JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

Riswan et al. 2013 : 2 (6) 2377-2382

STATISTICAL OPTIMIZATION OF MEDIUM COMPONENTS FOR HEMICELLULASE PRODUCTION USING TAPIOCA STEM

Riswan Ali S. B., Muthuvelayudham R , Viruthagiri T.*

Address(es): Mr. Riswan Ali S.B.
Annamalai University, Faculty of Engineering and Technology, Department of chemical Engineering, Annamalainagar,Tamilnadu 608002, India.

*Corresponding author: s.b.riswanali@gmail.com

ARTICLE INFO

Received 1. 12. 2012
Revised 3. 4. 2013
Accepted 4. 4. 2013
Published 1. 6. 2013

Regular article

ABSTRACT

In the present work the production of xylanase by *Cellulomonas fimi* in submerged fermentation (SmF) using tapioca stem as a sole carbon source was enhanced by medium optimization. A Plackett-Burman based statistical screening procedure was used to identify the most significant nutrient components which influence the xylanase production. From the experiments, twelve nutrient components were screened and the results revealed that peptone, yeast extract, KH_2PO_4 and $CaCl_2.2H_2O$ were the most significant nutrient components. Central composite design (CCD) was used to determine the optimal concentrations of these four screened nutrients components and the experimental results were fitted with a second-order polynomial model at 95% level ($P < 0.05$), the combined effects of these nutrients on production of xylanase was also studied. The optimum concentrations are peptone–0.957g/L, yeast extract–2.5g/L, KH_2PO_4–3.09g/L, and $CaCl_2.2H_2O$ –0.409g/L. Under these conditions, the production of xylanase was found to be 4.12 IU/ml. The results show a close agreement between the expected and obtained activity level.

Keywords: Xylanase, Plackett-Burman design, Tapioca stem, *Cellulomonas fimi*, central composite design,response surface methodology

INTRODUCTION

Lignocellulose is the most abundant and renewable biomass available on earth **(Kuhadand singh,1993).** The main components of lignocelluloses are cellulose, hemicelluloses and lignin. Hemicelluloses are classified into xylans, mannans, arabinans and galactans according to the main sugar components in their backbones. Xylan is the main carbohydrate found in hemicellulose, which is comprising a backbone of xylose residues linked by β-1,4-glycosidic bonds **(Wong *et al.*, 1988)**. Xylan is the second one in its distribution polysaccharide in nature after cellulose. It comprises 20–30% dry weight of the plants **(Saha, 2003)**. Hemicellulase mainly xylanase whose function is to hydrolyse β-1,4-glycosidic linkages in the xylan into xylose **(Gilbert and Hazlewood, 1993).** Xylanase are mainly classified according to their action on distinct substrates, endo-1,4-β-xylanase (EC 3.2.1.8) generates oligosaccharides from the cleavage of xylan and xylan 1,4-β- xylosidase (EC 3.2.1.37) produces xylose from oligosaccharides **(Jeffries, 1994).** The main industrial applications of xylanases are food and beverage industries like bakery goods, juice manufacture, coffee, plant oil and starch, and also for feedstock improvement(increasing animal feed digestibility) **(Coughlan and Hazlewood, 1993; Beg *et al.*, 2001).**Xylanase are produced by many microorganisms including bacteria, yeast and filamentous fungi **(Lakshmi *et al*., 2009).**

The main factor which determines the economics of a process is the enzyme cost. Reducing the costs of enzyme production by optimizing the fermentation medium and the process is the goal of basic research for industrial applications**.** Therefore, investigations on the ability of hemicellulose-hydrolyzing microbial strains to utilize cheap **(Biely, 1985; Park *et al.*, 2002)**. Lignocellulosic materials are cheap renewable resources available in large quantities, which are used as substrate for xylanase production.

