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Identification and Optimization of Critical Medium Components Using Statistical Experimental Designs for Enhanced Production of Xylanase from *Aspergillus flavus* DFR-6

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Summary

The Plackett-Burman screening design (PBSD) and Central composite rotatable design (CCRD) were used to optimize the fermentation parameters for enhanced xylanase production from *Aspergillus flavus* DFR-6 in submerged cultivation. The PBSD demonstrated that the positive main effects of yeast extract, wheat bran, Tween 80 and $NaNO_3$ were significant at 10 % level of significance. The interactive effects of these factors were deduced using CCRD and finally, the production medium was optimized using Design-Expert software. The optimized medium conditions with 97.2 % desirability and with respect to 'minimum organic nitrogen source' were (in g/L): $NaNO_3$ 4.09, K_2HPO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.25, KCl 0.25, $FeSO_4$ 0.005, wheat bran 24.99, yeast extract 10 and Tween 80 0.21 mL/L, pH=5.0. The enzyme was produced in 50 mL of medium working volume using 500-mL conical flask at 35 °C with 1 % (by volume) of inoculum size. The production titre of xylanase under aforementioned conditions was 31.4 U/mL after 6 days of static fermentation which is approx. fourfold higher than obtained from the unoptimized medium.

Key words: statistical design, optimization, xylanase, submerged fermentation, lignocellulose

Introduction

Lignocellulose is one of the most abundant natural complex organic carbons in the form of plant biomass, which mainly consists of three major components: cellulose, hemicellulose and lignin. Hemicellulose is a complex polymeric carbohydrate with xylan as its major component. Xylan is the second most abundant polysaccharide in nature, and it mainly consists of β-1,4-linked xylopyranosyl residues, which are further substituted, depending on plant source to a varying degree with glucuronopyranosyl, 4-O-methyl-D-glucopyranosyl, α-L-arabinofuranosyl and acetyl, as well as linked to feruloyl and coumaryl components of lignin (*1*). Nature is abundant with bacteria and fungi that can produce cell wall-degrading enzymes to solubilize these complex components to simple molecules for completing the carbon cycle (*2*).

Xylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) has gained increasing attention because of its various biotechnological applications (*3*). Potential applications of xylanase in biotechnology include biopulping of wood, pulp bleaching, treating animal feed to increase digestibility, processing food to increase clarification and converting lignocellulosic substances into feedstocks and fuel (*4,5*). Filamentous fungi are useful producers of xylanase because they are capable of producing high levels of extracellular enzymes and can be cultivated very easily. However, to reach commercial feasibility, enzyme production must be increased by introducing a more potent strain and/or by optimizing the culture conditions.

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For many microorganisms the production of xylanase has been described using `one-factor at a time' approach (*3*,*6*,*7*). The use of wheat straw as a carbon source for xylanase production has been reported by Ghanem *et al.* (*8*) and Bakri *et al.* (*3*,*9*). Other agro-residues such as rice straw (*10*), sugarcane bagasse (*11*), corncob (*12*) and oat spelt xylan (*13*) have also been reported as suitable substrates for xylanase production.

The traditional approach used for optimizing a multivariable system easily misses the interactive effects between the components. Response surface methodology (RSM) is a collection of statistical experimental designs suitable for identifying the individual and interactive effect of independent variables and for seeking the optimum conditions for a multivariable system efficiently (*14*).

Our laboratory is actively engaged in the studies on industrially important microbial proteins (*5*,*15–18*). Among the xylanase-producing microorganisms, isolated from 6 different soil samples, the initial xylanase titer of *Aspergillus flavus* DFR-6 was relatively high. Therefore, the strain was chosen to further enhance the enzyme yield through medium optimization. The identification of factors influencing xylanase production was carried out using Plackett-Burman screening design (PBSD) as a tool followed by determination of their optimum values using Central composite rotatable design (CCRD).

Materials and Methods

Microorganism and chemicals

The xylanase-producing microorganism used in the present study was isolated from a soil sample collected near fruit and vegetable debris at Mysore, India and identified as *Aspergillus flavus* at the Department of Plant Pathology, Indian Agricultural Research Institute, New Delhi, India. The isolate was named *Aspergillus flavus* DFR-6. The strain was grown on potato dextrose agar (PDA) slants at 30 °C for 5 days and subsequently stored at 4 °C. Inoculum was prepared by suspending the spores from PDA slants by adding sterile 0.1 % Tween 80 to give a final count of approx. 10^5 spores/mL. Unless indicated otherwise, all the chemicals used in the present investigation were of high quality analytical grade and were purchased from Sigma-Aldrich, Inc, St. Louis, MO, USA (cetyl trimethylammonium bromide – CTAB, Triton X-100, sodium dodecyl sulphate – SDS and Tween 80), Sisco Research Laboratories Pvt. Ltd, Mumbai, India ($NaNO_3$, KCl and KH_2PO_4), HiMedia Laboratories Pvt. Ltd, Mumbai, India (yeast extract and xylan) and Merck Limited, Mumbai, India ($MgSO_4{\cdot}7H_2O$ and $FeSO_4$).

