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Optimization of Media Component in Inulinase Production Using Garlic by *Penicillium Rugulosum*

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ABSTRACT

Penicillium rugulosum was found to secrete extracellular inulinase in to the medium. The optimization of inulinase production using garlic as carbon source was performed with statistical methodology based on experimental designs. The screening of eighteen nutrients for their influence on inulinase production was achieved using a Plackett–Burman design. Corn steep liquor, $FeSO_4.7H_2O$, and Urea were selected based on their positive influence on inulinase production. The selected components were optimized using Response Surface Methodology (RSM). The optimum conditions are: Corn steep liquor – 0.05813 (g/gds), $FeSO_4.7H_2O$ – 0.00011 (g/gds), and Urea – 0.0211 (g/gds). These conditions were validated experimentally which revealed an enhanced inulinase yield of 268 U/gds.

Keywords: Inulinase, Garlic, *Penicillium rugulosum*, Optimization, RSM

INTRODUCTION

Inulin is a linear *β*-(2, 1)-linked fructose polymer that occurs as a reserve carbohydrate in many plant families such as Jerusalem artichoke, dahlia tubers, or chicory root[1]. Inulinase has received much more attention recently as it can be widely applied to hydrolyze inulin for the production of fuel ethanol[2], fructose, and fructo-oligosaccharides, both of which are important ingredients in food and pharmaceutical industry[3]. Such inulin has recently received a great interest as it represents a relatively inexpensive and abundant substrate for the production of high fructose syrup, e.g. fructose syrup has beneficial effects in diabetic patients, increases the iron absorption in children, has high sweetening capacity so it can be used in the diet of obese persons [4], stimulates calcium absorption in postmenopausal women[5], stimulates growth of Bifidobacteria in large and small intestine[6], prevents colon cancer[7] and is used as dietary fibers because of its fat like texture[4]. Fructo-oligosaccharides were found to have good functional and nutritional properties such as low calorie diet, and source of dietary fiber in food preparations. These oligosaccharides, therefore, are now widely used to replace sugars in many food applications such as in confectionery, chocolate, and dairy products[8]. Besides, fructooligosaccharides were suggested to have growth inhibition effect of tumors[9]. One step enzymatic hydrolysis of inulin with inulinase is the best way to yield fructose and fructooligosaccharides. Inulinase can be produced by many microorganisms, such as yeast, fungi, and bacteria[10]. A number of fungal, yeast and bacterial strains have been used for inulinases production, like *Kluyveromyces*[11–15], *Aspergillus*[8, 16], *Staphylococcus*[15], *Xanthomonas*[17], and *Pseudomonas*[18]. These enzymes, (2, 1-β-D) fructan fructanohydrolase (EC 3.2.1.7), are usually inducible and exo-acting enzymes[19]. Inulinase can be extracted from many plants, but the yield is low, increasing productivity costs[14, 20, 21]. The possibility of using microorganisms to produce enzymes represents a good alternative to increasing

productivity, and in the present case, this would increase the potential for using inulinase in the industrial production of fructose from inulin[22].

MATERIALS AND METHODS

Fungi

Fungi used in this work are well preserved in the laboratory. Fungi *Penicillium rugulosum* MTCC-3487 is a stock of the Microbial Type Culture collection Centre (MTCC), Chandigarh, India. The strain was maintained on solid medium at 5°C. The medium composition was comprised off the followings: *Czapek concentrate, 10.0 ml; K_2HPO_4, 1.0g; Yeast extract, 5.0g; Sucrose, 30.0g; and Agar, 15.0g; Distilled water, 1.0 L. *Czapek concentrate: $NaNO_3$, 30.0g; Kcl, 5.0g; $MgSO_4.7H_2O$, 5.0g; $FeSO_4.7H_2O$, 0.1g; and Distilled water, 100.0ml. Cells were harvested from slants and used to inoculate liquid medium.