In order to optimize the nutrient medium for xylanase production, conventional methods based on the ''one- factor-at-a-time'' (OFAT) in which, one independent variable is studied while fixing all others at a specific level and does not allow for theinteraction of these variables with each other conclusion. This method is also time consuming and require large numbers of experiments to be carried out in order to determine the optimal level of each factor. Response surface methodology (RSM), the statistical method, it can be used to evaluate the significance of several factors, particularly when interactions exist among different factors. RSM has been widely used by many investigators in the field of bioprocessing and has been used to quantify the complex interplay of parameters affecting biological systems (**Bari *et al.*, 2010).**

In the present studyxylanase production of by *Cellulomonas fimi* was enhanced by medium optimization. The medium optimization of the bioprocess was carried out by 3 step procedure. Initially a large number of continuous factors (typically 12 medium components) were screened and insignificant ones were eliminated in order to obtain a smaller and more manageable set of factors. The remaining factors could be optimized by a response surface modeling. Finally, after model building and optimization, the predicted optimum was verified. To the best of our knowledge this is the first report which describes a 3-step procedure in order to optimize xylanase production using tapioca stem as a sole carbon source by *Cellulomonas fimi* strain.

MATERIAL AND METHODS

Microorganism and culture media

Cellulomonas fimi used in this study was purchased from the National Chemical Laboratory, Pune, India. Stock cultures were maintained on nutrient agar slants which contain 1 g of beef extract, 0.5 g of NaCl, 1 g of peptone, 2 g of agar, in 100 ml of distilled water,pH 7.0 to 7.5, at room temperature.The sub culturing was performed every 15 days to assure its viability. The inoculum was prepared by adding 5.0mL of sterile distilled water to the agar slants and shaking vigorously. The spore suspension that obtained was adjusted to 1 X 10^7 spores per mL and used as the inoculums.

Substrate preparation

100 g of the washed ground tapioca stem was treated separately with 2000 mL of 2% NaOH solution and autoclaved at 121°C for 30 minutes. Then it was filtered, washed with distilled water and excess alkali present was neutralized with phosphoric acid. Again it was filtered and the residue material was dried at 65°C to constant weight in a hot air oven. To the cellulosic material obtained, the same volume of distilled water was added and heated at 121°C for 30 minutes. The suspension was filtered and the solid material was dried at 65°C to constant weight in a hot air oven (**Muthuvelayudham and Viruthagiri, 2010**). The dried tapioca stem powder was used as the carbon source.

Cultivation of *Cellulomonas fimi*

Alkali pretreated tapioca stem powder was used as substrate for xylanase production. Fermentation was carried out in Erlenmeyer flasks (250 mL) with 10 g of alkali pretreated tapioca stem powder in 100 ml of enzyme production medium, the composition of media varied according to the experimental design described in this work. pH of the medium was adjusted to 7.0 with 1 mol NaOH or 1 mol HCl. Each flask was covered with hydrophobic cotton and autoclaved at 121 °C for 20 min. After cooling, each flask was inoculated with 10% v/v of inoculum incubated at 28 °C on rotary shaker (150 rpm).After incubation, the samples were withdrawn at regular time intervals and the samples were filtered through GD-120 glassfiber filter disks (Whatman) to remove the residual insoluble substrate. Then, the liquid content obtained after filtration was centrifuged at 10,000×g for 10 min at 4 °C to separate the cells. The cell-free supernatant was analyzed for enzyme activity.

During the preliminary screening process, the experiments were carried out for 96 hours, and it was found that at 72 hours the maximum enzymes production was obtained. Hence, experiments were carried out for 72 hours. All the experiments were carried out in triplicate and the average values are reported.

Xylanase assay

The reaction mixture containing 0.5 ml of suitably diluted enzyme solution, 1 ml citrate phosphate buffer (pH 7.0) and 0.5 ml of xylan (oat spelt) was incubated at 50 ^{0}C for 5 min (**Bailey *et al*., 1992**). Xylose released by xylanase was assayed by DNS (Dinitrosalicylic acid) method using xylose as standard (**Miller, 1959**). The enzyme activity was expressed in international units and was defined as the amount of enzyme required to release one µ mol of reducing sugar (xylose) per min.

Screening of medium components by Plackett-Burman (PB) experimental design

The main application of Plackett-Burman experimental design is to identify the most significant ingredients of the medium on the xylanase producing capability of *Cellulomonas fimi.* When this kind of statistical experimental design is employed it is assumed that no interactions between different factors occur in the range of variables under consideration (**Plackett and Burman, 1946**).A total of twelve variables were considered for screening (Table 1) in 20 experimental runs (Table 2) and insignificant variables were eliminated in order to obtain a smaller, manageable set of factors. Each factor examined in two levels: −1 for low level and +1 for high level of each factor are listed in Table 1. Twelve variables (Table 1) were screened.