Optimization of media

Standardization of basal media

Initially, a comparison between solid-state fermentation (SSF) and submerged fermentation (SmF) was made to determine the effect of fermentation type on xylanase production. Based on higher enzyme titre, SmF was selected for further standardization. For initial screening, 4 different media, *viz*. potato dextrose broth, PDB (in g/L: infusion from potato 200, glucose 20 and xylan 1), malt extract medium, MEM (in g/L: malt extract 20 and xylan 1), Czapek-Dox medium, CDM (in g/L: $NaNO_3$ 3.0, K_2HPO_4 1.0, $MgSO_4{\cdot}7H_2O$ 0.5, KCl 0.5, $FeSO_4$ 0.01 and xylan 1) and modified xylanase cultivation medium, mXCM (in g/L: yeast extract 5.0, KH_2PO_4 1.0, $MgSO_4{\cdot}7H_2O$ 0.2, NaCl 2.5 and xylan 1.0) (*19*) adjusted to pH=6.0, were used for xylanase production. Preliminary experiments led us to select fermentation period of 5 days at 35 °C under static conditions. Each medium was inoculated with 1 % inoculum and after due incubation, xylanase activity was monitored. Based on the optimum xylanase production, CDM was selected as basal medium for further optimization.

Effect of carbonaceous source

Various agricultural and industrial wastes such as orange peel, corn involucre, corncob, sugarcane bagasse, banana peel, pineapple peel as well as cheaper commercial sources such as wheat bran and oat bran were used to replace the xylan, a costly ingredient of basal medium at 1 % level. Different simple and readily metabolizable sugars such as xylose, glucose and fructose were also tried to test the inducible or constitutive nature of the enzyme.

Effect of supplementary organic nitrogen source

Organic nitrogen source is not a component of basal medium, so different commercially available nitrogen sources (proteose peptone, yeast extract, tryptone, skim milk, casein peptone, soy peptone, urea and beef extract) as well as agricultural and industrial wastes/cheaper nitrogen sources (cotton leaf residue and peanut cake) were added separately into the basal medium at 1 % level with the intention of enhancing xylanase production. Wheat bran at 1 % concentration was used as carbon source.

Effect of supplementary surfactants on xylanase production

Besides carbon and nitrogen sources, several other factors also have to be considered in designing the optimum cultivation conditions. In that line, various detergents/ surfactants (CTAB, Triton X-100, SDS and Tween 80) were added to xylanase-producing medium at 0.02 % level (*20*).

Fungal growth and xylanase assay

After 5 days of growth at 35 °C, the production medium was filtered through 4 layers of muslin cloth and centrifuged at 10 000 rpm for 20 min at 4 °C in refrigerated centrifuge. The supernatant was used to assay the enzyme activity. The enzyme activity was measured using the method described earlier (*21*) with slight modifications. The solution of xylan and the enzyme at appropriate dilution was incubated at 37 °C for 30 min and the reducing sugars were determined by the dinitrosalicylic acid method described by Miller (22), with xylose as standard. The released xylose was measured spectrophotometrically at 540 nm. One unit of xylanase is defined as the amount of enzyme required to release 1 μmol of reducing sugar as xylose equivalent per min under the assay conditions. The dry mass of fungal mycelium was measured to assess the cell growth and expressed as dry mycelial mass (mg/mL). All the experiments were done in triplicate and the results were expressed as mean values±S.D.

Optimization procedure and experimental design

Further optimization of CDM components for xylanase production in SmF fermentation was carried out using two statistical designs, *viz.* Plackett-Burman screening design (PBSD) and Central composite rotatable design (CCRD) of response surface methodology.

Identification of important physicochemical components using PBSD

The PBSD, a fractional factorial design (*23*) was used to reflect the relative importance of various physicochemical factors on xylanase production. Eleven independent variables were screened in twelve combinations and one center point experiment was organized according to the design matrix. Each variable was represented at two levels, high and low denoted by (+) and (–), respectively. The effect of each variable or factor is the difference between the average of the measurements made at the high level of that factor and the average of the measurements made at the low level of that factor, which was determined by the following equation:

$$E_{(xi)}=2(\Sigma P_{i+}-\Sigma P_{i-})/N \qquad /1/$$

where $E_{(xi)}$ is the effect of concentration on the tested variable, P_{i+} and P_{i-} are the xylanase activities from the trials where the measured variable (xi) is present at high and low concentration, respectively, and N is the number of trials excluding center point experiment (12 experiments).

