect of pH (medium) and inducer, effect of agitation rate and inducer, effect of pH and production time, effect of agitation rate and production time, effect of agitation rate and pH (Fig. 2a-f). The most suitable physicochemical conditions obtained by applying RSM are pH 7, inducer 3g/

Table 3. Quadratic fitted model analysis

Standard deviation	2.55
Mean	2.69
C.V. %	94.9
Press	134.07
R^2	0.7799
Adjusted- R^2	0.6542
Predicted-R^2	0.3532
Adequate Precision	8.140

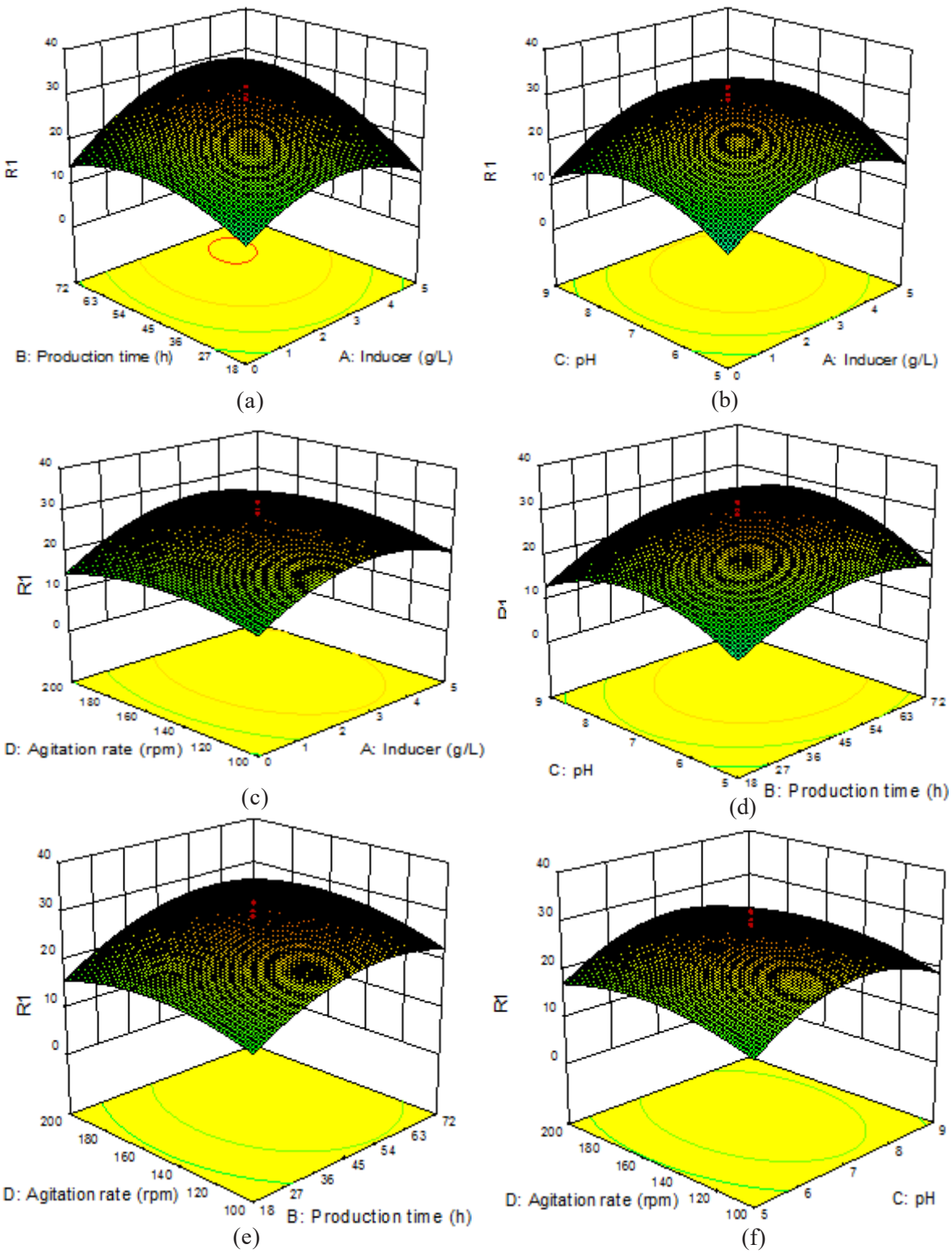

Fig. 2. Response surface plots for the yield of uricase from *A .faecalis*, variable components were (a) production time and inducer concentration, (b) pH and inducer concentration, (c) agitation rate and inducer concentration, (d) pH and production time (e) agitation rate and production time, (f) agitation rate and pH

L, agitation rate 150 rpm, production time 45 h with rest of the medium composition and conditions remain same. There was increase in the 2.5 time increase in the uricase activity. Abdel-fattah *et al*.,[2] has optimized medium component for the production of uricase from *Pseudomonas aeruginosa* through RSM of the following composition (pH 6): sucrose 3 %, uric acid 0.15 %, soy bean flour 0.4 %, KH_2PO_4 0.5 %, K_2HPO_4 0.5 %, $CuSO_4$, 10^{-3} M; Fe_2SO_4, 10^{-3} M, $ZnSO_4$ 10^{-3} M, $MnSO_4$ 10^{-3} M.

Validation of model

The maximum uricase activity obtained by performing experiment on the bases of the above statistical information was 31.9 IU which was more than the predicted value (28.4 IU) calculated by the empirical model (Eq. 2).