Table 1 Nutrient screening using a Plackett-Burman design

Nutrient Code	Nutrient (g/L)	Low (-1)	High (+1)
A	Peptone	0.25	1.25
B	Yeast extract	0.5	3.5
C	$MnSO_4.7H_2O$	0.001	0.002
D	$KH_2 PO_4$	2.0	4.0
E	$K_2H PO_4$	1.0	5.0
F	$NaNO_3$	0.5	1.0
G	$CoCl_2 .6H_2O$	0.02	2.0
H	$MgSO_4 .7H_2O$	0.3	0.5
I	$CaCl_2.2H_2O$	0.3	0.5
J	$(NH_4)_2SO_4$	0.06	0.1
K	Corn steep liquor	0.4	0.8
L	$FeSO_4.7H_2O$	0.005	0.1

The PB design is a fractional factorial design and the main effect (the contrast coefficient) of such a design may be simply calculated as the difference between the average of measurements made at the high level (+1) of the factor and the average of measurements made at the low level (-1). Contrast coefficients allow the determination of the effect of each constituent. A large contrast coefficient either positive or negative indicates that a factor has a large effect, while a coefficient close to zero means that a factor has little or no effect.

The P-value is the probability that the magnitude of a contrast coefficient is due to random process variability. A low P-value indicates a ‘‘real’’ or significant effect. The significance of each variable was determined by applying the Student’s t-test. All the 20 experimental runs were carried out in duplicate and the averages of the xylanase activity was taken as the response (Table 2). From the regression analysis the variables, which were significant at or above 95% level ($P < .05$), were considered to have greater impact on xylanase activity and were further optimized by central composite design.

Central Composite Design and Statistical Analysis

Once Significant nutrient components for xylanase production such as nitrogen source, and inorganic salts were screened and identified by the Plackett-Burman design using statistical software package MINITAB (Release 15.1, PA, USA).The screened nutrients were further optimized by Response surface methodology based central composite design.It consists of a complete 2^k factorial design, where k is the number of the test variables and is equal to 4, six replications of the center points to estimate the experimental error and have a satisfactory orthogonality for the coefficients estimation (all factors at level 0), six star points (2 axis points on the axis of each variable at a distance of α (= $2^{k/4}$, = 2 for k = 4), whereas the other two factors are at level 0. Hence, the total number of design points is $N = 2^k + 2k + n_0 = 30$ experiments. Where n_0 the number of replicate runs at center point of the variables. The central composite design along with the experimental and predicted values of xylanase production is shown in Table 3. The relationships and interrelationships of the variables were determined by fitting the second-order polynomial equation to data obtained from 30 experiments. The response values (Y) used in each trial was the average of the duplicates.

A second-order polynomial equation is

$$Y=\beta 0+\sum_{i=1}^{k}\beta_i X_i+\sum_{i=1}^{k}\beta_{ii} X_i^2+\sum_{i=1,i<j}^{k-1}\sum_{j=2}^{k}\beta_{ij} X_i X_j \quad \ldots(1)$$

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 X_i and X_j are coded independent variables. The following equation was used for coding the actual experimental values of the factors in the range of (−1 to +1):

$$x_i=\frac{X_i-X_0}{\Delta X_i} \quad \ldots (2)$$

$\Delta X_i, \quad i = 1, 2, 3 \ldots K,$

Where x_i is the dimensionless value of an independent variable, X_i is the real value of an independent variable, X_0 is the value of X_i at the center point, and ΔX_i is the step change. Statistical analysis of the data was performed by design package Design Expert 7.1.5 to evaluate the analysis of variance (ANOVA) to determine the significance of each term in the equations fitted and to estimate the goodness of fit in each case. The fitted polynomial equation was then expressed in the form of three-dimensional response surface plots to illustrate the main and interactive effects of the independent variables on the dependent ones. The combination of different optimized variables, which yielded the maximum response, was determined to verify the validity of the model. In order to verify the accuracy of the predicted model an experiment was conducted with initial and optimized media. 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).

The minimum and maximum ranges of variables investigated are listed in Table 4. Upon the completion of experiments, the average maximum xylanase was 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.