Central composite rotatable design (CCRD)

Based on the results from PBSD experiments, 4 factors that significantly affected the xylanase production were identified and further optimized using CCRD. The CCRD with 4 factors and 5 levels including 6 replicates at the centre point was used to fit the second order response surface. To design the experiments, the test factors were coded according to the following equation:

$$x_i=X_i-X_0/\delta X_i \qquad /2/$$

where x_i is the dimensionless coded value of the ith independent variable, X_i is the natural value of the ith independent variable, X_0 is the natural value of the ith independent variable at the center point and δX_i is the step change value. Once the experiments were performed, the experimental results were fitted with a 2nd order polynomial equation:

$$Y=b_0+b_1x_1+b_2x_2+b_3x_3+b_4x_4+b_{11}x_1^2+b_{22}x_2^2+b_{33}x_3^2+ \\ +b_{44}x_4^2+b_{12}x_1x_2+b_{13}x_1x_3+b_{14}x_1x_4+b_{23}x_2x_3+b_{24}x_2x_4+b_{34}x_3x_4 \qquad /3/$$

where Y is the predicted response, b_0 is the intercept, b_1, b_2, b_3 and b_4 are the linear coefficients, b_{11}, b_{22}, b_{33} and b_{44} are the squared coefficients, and b_{12}, b_{13}, b_{14}, b_{23}, b_{24} and b_{34} are the interaction coefficients.

Data analysis

All statistical experimental designs and analysis results were carried out using Design-Expert and Minitab software. The quality of fit of the polynomial model equation was expressed by the coefficient of determination, R^2, and its statistical significance was checked by Fisher's F-test. The significance level of each regression coefficient was determined by Student's t-test. The level of significance was given as p-value.

Results and Discussion

To achieve maximum xylanase production by *Aspergillus flavus* DFR-6, a proper combination of various cultural conditions and nutrients was established using `one--at-a-time' approach followed by RSM.

Media optimization for xylanase production

Even though PDA medium was used for the isolation of *A. flavus* DFR-6, further screening of media showed that CDM was suitable to be used as basal medium since comparatively higher titre of xylanase was observed using this medium (8.1 U/mL). The initial production of xylanase from *A. flavus* DFR-6 was approx. 3.5-fold higher than reported earlier in *Sclerotinia sclerotiorum* S2 (*24*). CDM that contains minerals like K^+, Fe^{2+} and Mg^{2+} probably supported better xylanase production as compared to other media which contain either fewer (Na^+ and Mg^{2+} containing mXCM 7.3 U/mL) or no metal ions (MEM 6.7 U/mL or PDM 5.3 U/mL). Hence CDM was used to optimize different cultural and environmental parameters for xylanase production.

Effect of carbonaceous substrate on xylanase production

The carbon source in the medium appears to exert a profound effect on the enzyme production behaviour of the fungus because it is one of the essential constituents of the microbial fermentation medium and has major role on overall cellular growth and metabolism. A slightly higher quantity of xylanase was produced by the strain in wheat bran-supplemented medium (9.5 U/mL) followed by oat bran (9.2 U/mL) and pineapple peel (8.9 U/mL) (data not shown). None of the tested monosaccharides and disaccharides, *viz.* sucrose, maltose, dextrose, fructose, galactose, lactose and xylose was found suitable for xylanase production. Since no xylanase activity could be observed in the absence of xylanaceous substance in the culture medium, it could be suggested that xylanase production is inducible for *A. flavus* DFR-6. Induction of the synthesis of xylan-degrading enzymes by xylanolytic organisms cultured with xylan as a carbon source is well documented (*25,26*). Suvarna Laxmi *et al.* (*27*) have, however, reported that xylanase production by *Aspergillus* sp. RSP-6 is constitutive in nature.

The highest level of xylanase activity as well as extracellular protein using wheat bran has been reported by Okafor *et al.* (*28*) in *A. niger* ANL 301. The xylanase titre produced by the studied strain is approx. 2.5-fold higher than that by *Penicillium oxalicum* in the presence of wheat bran (*13*). However, higher yields of xylanase have been reported by Gasper *et al.* (*29*) and Bakri *et al.* (*30*).

Effect of supplementary organic nitrogen source on xylanase production

The effect of supplementation of different organic as well as cheaper nitrogen sources on xylanase production by *A. flavus* DFR-6 was studied with 1 % wheat bran as carbon source. Of all the nitrogen sources investigated, yeast extract was the most promising and the corresponding xylanase production was 13.8 U/mL, which

was approx. 45 % higher than that of control (9.5 U/mL) medium lacking organic nitrogen (data not shown). The results obtained during the present investigation are in close agreement with those reported by Suvarna Laxmi *et al.* (*27*), where yeast extract has been reported to be the best nitrogen source for xylanase production by *Aspergillus* sp. RSP-6 and other nitrogen meals were found to be poor nitrogen sources. In contrast to our results, Bakri *et al.* (*9*) and Gupta *et al.* (*31*) reported peptone as the best source of organic nitrogen for the production of xylanase from *A. niger* and *F. solani*, respectively.