Pretreatment of substrate

Garlic (bulbs) were washed thoroughly with cold water, sliced and then dried at 100 °C for 72h. The dried slices were then milled to a fine powder with a hammer mill. After milling the resultant powder was used directly as a carbon source[21].

Solid state fermentation

Pretreated garlic was used as substrate for inulinase production. Fermentation was carried out in Erlenmeyer flasks (250 ml) with 10g of pretreated garlic powder, supplemented with nutrients concentrations defined by the experimental design. Moisture was adjusted to 65%; each flask was covered with hydrophobic cotton and autoclaved at 121°C for 20 min. After cooling, each flask was inoculated with 2 ml of the suspension previously prepared and incubated for 120 hrs in a chamber with temperature and humidity control. During the preliminary screening process, the experiments are carried out for 5 days and it was found that at the 48 hr, the maximum production occurs. Hence experiments are carried out for 48 hrs.

Extraction of Inulinase

After fermentation, 10 volumes of distilled water were added to the fermented matter and the contents were agitated for 30 minutes at 200 rpm on a rotary shaker (at 28°C). Then the sample was centrifuged at 15000 rpm for 20 minutes and the supernatant were analyzed by DNS method[23].

Optimization of Inulinase production

Once the critical factor were identified through the screening, the central composite design (CCD) was used to obtain a quadratic model, consisting of factorial trials and star points to estimate quadratic effects and central points to estimate the pure process variability with inulinase production as response. Response surface methodology (RSM) was employed to optimize the selected three RSM consist of a group of empirical techniques used for evaluation of relationship between cluster of controlled experimental factors and measured response. A prior knowledge with understanding of the related bioprocesses is necessary for a realistic modeling approach. To determine which variables significantly affect inulinase production by Penicillium rugulosum, Plackett–Burman design was used. Eighteen variables in Table 1 were screened in 20 experimental runs in Table 2 and insignificant ones were eliminated in order to obtain a smaller, manageable set of factors. The low level (-1) and high level (+1) of each factor are listed in Table 1. The statistical software package 'Design Expert 7.1.5', was used for analyzing the experimental data.

Significant nutrient components viz., Corn steep liquor, $FeSO_4.7H_2O$, and Urea, which enhances the inulinase production. The three independent variables were studied at five different levels in Table - 3 and sets of 20 experiments were carried out in Table 4. The statistical software package 'Design Expert 7.1.5 was used to analyze the experimental data. All variables were taken at a central coded value of zero. The minimum and

maximum ranges of variables investigated are listed in Table 3. Upon the completion of experiments, the average maximum inulinase were taken as the response (Y). A multiple regression analysis of the data was carried out for obtaining an empirical model that relates the response measured to the independent variables. A second order polynomial equation is:

$$Y = \beta_O + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1,i<j}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j$$

Where Y is the measured response, β_0 is the intercept term, β_i are linear coefficients, β_{ii} are quadratic coefficient, β_{ij} are interaction coefficient and Xi and X_j are coded independent variables. The optimal concentrations of the critical variables were obtained by analyzing 3D plots. The statistical analysis of the model was represented in the form of analysis of variance (ANOVA).

Variable		Levels (g/10gds)	
Nutrient Code	**Nutrient**	**Low (-1)**	**High (+1)**
A	Yeast extract	0.01	0.05
B	Beef Extract	0.05	0.15
C	$MnSO_4.7H_2O$	0.1	0.5
D	$K_2 HPO_4$	0.02	0.07
E	Soya bean cake	0.4	0.8
F	$MgSO_4.7H_2O$	0.002	0.012
G	NH_4Cl	0.01	0.03
H	KCl	0.005	0.015
J	$(NH_4)_2 HPO_4$	0.05	0.3
K	NH_4NO_3	0.05	0.1
L	$ZnSO_4.7H_2O$	0.1	0.5
M	$(NH_4)_2SO_4$	0.06	0.1
N	Corn steep liquor	0.4	0.8
O	Peptone	0.05	0.15
P	Dextrose	0.1	0.3
Q	$FeSO_4.7H_2O$	0.0005	0.002
R	KH_2PO_4	0.1	0.6
S	Urea	0.1	0.3