Table 2 Plackett–Burman design for medium optimization and measured response

RunOrder	A	B	C	D	E	F	G	H	I	J	K	L	Xylanase IU/mL
1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1.87
2	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	1.96
3	-1	1	1	1	1	-1	-1	1	1	-1	1	1	3.39
4	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1.91
5	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	3.16
6	1	1	1	1	-1	-1	1	1	-1	1	1	-1	3.52
7	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	3.12
8	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1.27
9	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	3.17
10	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	2.7
11	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	1.29
12	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	3.46
13	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	2.97
14	1	-1	1	-1	1	1	1	1	-1	-1	1	1	1.87
15	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	2.95
16	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	1.92
17	1	-1	1	1	1	1	-1	-1	1	1	-1	1	2.98
18	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	3.21
19	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1.84
20	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	3.01

RESULTS AND DISCUSSION

Plackett-Burman experiments (Table 2) is used to screen the important medium components for maximum productivity of xylanase. The huge variation in xylanase activity reflected the importance of optimization to attain higher productivity using Central Composite Design. Table3 shows the analysis of regression coefficients, the effect, standard error, t-value and P values of 12 ingredients. Generally, a large *t*-value associated with a low *P*-value of a variable indicates a high significance of the corresponding model term. The components were screened at the confidence level of 95% on the basis of their effects. Statistical analysis (*t*-value and p-value) demonstrated that among the positive main effects ofpeptone, yeast extract, KH_2PO_4 and $CaCl_2.2H_2O$ showed confidence level above 95% and these nutrients were considered to be significant. The rest of the components showed confidence level below 95% and were considered to be insignificant. The variables which are insignificant were neglected.

A standardized Pareto chart consists of bars with a length proportional to the absolute value of the estimated effects, divided by the standard error. The bars are exhibited in the order of the size of the effects, with the largest effect on top. The Pareto chart illustrates the order of significance of the variables affecting xylanase production at $p \leq 0.05$ (Figure 1). From the Pareto chart we found that the variables namely peptone, yeast extract, KH_2PO_4 and $CaCl_2.2H_2O$ was significant. These nutrients were selected for further optimization to attain a maximum production of xylanase.

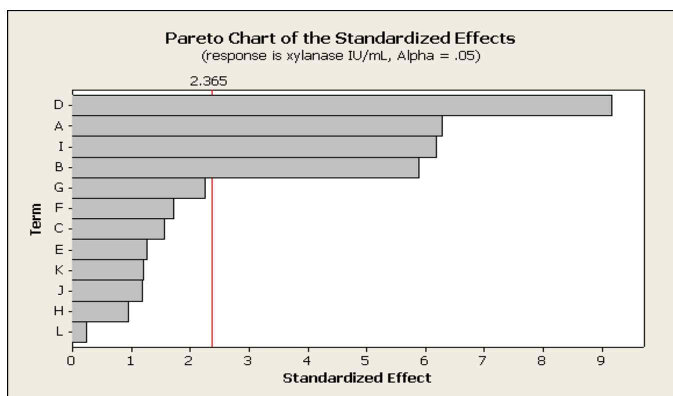

Figure 1 Pareto chart showing the effect of different media components significance at $p \leq 0.05$ in xylanase production

Table 3 Statistical data for the determination of variable significance in the Plackett–Burman design

Term	Effect	Coef	Standard Error	T	P	Confidence level(%)
Constant	2.58	0.05	52.6	0		
A	0.62	0.31	0.05	6.27	0	100
B	0.58	0.29	0.05	5.89	0.001	99.9
C	-0.15	-0.08	0.05	-1.56	0.16	83.7
D	0.9	0.45	0.05	9.17	0	100
E	-0.12	-0.06	0.05	-1.25	0.25	75
F	-0.17	-0.08	0.05	-1.72	0.13	87.2
G	0.22	0.11	0.05	2.25	0.06	94.1
H	0.1	0.05	0.05	0.95	0.37	62.6
I	0.6	0.30	0.05	6.19	0	100
J	0.12	0.06	0.05	1.19	0.27	72.8
K	0.12	0.06	0.05	1.21	0.26	73.6
L	-0.02	-0.01	0.05	-0.23	0.82	17.9

The central composite design (CCD) of RSM was employed to optimize the selected four significant nutrient components namelypeptone, yeast extract, KH_2PO_4 and $CaCl_2.2H_2O$ which enhances the xylanase production. The four independent variables were studied at five different levels (Table 4), and set of 30 experiments were carried out (Table 5), the results were analyzed by ANOVA (Table 6).