Effect of supplementation of surfactants on xylanase production

Fig. 1 shows the effect of different surfactants on xylanase production by *A. flavus* DFR-6. The results show that all the surfactants used at 0.02 % enhanced xylanase production but the stimulatory effect of Tween 80 was more remarkable, increasing the enzyme yield by a factor of 15.6 %. The positive effect of surfactants on enzyme yield has been found in many studies (*32–35*), but the exact mechanism is still unclear. However, researchers believe that surfactants decrease the phospholipid content of the cell membrane to increase its porosity, which in turn facilitates the release of enzymes (*36*). In contrast, Helle *et al.* (*37*) postulated that surfactants improve the enzyme stability and prevent its denaturation during hydrolysis by desorbing it from the substrate.

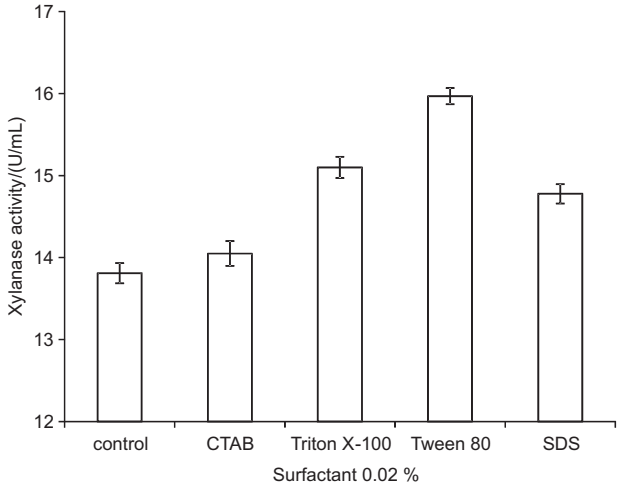

Fig. 1. Effect of surfactant on xylanase production by *A. flavus* DFR-6

Factors affecting xylanase production (PBSD)

For elucidation of medium components affecting xylanase production, the independent variables examined in the PBSD experiment and their settings are shown in Table 1. The experimental data of xylanase production in the screening design experiments illustrate a wide variation in xylanase activity from 11.4 to 22.3 U/mL (Table 2), which reflects the importance of medium optimization to attain higher yields. The main effect of each variable was calculated. The positive value indicates that the high concentration of this variable is near optimum and a negative value indicates that the low concentration of this variable is near optimum. The obtained data showed a range of positive main effect values, indicating that the presence of high levels of yeast extract, $NaNO_3$, wheat bran, Tween 80, KCl and K_2HPO_4 in the growth medium positively affects xylanase production by the studied strain. On the other hand, the presence of $MgSO_4$, $FeSO_4$, temperature, pH and agitation at their lowest levels results in higher xylanase production. The Pareto graph was used to show the effect of all variables on xylanase production (Fig. 2). Statistical analysis (*t*-value and p-value) demonstrated that among the positive main effects yeast extract, wheat bran, Tween 80 and $NaNO_3$ had significant effects at 10 % level (Table 1). The factors resulting in a negative effect on xylanase production were no longer taken into account (they were kept at their –1 level), while the 4 positive factors showing significant effect at 10 % level of significance were included in the next optimization strategy.

Optimization using central composite rotatable design

The variables, selected based on the results of PBSD, were further optimized using CCRD. The experimental design matrix is presented in Tables 3 and 4. Thirty experiments were performed in triplicate. The relative deviation (%) was calculated using the formula given below:

Table 1. Factors examined as independent variables affecting xylanase production and their levels in the Plackett-Burman experiment

Variable	Symbol	–1	0	+1	Effect	*t*-value	p-value
φ(Tween 80)/(mL/L)	T80	0.1	0.2	0.3	1.873	8.01	0.079
$\gamma(FeSO_4)$/(g/L)	FeS	0.005	0.01	0.015	–1.427	–6.10	0.103
γ(KCl)/(g/L)	KCl	0.25	0.5	0.75	0.860	3.68	0.169
Agitation/rpm	Agit	0	50	100	–3.643	–15.58	0.041
Temperature/°C	Temp	35	40	45	–2.123	–9.08	0.070
γ(yeast extract)/(g/L)	YE	5	10	15	3.270	13.98	0.045
$\gamma(NaNO_3)$/(g/L)	NaN	1.5	3.0	4.5	3.020	12.91	0.049
$\gamma(MgSO_4{\cdot}7H_2O)$/(g/L)	MgS	0.25	0.5	0.75	–1.633	–6.98	0.091
pH	pH	5	6	7	–0.610	–2.61	0.233
$\gamma(K_2HPO_4)$/(g/L)	K_2H	0.5	1.0	1.5	0.360	1.54	0.367
γ(wheat bran)/(g/L)	WB	5	10	15	1.890	8.08	0.078