Table – 1: Nutrient screening using a Plackett-Burman design

Enzymes were assayed by measuring the concentration of reducing sugars released from inulin or sucrose. The reaction mixture containing 1 ml of diluted crude enzyme and 4 ml of 2% inulin or 2% sucrose (dissolved in 0.1 M acetate buffer, pH 5.0) was incubated at 50°C. After incubating for 30 min, aliquots of 0.5 ml were withdrawn and increase in reducing sugar was estimated by a 3, 5-dinitrosalicylic acid method[23] using calibration curve obtained with a standard solution of fructose[24]. Absorbance was read at 575 nm. A higher absorbance indicated a high level of reducing sugar produced and consequently, a high enzyme activity. One unit of inulinase activity (U) was defined as the amount of enzyme, which forms 1 µ mol fructose per min. Results of the determination of inulinase activity were presented in units of activity/gram of dry substrate (U/g.d.s.).

Run No.	A	B	C	D	E	F	G	H	J	K	L	M	N	O	P	Q	R	S	Inulinase Activity (U/gds)
1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	99
2	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	151
3	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	65
4	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	52
5	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	93
6	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	62
7	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	137
8	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	113
9	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	84
10	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	103
11	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	60
12	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	73
13	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	75
14	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	55
15	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	164
16	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	52
17	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	61
18	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	145
19	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	112
20	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	103

Table – 2: Plackett–Burman experimental design matrix for screening of important variables for inulinase production

Variable		Levels (g/10gds)				
	Code	-1.68	-1	0	+1	+1.68
Corn steep liquor	X_1	0.4	0.5	0.6	0.7	0.8
$FeSO_4.7H_2O$	X_2	0.0005	0.0008	0.0012	0.0016	0.002
Urea	X_3	0.1	0.15	0.2	0.25	0.3

Table – 3: Ranges of the independent variables used in RSM

Run. No	X_1	X_2	X_3	Inulinase Activity(U/gds)	
				Experimental	Predicted
1	0.00000	1.68179	0.00000	120	129.800
2	1.00000	1.00000	1.00000	101	87.339
3	1.00000	-1.00000	-1.00000	130	139.428
4	0.00000	0.00000	0.00000	210	209.503
5	0.00000	0.00000	-1.68179	120	102.342
6	0.00000	0.00000	0.00000	205	209.503
7	0.00000	0.00000	0.00000	210	209.503
8	0.00000	0.00000	0.00000	207	209.503
9	0.00000	0.00000	0.00000	212	209.503
10	-1.00000	-1.00000	-1.00000	88	114.105
11	1.00000	-1.00000	1.00000	103	114.478
12	0.00000	0.00000	0.00000	210	209.503
13	0.00000	-1.68179	0.00000	202	174.603
14	1.00000	1.00000	-1.00000	99	102.289
15	-1.00000	1.00000	1.00000	150	153.015
16	0.00000	0.00000	1.68179	136	136.061
17	-1.00000	1.00000	-1.00000	87	87.965
18	1.68179	0.00000	0.00000	90	89.735
19	-1.68179	0.00000	0.00000	141	123.667
20	-1.00000	-1.00000	1.00000	160	169.154

Table – 4: Central composite design (CCD) of factors in coded levels with Enzyme activity as response. Assay of enzyme activity

RESULTS AND DISCUSSION

Plackett–Burman experiments in Table 2 showed a wide variation in inulinase activity. This variation reflected the importance of optimization to attain higher productivity. From the Pareto chart in Fig. 1, the variables viz., Corn steep liquor, $FeSO_4.7H_2O$, and Urea were selected for further optimization to attain a maximum production of inulinase.

The levels of factors (Corn steep liquor, $FeSO_4.7H_2O$, and Urea) and the effect of their interactions on inulinase production were determined by central composite design of RSM. Twenty experiments were performed at different combinations of the factors shown in Table 3. The predicted and observed responses along with design matrix are presented in Table 4 and the results were analyzed by ANOVA.