Table 4 Ranges of the independent variables used in RSM

	Variable	Code	Levels (g/L)				
			-2	-1	0	1	2
1.	Peptone	X_1	0.25	0.5	0.75	1.0	1.25
2.	Yeast extract	X_2	0.5	1.25	2.0	2.75	3.5
3.	$KH_2 PO_4$	X_3	2.0	2.5	3.0	3.5	4.0
4.	$CaCl_2.2H_2O$	X_4	0.3	0.35	0.4	0.45	0.5

The relationship and interrelationship of the variables were determined by fitting the second-order polynomial equation to data obtained from 30 experiments. The response values (*Y*) used in each trial was the average of the duplicates. The second-order regression equation provided the levels of xylanase as a function of peptone, yeast extract, KH_2PO_4 and $CaCl_2.2H_2O$ which can be represented in terms of coded factors as in the following equation.

$$Y = 3.6 + 0.41X_1 + 0.44X_2 + 0.074X_3 + 0.036X_4 - 0.074X_1X_2 - 8.125\ 10^{-3}X_1X_3 + 0.017X_1X_4 - 9.375 * 10^{-3}X_2X_3 - 0.140X_2X_4 - 0.056X_3X_4 - 0.16X_1^2 - 0.15X_2^2 - 0.19X_3^2 - 0.19X_4^2 \quad \ldots (3)$$

Where Y is the predicted xylanase activity (IU/mL), whereas X_1, X_2, X_3 and X_4 are the coded values of peptone, yeast extract, KH_2PO_4 and $CaCl_2.2H_2O$ respectively.

The independent variables were fitted to the second order model equation and examined for the goodness of fit. Several indicators were used to evaluate the adequacy of the fitted model and the results are shown in Table 6. The determination coefficient R^2 value, correlation coefficient R^2value, coefficients of variation (CV) and model significance (F-value) were used to judge the adequacy of the model. The F-value is the ratio of the mean square due to regression to the mean square due to error and indicates the influence (significance) of each controlled factor on the tested model.

The Model F-value of 45.77 for xylanase implies the model is significant. There is only 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.05 indicate that model terms are significant. Values greater than 0.1 indicate that the model terms are not significant. The coefficient of variation (CV) is the ratio of the standard error of estimate to the mean value of the observed response, expressed as a percentage. A model can be considered reasonably reproducible if the CV is not greater than 10%. Usually, the higher the value of CV, lower is the reliability of experiment. Here, a lower value of CV (4.36%) for xylanase indicated a greater reliability of the experiments performed (**Box*et al.*, 1978; Lee *et al.*, 2003**).

Table 5 Central composite design (CCD) of factors in coded levels with xylanase activity as response

Run	X_1	X_2	X_3	X_4	Xylanase IU/mL	
					Observed	Predicted
1	1	-1	-1	-1	2.64	2.74
2	0	0	0	0	3.6	3.6
3	0	0	0	0	3.6	3.6
4	0	0	0	0	3.6	3.6
5	0	0	0	-2	2.75	2.75
6	-1	-1	1	1	2.18	2.05
7	-2	0	0	0	1.94	2.15
8	-1	-1	1	-1	2.14	2.11
9	-1	-1	-1	1	2.06	1.98
10	1	-1	1	1	3.11	3.03
11	1	1	1	1	3.71	3.71
12	0	0	0	0	3.6	3.6
13	0	-2	0	0	1.95	2.11
14	1	1	-1	1	3.74	3.71
15	0	0	-2	0	2.67	2.68
16	0	2	0	0	3.81	3.85
17	-1	1	1	1	3.21	3.02
18	0	0	0	2	2.69	2.89
19	-1	1	-1	-1	2.87	2.87
20	-1	1	1	-1	3.17	3.12
21	0	0	0	0	3.6	3.6
22	1	-1	1	-1	3.11	3.01
23	-1	1	-1	1	3.01	2.99
24	1	1	1	-1	3.74	3.74
25	2	0	0	0	3.8	3.78
26	1	-1	-1	1	3.07	2.99
27	0	0	0	0	3.6	3.6
28	-1	-1	-1	-1	1.92	1.8
29	1	1	-1	-1	3.51	3.51
30	0	0	2	0	2.78	2.97