Table 2. Plackett-Burman experimental design matrix and the observed response

T80	FeS	KCl	Agit	Temp	YE	NaN	MgS	pH	K_2H	WB	U/mL
–1	–1	1	1	1	–1	1	1	–1	1	–1	13.3±0.45
1	1	1	–1	1	1	–1	1	–1	–1	–1	17.3±0.52
–1	1	–1	–1	–1	1	1	1	–1	1	1	22.0±0.38
1	1	–1	1	–1	–1	–1	1	1	1	–1	11.4±0.57
–1	1	1	–1	1	–1	–1	–1	1	1	1	15.4±0.48
1	1	–1	1	1	–1	1	–1	–1	–1	1	16.1±0.41
–1	–1	–1	1	1	1	–1	1	1	–1	1	13.6±0.51
1	–1	1	1	–1	1	–1	–1	–1	1	1	21.1±0.62
1	–1	1	–1	–1	–1	1	1	1	–1	1	21.9±0.49
–1	–1	–1	–1	–1	–1	–1	–1	–1	–1	–1	16.5±0.53
1	–1	–1	–1	1	1	1	–1	1	1	–1	22.3±0.64
–1	1	1	1	–1	1	1	–1	1	–1	–1	17.9±0.55
0	0	0	0	0	0	0	0	0	0	0	17.8±0.47

$$\text{Relative deviation} = \frac{\text{Predicted response} - \text{Observed response}}{\text{Predicted response}} \times 100 \quad /4/$$

The results of the second-order response surface model in the form of analysis of variance (ANOVA) are given in Table 5. Using Design–Expert software, the data were analyzed for their linear and quadratic effects and the following model equation (in uncoded factors) was proposed for xylanase production:

Xylanase activity/(U/mL)=–70.86+5.20 (wheat bran)+ 2.48 ($NaNO_3$)+72.16 (Tween 80)+3.43 (yeast extract)–0.079 (wheat bran^2)–0.43 ($NaNO_3^2$)–33.78 (Tween 80^2)–0.053 (yeast extract^2)+0.022 (wheat bran·$NaNO_3$)–2.07 (wheat bran·Tween 80)–0.064 (wheat bran·yeast extract)+1.99 ($NaNO_3$·Tween 80)+0.01 ($NaNO_3$·yeast extract)–1.50 (Tween 80·yeast extract) /5/

The significance of each coefficient was determined by student's *t*-test and p-value, which are listed in Table 6. The larger the magnitude of *t*-test and smaller the p-value, the more significant the corresponding coefficient (*38*). Wheat bran had a strong positive linear effect on the response ($p<0.0001$). Yeast extract and Tween 80 also showed a significant effect, whereas the effect of $NaNO_3$ was not significant. Significant interactions were noted between wheat bran and yeast extract. Wheat bran also showed significant interaction with Tween 80. The above four variables indicated negative quadratic effects on xylanase production at various levels of significance.

The Fisher's F-test ($F_{(14, 15)}=12.99 > Ft_{(14, 15)}=3.56$) with a very low probability value ($p<0.0001$) indicated the model was highly significant. The goodness of fit of the model was examined by determination coefficient ($R^2=0.905$), which implied that the sample variation of more than 90 % was attributed to the variables and only less than 10 % of the total variation could not be explained by the model. Further proof of the high significance of the model obtained for xylanase production is the plot representing predicted *versus* experimental value. Since the plot (not shown) is very close to y=x (R^2~0.90), it can be assumed that the prediction of experimental data by the applied model is satisfactory. A lower value of the coefficient of variation (CV=5.96 %) showed that the conducted experiments were precise and reliable (*38*).

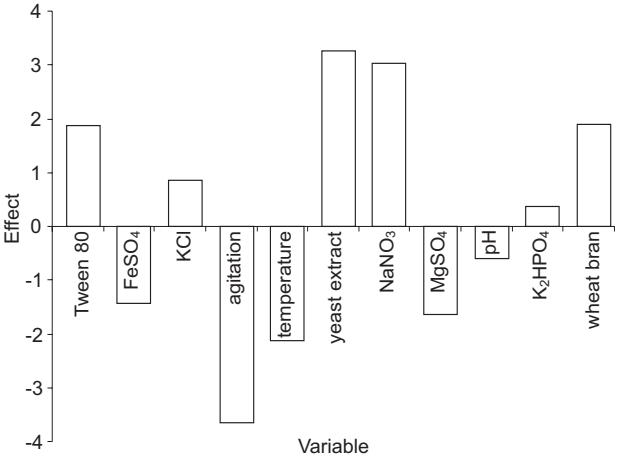

Fig. 2. Pareto graph showing the effect of independent variables on xylanase production by *A. flavus* DFR-6

Table 3. Levels of variables used in the CCRD

Variables	Range and levels				
	–2	–1	0	+1	+2
X_1 (wheat bran)/(g/L)	10	15	20	25	30
X_2 ($NaNO_3$)/(g/L)	1.5	3.0	4.5	6.0	7.5
X_3 (Tween 80)/(mL/L)	0.1	0.2	0.3	0.4	0.5
X_4 (yeast extract)/(g/L)	5	10	15	20	25