A high degree of similarity was observed between the predicted data of the response (28.4 IU) from the empirical model and the experimental values (31.9 IU) in the range of the operating variables reflecting the usefulness of RSM to determine the most suitable parameters of uricase production (Fig. 3). A regression model could be used to predict future observations on the response Y (uricase activity) corresponding to particular values of the variables. Fig. 4 illustrates the deviation of different variable for the production of uricase from the reference point.

Depending on the conditions of experiments, the uricase activity ranged from 0 to 31.9 IU (Table 1). The statistical testing of the model was done by Fisher's F test for analysis of variance (ANOVA). Poreto chart showed that inducer is the most effective variable, followed by production time, pH and agitation rate. The value of p > F for the model is less than 0.001, which indicate that it is highly significant and desirable model. The 'Lack of Fit F-value' of 2.82 implies that 'Lack of Fit' is not significant relative to pure error (Table 4). There is only a 1.87 % chance that an F-value this large could occur due to noise. Non-significant lack of fit is good for experiment model as it is desirable always for model to fit.

Conclusion

The major disadvantages of one parameter at a time during optimization of process parameter are time-consumption, incapability to describe the gross effects of the parameters in the process and omit the combined interactions between physico-chemical parameters. The RSM has been widely accepted statistical experimental design in biotechnology to explore the culture conditions of various fermentation processes. In present study, response surface methodology eases the analysis of experimental data to find out the suitable physicochemical conditions for the uricase production from *Alcaligenes faecalis*. It also helped in know-

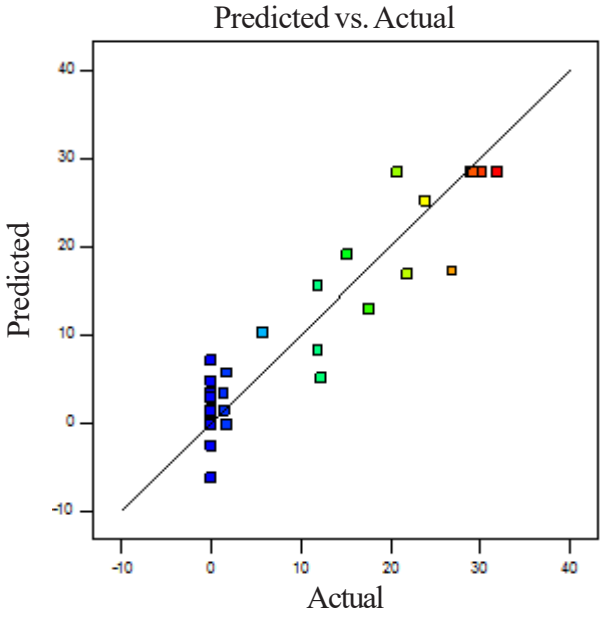

Fig. 3. Observed verses predicted value of uricase activity

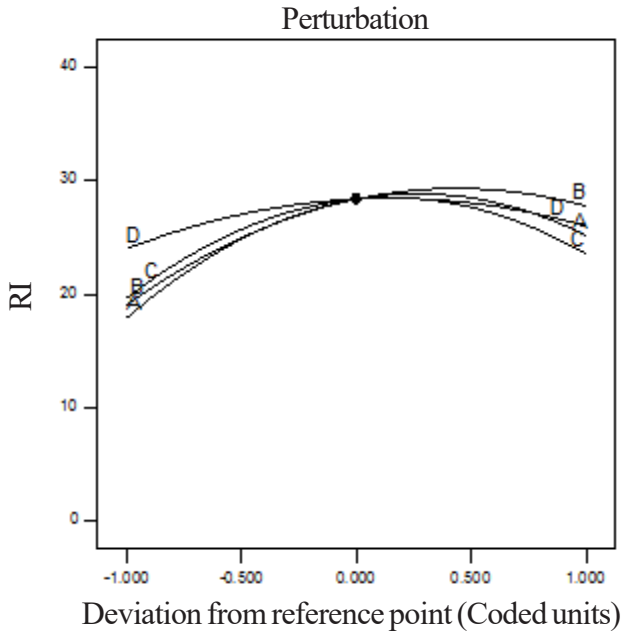

Fig. 4. Perturbation plot showed the optimum value for four variables

Table 4. Lack of fit test of central composite design (CCD) calculated value for deriving effective model

Source	Sum of squares	df	Mean square	F-value	p-value	Prob>F
Model	3707.06	14	264.79	7.97	0.0001	Significant
A-Inducer	314.65	1	314.65	9.47	0.0077	
B-Production time	451.53	1	451.53	13.59	0.0022	
C-pH	88.55	1	88.55	2.66	0.1234	
D-Agitation rate	23.40	1	23.40	0.70	0.4145	
AB	158.13	1	158.13	4.76	0.0455	
AC	28.89	1	28.89	0.87	0.3659	
AD	14.25	1	14.25	0.43	0.5225	
BC	28.89	1	28.89	0.87	0.3659	
BD	13.51	1	13.51	0.41	0.5334	
CD	0.60	1	0.60	0.018	0.8948	
A^2	1284.28	1	1284.28	38.64	<0.0001	
B^2	676.89	1	676.89	20.37	0.0004	
C^2	1265.58	1	1265.58	38.08	<0.0001	
D^2	315.72	1	315.72	9.50	0.0006	
Residual	498.51	15	33.23			
Lack of fit	423.61	10	42.35	2.82	0.1317	Not
Pure error	75.00	5	15.00			Significant
Cor total	4205.57	29				

ing the contribution of individual variables to assess the response (uricase activity).

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