The second-order regression equation provided the levels of inulinase activity as the function of Corn steep liquor, $FeSO_4.7H_2O$, and Urea which can be presented in terms of coded factors as in the following equation:

$$Y = 209.503 - 10.0881X_1 - 13.3198X_2 + 10.0249X_3 - 36.3459X_1^2 - 20.2592X_2^2 - 31.9265X_3^2 - 2.75000X_1X_2 - 20.0000X_1X_3 + 2.50000X_2X_3$$

Where Y is the inulinase activity (U/gds), X_1, X_2, and X_3 are Corn steep liquor, $FeSO_4.7H_2O$, and Urea respectively. ANOVA for the response surface is shown in Table - 5. The Model F-value of 17.42 implies the model is significant. Values of "Prob > F" less than 0.05 indicate model terms are significant. Values greater than 0.1 indicate the model terms are not significant. In the present work, all the linear, interactive effects of X_1X_3 and square effects of X_1, X_2 and X_3 were significant for inulinase production. The coefficient of determination (R^2)

for inulinase activity was calculated as 0.9401, which is very close to 1 and can explain up to 94.01% variability of the response. The predicted R^2 value of 0.7935 was in reasonable agreement with the adjusted R^2 value of 0.8861. An adequate precision value greater than 4 is desirable. The adequate precision value of 10.542 indicates an adequate signal and suggests that the model can be used to navigate the design space.

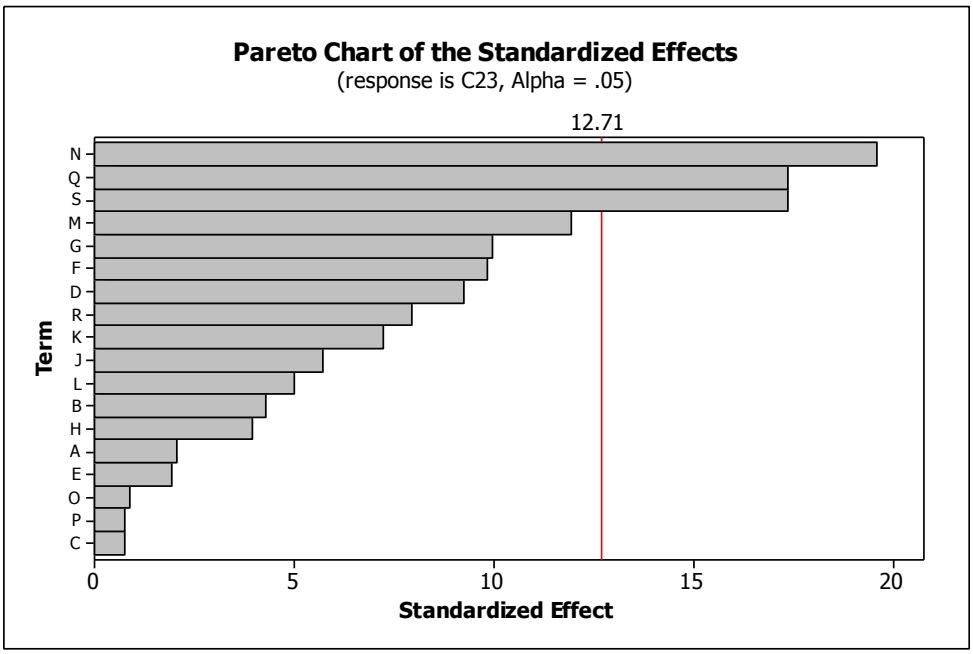

Fig. 1. Pareto chart showing the effect of media components on inulinase activity.