Figs. 3 and 4 represent some of the 3D surface graphics showing the interactive effects of medium components on xylanase production. Each figure presents the effect of two variables on the production of xylanase, while other two variables were held at zero level. The effect of variations in the level of independent factors on xylanase production can be seen in the perturbation graph

Table 4. Experimental design with observed and predicted values of xylanase activity

Coded level				Response/(U/mL)		Relative deviation
X_1	X_2	X_3	X_4	observed	predicted	%
–1.00	–1.00	–1.00	–1.00	19.7±0.62	19.2	–2.6
1.00	–1.00	–1.00	–1.00	29.5±0.51	29.1	–1.4
–1.00	1.00	–1.00	–1.00	17.5±0.39	17.0	–2.7
1.00	1.00	–1.00	–1.00	29.2±0.48	28.1	–3.8
–1.00	–1.00	1.00	–1.00	19.2±0.46	21.0	8.6
1.00	–1.00	1.00	–1.00	28.2±0.38	27.3	–3.3
–1.00	1.00	1.00	–1.00	19.1±0.41	20.6	–5.2
1.00	1.00	1.00	–1.00	28.1±0.52	27.5	–2.3
–1.00	–1.00	–1.00	1.00	24.7±0.40	24.8	0.5
1.00	–1.00	–1.00	1.00	30.7±0.58	28.9	–6.2
–1.00	1.00	–1.00	1.00	22.8±0.44	23.4	2.6
1.00	1.00	–1.00	1.00	30.4±0.63	28.1	–8.3
–1.00	–1.00	1.00	1.00	23.4±0.45	24.2	3.3
1.00	–1.00	1.00	1.00	24.2±0.51	24.1	–0.2
–1.00	1.00	1.00	1.00	24.0±0.37	23.9	–0.3
1.00	1.00	1.00	1.00	23.8±0.48	24.5	2.8
–2.00	0.00	0.00	0.00	17.6±0.39	15.6	–13.0
2.00	0.00	0.00	0.00	23.8±0.29	26.7	10.7
0.00	–2.00	0.00	0.00	25.6±0.42	25.9	1.1
0.00	2.00	0.00	0.00	24.1±0.39	24.6	2.0
0.00	0.00	–2.00	0.00	25.6±0.46	28.3	9.7
0.00	0.00	2.00	0.00	23.8±0.36	27.1	12.0
0.00	0.00	0.00	–2.00	22.1±0.28	22.2	0.7
0.00	0.00	0.00	2.00	24.7±0.57	25.3	2.5
0.00	0.00	0.00	0.00	29.1±0.51	29.1	–0.2
0.00	0.00	0.00	0.00	29.0±0.39	29.1	0.1
0.00	0.00	0.00	0.00	29.1±0.48	29.1	–0.2
0.00	0.00	0.00	0.00	29.0±0.42	29.1	0.1
0.00	0.00	0.00	0.00	28.9±0.53	29.1	0.5
0.00	0.00	0.00	0.00	29.2±0.40	29.1	–0.5

Table 5. ANOVA for full quadratic model

Source	SS	DF	MS	F-value	Prob>F
Model	410.85	14	29.35	12.99	<0.0001
Residual	33.90	15	2.26		
Total	444.75	29			

CV=5.96 %, R^2=0.923

Table 6. Model coefficient estimated by multiple linear regression

Factor	Coefficient	Computed *t*-value	p-value
Intercept	29.055	45.360	0.000
X_1	2.763	8.627	0.000
X_2	–0.323	–1.008	0.330
X_3	–0.706	–1.863	0.084
X_4	0.779	2.432	0.029
X_1^2	–1.886	–6.206	0.000
X_2^2	–0.862	–2.836	0.013
X_3^2	–0.830	–2.117	0.053
X_4^2	–1.224	–4.029	0.001
X_1X_2	0.164	0.419	0.682
X_1X_3	–1.037	–2.643	0.019
X_1X_4	–1.588	–4.049	0.001
X_2X_3	0.298	0.760	0.460
X_2X_4	0.054	0.139	0.892
X_3X_4	–0.749	–1.910	0.077

(Fig. 5). The plot revealed that the produced xylanase activity was most sensitive to wheat bran, followed by yeast extract.

Optimization and validation of the model equation

Xylanase production medium was numerically optimized using Design-Expert software with the constraint of minimum concentration of yeast extract (organic nitrogen source) in optimized medium so that further down streaming of xylanase can be made easy without losing its titre. The significant interactions of yeast extract with