To test the fit of the model equation, the regression-based determination coefficient R^2 was evaluated which is the proportion of variation in the response attributed to the model rather than to random error. The closer the values of R^2 to 1, the better the model would explain the variability between the experimental and the model predicted values (**Sayyad *et al.*, 2007**). The coefficient of determination (R^2) for xylanase activity was calculated as (R^2=0.9771which are nearly equal to 1) 97.71% variability of the response, and only about 2.29% of the total variation cannot be explained by the model. The predicted R^2 value of xylanase activity 86.83 %was in reasonable agreement with the adjusted R^2 value of xylanase activity 95.58%."Adequateprecision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Adequate precision of 21.877 for xylanase indicates an adequate signal. These models can be used to navigate the design space.

Table 6 Analysis of Variance (ANOVA) for Xylanase

Source	Sum of Squares	Degree of freedom	Mean Square	F Value	p-value Prob > F
Model	11.26	14	0.80	45.77	< 0.0001
X_1	3.99	1	3.99	227.31	< 0.0001
X_2	4.55	1	4.55	258.99	< 0.0001
X_3	0.13	1	0.13	7.43	0.016
X_4	0.03	1	0.03	1.8	0.2
$X_1 X_2$	0.089	1	0.09	5.04	0.04
$X_1 X_3$	0.001	1	0.001	0.06	0.81
$X_1 X_4$	0.005	1	0.005	0.26	0.62
$X_2 X_3$	0.001	1	0.001	0.08	0.78
$X_2 X_4$	0.003	1	0.003	0.19	0.67
$X_3 X_4$	0.05	1	0.05	2.82	0.11
X_1^2	0.68	1	0.68	38.68	< 0.0001
X_2^2	0.66	1	0.66	37.46	< 0.0001
X_3^2	1.03	1	1.03	58.55	< 0.0001
X_4^2	1.04	1	1.04	59.30	< 0.0001
Residual	0.26	15	0.02		
Lack of Fit	0.26	10	0.03		
Pure Error	0	5	0		
Cor Total	11.52	29			

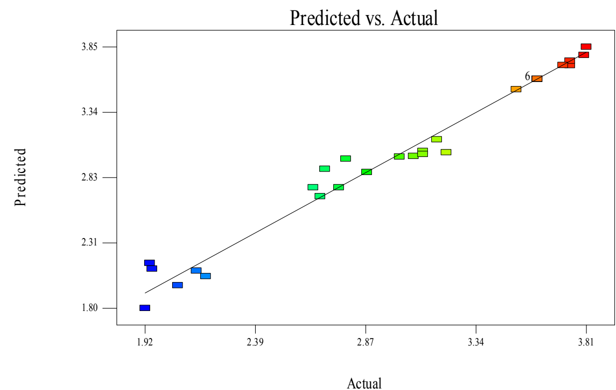

Figure 2 Predicted response Vs Actual value for xylanase

The statistical significance of (equ.3) was checked by F- test, the results of ANOVA are shown in Table 6. The results demonstrated that the model is highly significant and is evident from Fischer's F- test with a low probability value (P model $>F$ less than 0.05) (Table 6). Model coefficients estimated by regression analysis for each variable is shown in Table 6. The significance of each coefficient was determined by t-values and P-values. The larger the magnitude of t-test value and smaller the P-value indicates the high significance of the corresponding coefficient (**Purama and Goyal**, **2008**).

The above models can be used to predict the xylanase production within the limits of the experimental factors. Figure 2 shows that the actual response values agree well with the predicted response values of xylanase. According to Table 6, values of "Prob >F" less than 0.05, the linear effects of X_1, X_2, X_3,the interactive effects of $X_1 X_2$, and square effects of X_1^2, X_2^2, X_3^2, $X_4^2$are significant model terms for xylanase production. This interaction effect on the variables on xylanase production was investigated by plotting the 3D response surfaces with the vertical (Z) axis representing enzyme activity (response) yield and two horizontal axes representing the coded levels of two explanatory factors, while maintaining other variables at their median levels shown in Figure 3(A)to Figure 3(F). The contour plots might be circular, saddle, elliptical or rising ridges (**Muralidhar *et al.*, 2001**). The response surface having circular contour plot indicate no interaction, whereas, an elliptical or saddle nature of the contour plot indicates a significant interaction between the corresponding variables. Figure 3 contour plot is in elliptical shape indicates there is a significant interaction between X_1 X_2,where as from Figure 4 to Figure 8 contour plot slows nearly circular plots indicate no interaction.

