Short Communication

Optimization of xylanase production from *Aspergillus foetidus* MTCC 4898 by solid state fermentation using statistical methods

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Xylanolytic enzymes consist of endo-1, 4- β -xylanase, β -xylosidase, α -glucuronidases, α -L-arabinofuranosidase and acetylxytan esterase (Christov and Prior, 1993). Their potential applications are mainly in the pulp and paper industry and also in the bioconversion of lignocellulosic materials to fuels and chemicals (Bajpai, 1999; Bajpai et al., 1994). As the structure of xylan is complex, a group of enzymes known as “xylanolytic enzymes” are required to hydrolyze it. Xylosidic linkages in lignocellulose are not all equivalent and equally accessible to xylanolytic enzymes (Wong et al., 1988). The accessibility of some linkages also changes during the course of hydrolysis. Multiple xylanases have been reported in numerous microorganisms.

A locally isolated strain of *Aspergillus foetidus* MTCC 4898 was studied for xylanase (EC 3.2.1.8) production using lignocellulosic substrates under solid state fermentation. *Aspergillus foetidus* is a filamentous and ubiquitous fungus belonging to the Trichomaceae family. Xylanases from *Aspergillus foetidus* have shown improvement in the quality of whole wheat bread (Shah et al., 2006). The development of proper fermentation

media is a necessary and important step in efficient utilization of fermentation technology (Margaritis and Pace, 1985). In the present study, different parameters like solid substrates, moisture content, nitrogen source, pH and temperature were optimized (Shah and Madamwar, 2005). The conventional method of medium optimization involves varying one parameter at a time and keeping the others constant. It is extremely time consuming and often does not bring about the effect of interaction of various parameters. Moreover, it seldom guarantees the determination of optimal conditions (Survase et al., 2006). These limitations of the single factor optimization process can be overcome by using statistical methods.

In statistics-based approaches, response surface methodology (RSM) has been extensively used in fermentation media optimization (Balusu et al., 2005; Kallil et al., 2000; Rao et al., 2000; Wei et al., 1998; Ya-Hong et al., 2004). It is a statistically designed experimental protocol in which several factors are simultaneously varied (Rao et al., 2000). In RSM, the experimental responses to the design of experiments (DOE) are fitted to a quadratic function. Thus, RSM can be employed to optimize the medium components (Balusu et al., 2005). RSM and factorial design are important tools to study the effect of both the primary factors and their mutual interactions to determine the optimal process conditions. The objective of the present work was to maximize the solid state fermentative

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yield of xylanase using statistics and artificial intelligence-based optimization techniques.

All media components, viz., peptone, potassium dihydrogen phosphate, ammonium sulphate, calcium chloride, magnesium sulphate, fructose and assay reagent 3,5-dinitrosalicylic acid, used in the study were purchased from S.D. Fine Chemicals, Mumbai, India. Brick wood xylan was purchased from Sigma-Aldrich Chemicals, USA. Different agricultural waste materials were used as solid substrates in this study. Lignocellulosic substrates like wheat straw (30% cellulose, 50% hemicellulose, and 15% lignin) and corncob (45% cellulose, 35% hemicellulose, and 15% lignin) were procured from local farms.

Aspergillus foetidus MTCC 4898 was procured from NCL, Pune, India. The strain was grown on potato dextrose agar for 4–6 days and stored at 4°C and sub-cultured every 20 days. The moistening media used was Mendels-steinberg medium containing (g/L): Peptone 1, potassium dihydrogen phosphate 2, urea 0.3, calcium chloride 0.3, magnesium sulphate 0.3 and some micronutrients (mg/L): ferrous sulphate 5.0, manganese sulphate 1.6, zinc sulphate 1.4, cobalt chloride 2, and tween 80 at 0.1% (v/v). The pH of the medium was maintained at 6.25 ± 0.05 before autoclaving.