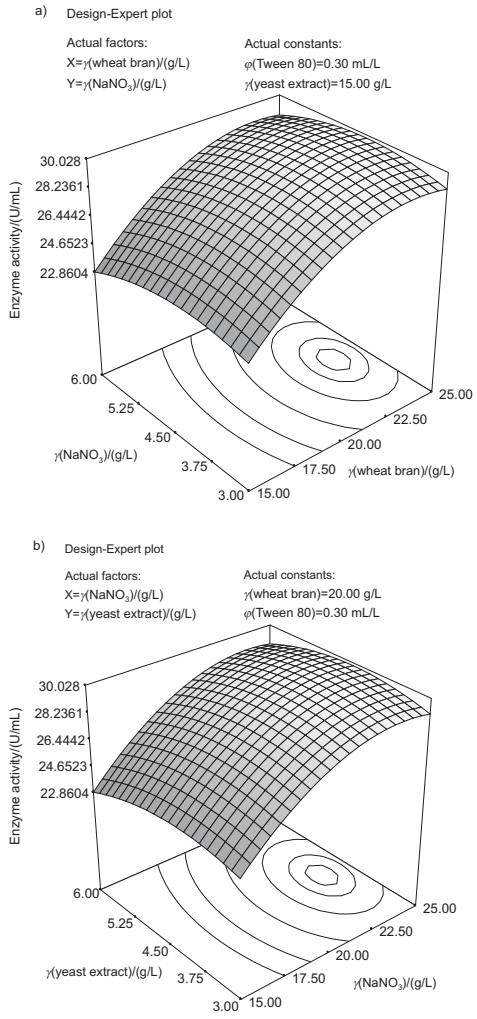

Fig. 3. Three-dimensional graphs showing the effects of: a) wheat bran and $NaNO_3$, and b) yeast extract and $NaNO_3$ on xylanase production by *A. flavus* DFR-6

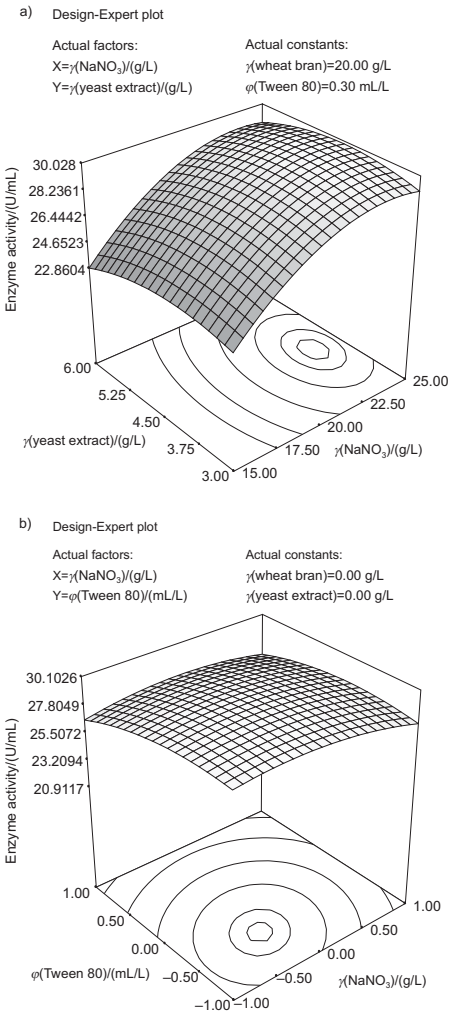

Fig. 4. Three-dimensional graph showing the effects of: a) yeast extract and $NaNO_3$, and b) Tween 80 and $NaNO_3$ on xylanase production by *A. flavus* DFR-6

other components (Table 6) made it possible to keep the amount of yeast extract minimum in the finalized medium without losing xylanase activity. To the best of our knowledge, this is the first report of this kind where cultivation medium has been finalized with this concept. The criteria used for optimization along with the predicted and actual/observed response values are presented in Table 7. Using the set criteria, a solution having 97.2 % desirability was selected and experiments were conducted. The closeness of the observed response (28.8 U/mL) to the predicted one (29.6 U/mL) strongly advocates the adequacy of the developed model. The production of xylanase using RSM optimized medium was approx. 4-fold higher than the original medium, which is again 4 times higher than xylanase extracted earlier from *Bacillus circulans* BL53 using RSM (*39*).

Xylanase production using optimized medium

The time course of fungal growth and xylanase production was investigated using optimized medium. The effect of incubation period on the biomass production revealed that the yield increased up to 7 days and then

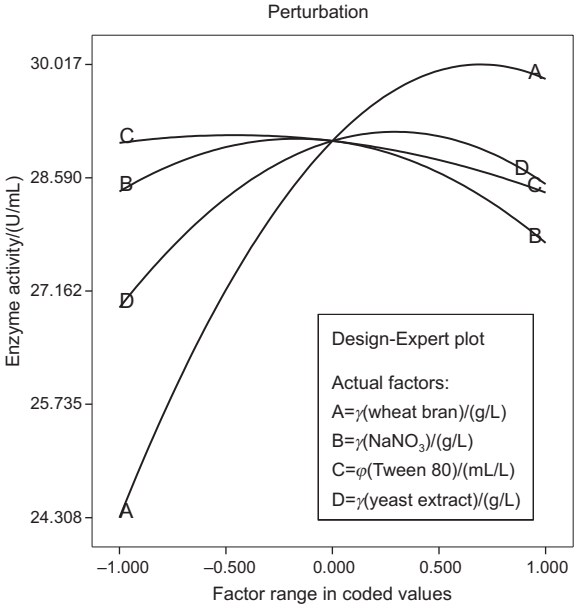

Fig. 5. Perturbation graph showing the effect of variables on xylanase production by *A. flavus* DFR-6