Source	Coefficient factor	Sum of squares	DF	F	P > F
Model	209.503	42119.12	9	17.42	< 0.0001
X_1	-10.0881	*1389.85*	1	5.17	0.0462
X_2	-13.3198	*2422.97*	1	9.02	0.0133
X_3	10.0249	*1372.50*	1	5.11	0.0473
X_1* X_2	-2.75000	*60.50*	1	0.23	0.6453
X_1* X_3	-20.0000	*3200.00*	1	11.91	*0.0062*
X_2* X_3	2.50000	*50.00*	1	0.19	*0.6753*
X_1* X_1	-36.3459	*19037.67*	1	70.88	*< 0.0001*
X_2* X_2	-20.2592	*5914.91*	1	22.02	*0.0009*
X_3* X_3	-31.9265	*14689.43*	1	54.69	*< 0.0001*
Residual		2685.83	10		
Lack of fit		*2653.83*	5	82.93	*< 0.0001*
Pure Error		*32.00*	5		
Cor Total		44804.95	19		

Std. Dev.-16.39; R^2-94.01%; Mean-149.05; Adj R^2-88.61%; C.V. %-11.00; Pred R^2 – 79.35%; Adeq Precision –10.542

Table – 5: Analysis of Variance (ANOVA) for response surface quadratic model for the production of inulinase.

The above model can be used to predict the inulinase production within the limits of the experimental factors. Fig. 2 shows that the actual response values agree well with the predicted response values.

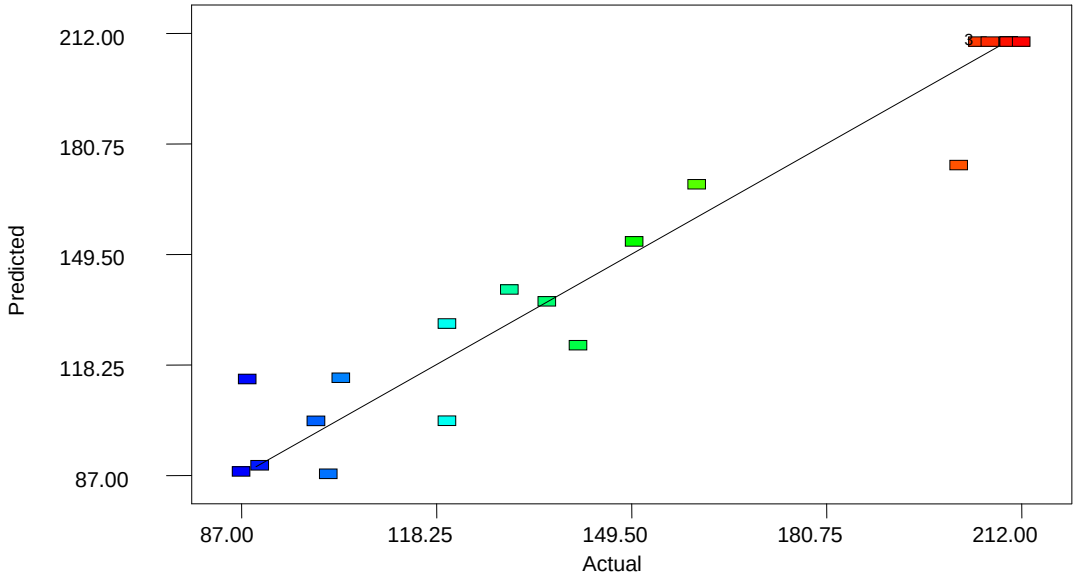

Fig.2. Predicted response versus actual value.