Effect of peptone on xylanase activity

Figures 3(A), 3(B) and 3(C) show the dependency of xylanase on peptone. The xylanase activity increases with increase in peptone up to 0.957g/L and thereafter xylanase activity decreases with further increase in peptone. The results were almost similar with findings of **Muthechilan *et al.* (2007)** when peptone used as the nitrogen source for production of xylanase. **Murugan *et al.***

(2011) found that that 1% of peptone and beef extract are good supplements for the production of xylanase enzyme.

Effect of yeast extract on xylanase activity

The variation of xylanase productivity may be due to nitrogen source mediated regulation of microbial growth and metabolism.Since nitrogen is an essential medium components requirement for growth and production of an enzyme and is an important cellular component mostly as part of the protein. The nitrogen may also significantly affect the pH of the medium during the course of fermentationthereby influences the xylanase productivity**.**
The same trend was observed in Figures 3(A), 3(D) and 3(E) which show the dependency of xylanase on yeast extract. The xylanase and activity increases with increase in yeast extract up to 2.5g/L and thereafter xylanase activity decreases with further increase in yeast extract. **Suvarna Laxmi *et al.* (2008)** reported thatyeast extract to bethe best nitrogen source for xylanase production.

Effect of KH_2PO_4 on xylanase activity

Figures 3(B), 3(D), and 3(F) show the effect of KH_2PO_4 on xylanase activity. The xylanase activity increases with increase in KH_2PO_4 up to 3.09g/L and thereafter xylanase activity decreases with further increase in KH_2PO_4. **Liu *et al.* (2008)** found that KH_2PO_4, one of the three screened significant variables which enhance xylanase production.

Effect of $CaCl_2.2H_2O$on xylanase activity

The influence of $CaCl_2.2H_2O$ on xylanase activity shows from the figures 3(C), 3(E) and 3(F). The xylanase activity increases with increase in $CaCl_2.2H_2O$ up to 0.409g/L and thereafter xylanase activity decreases with further increase in $CaCl_2.2H_2O$. Calcium may be responsible for some changes in the permeability of the cell wall that result in a more rapid excretion of the enzymes, which in turn, improves enzyme synthesis (**Chen and Wayman, 1992**). Similar results obtained by **Cesar and Mrša (1996)** for *T. lanuginosus* showed that Ca^{2+} enhanced xylanase activity.

The optimum conditions for the maximum production of xylanase were determined by response surface analysis using statistical software package "Design Expert 7.1.5". The optimum conditions are peptone–0.957g/L, yeast extract–2.5g/L, KH_2PO_4–3.09g/L and $CaCl_2.2H_2O$ –0.409g/L. The predicted results are shown in Table 5.

Validation of the experimental

Validation of the experimental model was carried in experiment under optimal operating conditions. The experiments were done in triplicate and the results were compared. The xylanase 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 xylanase activity was found to be 4.12 IU/ml.