Table 7. Constraints, criteria for optimization and solution along with the predicted and observed response values

Constraints	Goal	Importance	Solution	Observed response
X_1/(g/L)	maximize	4	24.99	–
X_2/(g/L)	in range	3	4.09	–
X_3/(mL/L)	in range	3	0.21	–
X_4/(g/L)	minimize	4	10.00	–
Response/(U/mL)	maximize	4	29.64	28.82±0.52

declined (Fig. 6a). The increase in biomass indicates the efficient utilization of substrate/medium constituents by the strain, while the decline points out the autolysis of cells (due to exhaustion of nutrients) resulting in low biomass recovery. A progressive increase in xylanase production was recorded with longer incubation period. The increase in xylanase activity in optimized medium was parallel with growth, and the activity was maximum (31.4 U/mL) at 6 days. A decrease in enzyme titre with further incubation was observed. This may be attributed to the exhaustion of nutrients and/or catabolic repression during prolonged incubation. The low enzyme activity after 6 days may also be due to the partial degradation of xylanase by nonspecific proteolytic enzymes. A plot between fungal biomass and xylanase activity shows that data were very close to the straight line and situated at both sides of it, indicating a high degree of correlation (R^2= 0.947) between them (Fig. 6b). From the plot, it can also be concluded that approx. 95 % increase in xylanase activity during incubation can be attributed to increased fungal biomass.

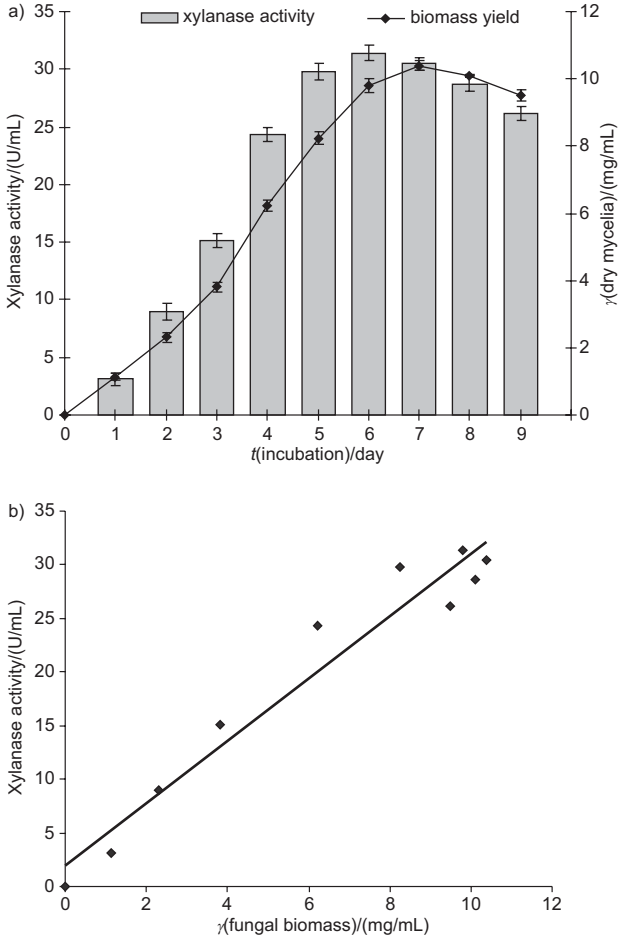

Fig. 6. Xylanase production in an optimized medium: a) effect of fermentation time on growth and xylanase production by *A. flavus* DFR-6, and b) plot between fungal biomass and xylanase activity

Conclusion

From the present study, it is clear that PBSD and CCRD can be used effectively, one after the other, to determine the significant variables and their optimum conditions for enhanced xylanase production. Using PBSD, wheat bran, yeast extract, $NaNO_3$ and Tween 80 were identified as the most influencing parameters affecting xylanase production, and CCRD helped to study their interactive effects on enzyme yield. The medium designed using a limited number of experiments, minimum efforts and time resulted in approx. 4-fold increase in xylanase production. This systematic and statistical optimization carried out using PBSD and CCRD has the advantages over conventional method of optimization which is incapable of reaching the true optimum conditions. The traditional method assumes that various fermentation parameters do not interact and that the process response is a direct function of the single varied parameter. In contrast, we have seen that the observed behaviour of fermentation results from the interactive influences of various variables. The statistical strategy as a whole proved to be adequate for the design and optimization of the bioprocess for enhanced xylanase production. Further studies on the purification and detailed characterization of xylanase from *A. flavus* DFR-6 using optimized medium are in progress.

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