The interaction effects of variables on inulinase production were studied by plotting 3D surface curves against any two independent variables, while keeping another variable at its central (0) level. The 3D curves of the calculated response (Inulinase production) and contour plots from the interactions between the variables are shown in Figs. 3-5. Fig. 3 shows the dependency of inulinase on corn steep liquor and $FeSO_4.7H_2O$. The inulinase activity increased with increase in corn steep liquor to about 0.05813 g/gds and thereafter inulinase activity decreased with further increase in corn steep liquor. The same trend was observed in Fig.4. Increase in $FeSO_4.7H_2O$ resulted increase in inulinase activity up to 0.00011 g/gds. This is evident from Figs.3 and 5. Fig. 4 and 5 shows the dependency of inulinase activity on urea. The effect of urea on inulinase observed was similar to other variables. The maximum inulinase activity was observed at 0.0211g/gds of urea. The optimum conditions for the maximum production of inulinase were determined by response surface analysis and also estimated by regression equation. The optimum conditions are: Corn steep liquor- 0.05813 (g/gds), $FeSO_4.7H_2O$ – 0.00011 (g/gds) and Urea – 0.0211 (g/gds). The predicted results are shown in Table 4. The predicted values from the regression equation closely agreed with that obtained from experimental values. Validation of the experimental model was tested by carrying out the batch experiment under optimal operation conditions. Three repeated experiments were performed and the results are compared. The inulinase activity obtained from experiments was very close to the actual response predicted by the regression model, which proved the validity of the model. At these optimized conditions the maximum inulinase activity was found to be 268 U/gds.

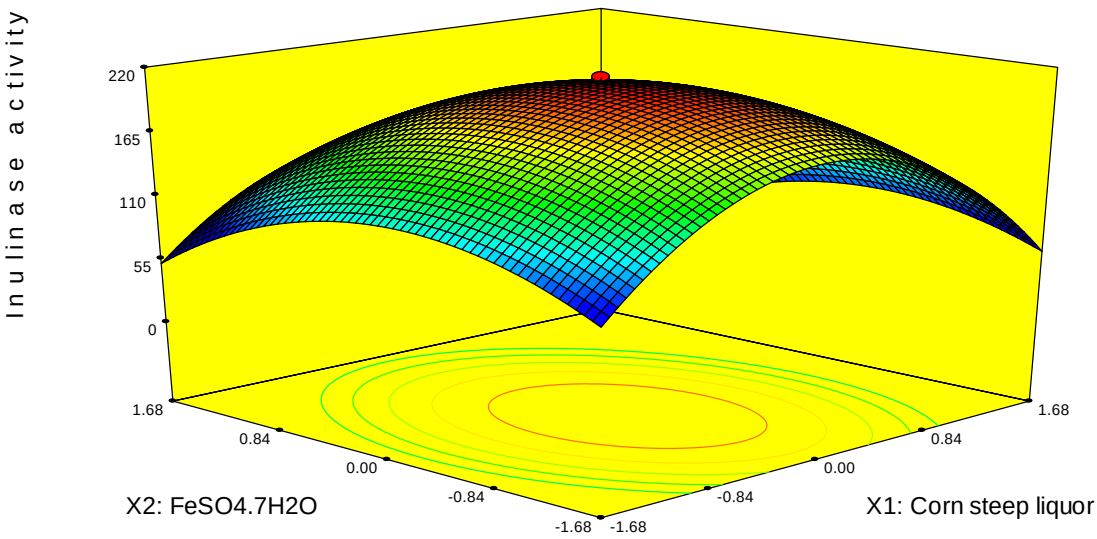

Fig.3. 3D plot showing the effect of Corn steep liquor and $FeSO_4.7H_2O$ on inulinase activity.