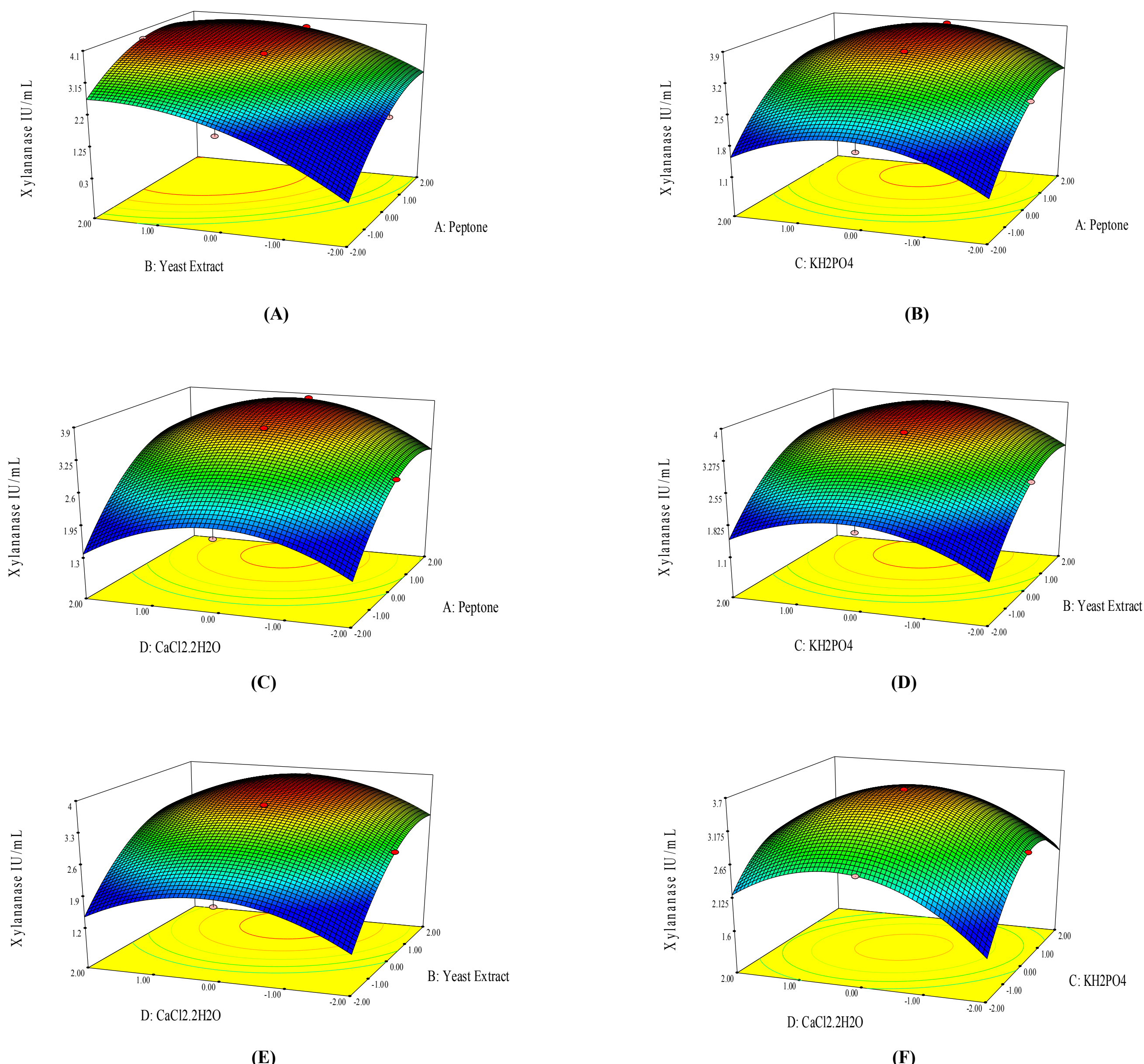

Figure 3 Three-dimensional response surface plot for xylanase production by *Cellulomonas fimi* usingtapioca stem showing the interactive effects of (A) Peptone and yeast extract; (B) Peptone and KH_2PO_4; (C) Peptone and $CaCl_2.2H_2O$; (D) yeast extract and KH_2PO_4; (E) yeast extractand $CaCl_2.2H_2O$; (F) KH_2PO_4 and $CaCl_2.2H_2O$

CONCLUSION

In this work medium components for higher xylanase activity from *Cellulomonas fimi* were optimized by RSM. Using Placket-Burman design peptone, yeast extract, $KH_2PO_4$and $CaCl_2.2H_2O$were found to be the most significant variables, which significantly enhanced xylanase activity. Central composite design was applied to study the combined effects of these nutrients. The optimal levels of components were obtained as peptone–0.957g/L, yeast extract–2.5g/L, KH_2PO_4–3.09g/L, and $CaCl_2.2H_2O$–0.409g/L. This study showed that the tapioca stem constitutes a good carbon source for the production of xylanase. Using the optimized conditions, the enzyme activity 4.12 IU/mlof xylanase was obtained. These results show a close agreement between the expected and obtained activity level.

Acknowledgments: The authors gratefully acknowledge UGC, New Delhi, for providing financial support to carry out this research work under UGC –Major Research Project Scheme .The authors also wish to express their gratitude for the support extended by the authorities of Annamalai University, Annamalainagar, India, in carrying out the research work in Bioprocess Laboratory, Department of Chemical Engineering.

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