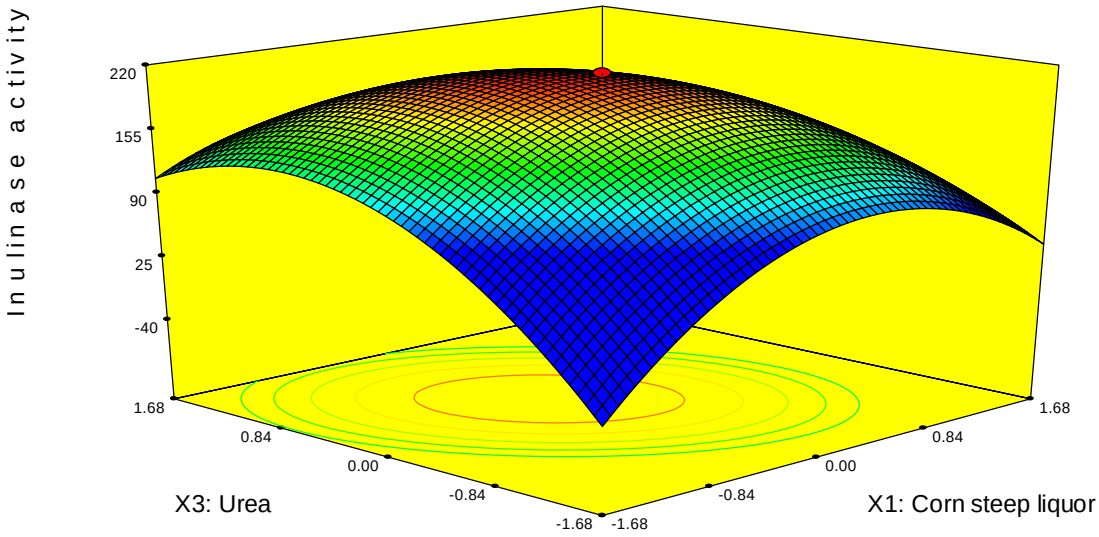

Fig.4. 3D plot showing the effect of Corn steep liquor and Urea on inulinase activity.

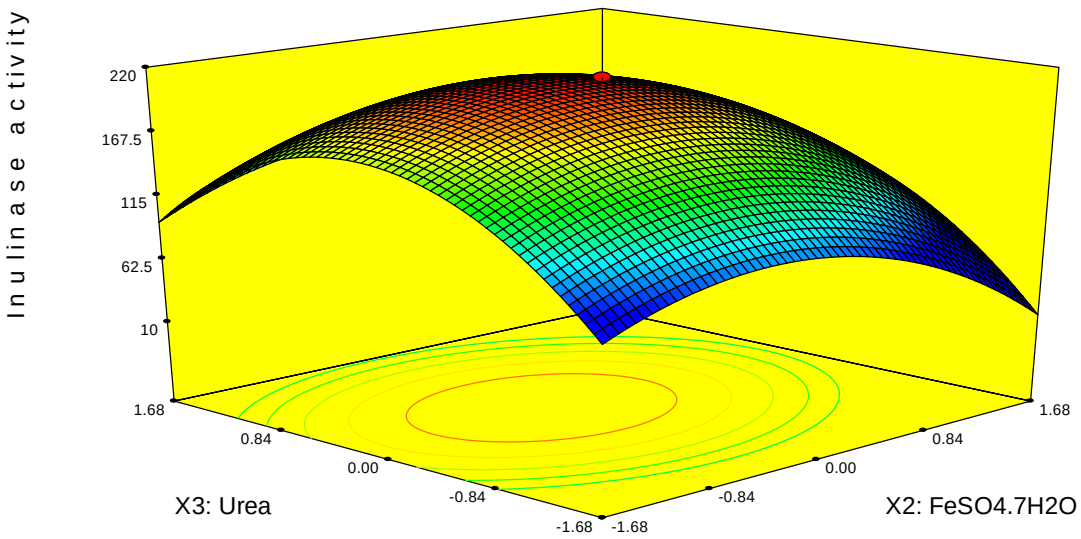

Fig.5. 3D plot showing the effect of $FeSO_4.7H_2O$ and Urea on inulinase activity.

CONCLUSION

In this work, Plackett Burman design was used to test the relative importance of medium components on inulinase production. Among the variables, Corn steep liquor, $FeSO_4.7H_2O$, and Urea were found to be the most significant variables. From further optimization studies the optimized values of the variables for inulinase production were as follows: Corn steep liquor – 0.05813 (g/gds), $FeSO_4.7H_2O$ – 0.00011 (g/gds), and Urea - 0.0211 (g/gds). This study showed that the garlic constitutes a good carbon source for the production of inulinase. Using the optimized conditions, the produced activity reaches 268 U/gds. The results show a close concordance between the expected and obtained activity level.

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