Saline solution (10 ml containing 0.1% tween 80) was added to the growing slants and scrubbed to release the spores. From slant suspension, 100 times diluted, 2 ml of inoculum (0.91×10^8 spores/ml) was inoculated on solid substrate moistened with Mendels-steinberg medium (initial pH 6.25). Cultures were grown at $28 \pm 2^\circ\text{C}$ for 4 days. Xylanase assay was performed by the dinitrosalicylic acid method (DNSA) (Miller, 1959). The selection of a substrate is an important step in SSF and mainly depends on the cost and availability of the substrate. Different solid substrates like corncob, wheat bran, sugarcane baggase, wheat straw, red gram bran, rice husks and combinations of these substrates were used for screening xylanase production from *Aspergillus foetidus* MTCC 4898. The water activity, moisture content and particle size of the substrate are the main factors affecting SSF. In the current study Mendels-steinberg medium (pH 6.25) was used to moisten the substrate and the moisture content was maintained at 70.5% at $28 \pm 2^\circ\text{C}$ for 4 days. In the study cellulase activity was determined for each of the substrates tried in order to select the substrate with maximum xylanase activity and minimum cellulase

Fig. 1. Screening of substrate for xylanase production by SSF from *Aspergillus foetidus* MTCC 4898.

activity. Amongst the different solid substrates tested, combinations of corncob and wheat bran (3 : 2) gave the maximum xylanase yield 2,206.6 U/gds (Fig. 1). Best results for xylanase productivity from *Aspergillus foetidus* MTCC 4898 were obtained at 75% moisture content of substrate, pH 5.5, at 27°C with 0.3% (v/v) tween 80 and fructose as an additional carbon source in medium. In the study of inoculum age optimization, the maximum yield of xylanase was found with a 5-day-old slant. For inoculum size, best results were with 2 ml of inoculum (0.91×10^8 spores/ml).

Xylanase activity measurements were performed in culture supernatant. Aliquots of culture fluids were centrifuged ($1,957 \times g$) and supernatants were collected for enzymatic activity studies. Xylanase was assayed using a 1% solution of brick wood xylan as a substrate (Bailey et al., 1992). The release of reducing sugars in 5 min at 50°C was measured as xylose using the dinitrosalicylic acid method (Miller, 1959). The reaction mixture contained 0.5 ml xylan and 0.5 ml of appropriately diluted enzyme in 50 mM sodium citrate buffer, pH 4.5. The reaction was terminated by adding 1 ml of dinitrosalicylic acid. The whole solution was then boiled for 5 min. Absorbance was measured at 540 nm. One unit of xylanase is defined as amount of enzyme that 1 μmol of xylose reducing equivalents produced per minute under the assay conditions. Xylose was used as a standard.

Calculations:

Enzyme activity (IU/ml)=

$$\left(\frac{\text{concentration (mg)} \times 1,000}{150.13 \times 0.5 \text{ ml} \times 5} \right) \times \text{Dilution}$$

To examine the interactions and optimize the concentrations of components of production medium optimized by the one-factor-at-a-time approach, two statistical approaches, orthogonal array (Taguchi's design) and response surface methodology (RSM), were carried out to get the maximum yield of xylanase. Experimental designs and analysis of experimental data were

performed by using two software programs MINITAB 13.30 and Design-Expert Version 6.0.10 (Stat-Ease Inc., Minneapolis, USA). The eight media variables used for the L_{16} -orthogonal array design were ferrous sulphate, calcium chloride, potassium dihydrogen phosphate, fructose, peptone, tween 80, ammonium sulphate, and magnesium sulphate. Each variable was represented at two levels, high and low. The full experimental plan with respect to their coded forms is listed in Table 1. Table 2 shows the response table for signal-to-noise ratio obtained using the L_{16} -orthogonal array. The last two rows in the tables show delta values and ranks for the system. Ranks and delta values help in assessing factors having greatest effect on the response characteristics of interest. Delta measures the

Table 1. Orthogonal project design for two levels of eight factors used for media optimization for xylanase production.

Run	A	B	C	D	E	F	G	H	Activity (U/gds)*
1	1	1	1	1	1	1	1	1	1,922.5±21.2
2	1	1	1	1	1	1	1	2	2,012.3±14.5
3	1	1	1	2	2	2	2	1	1,854.6±19.5
4	1	1	1	2	2	2	2	2	1,975.4±23.2
5	1	2	2	1	1	2	2	1	2,100.4±12.4
6	1	2	2	1	1	2	2	2	1,942.6±24.5
7	1	2	2	2	2	1	1	1	1,854.2±15.2
8	1	2	2	2	2	1	1	2	1,864.2±12.1
9	2	1	2	1	2	1	2	1	1,847.6±21.3
10	2	1	2	1	2	1	2	2	1,975.12±22.1
11	2	1	2	2	1	2	1	1	1,997.65±62.4
12	2	1	2	2	1	2	1	2	2,143.5±25.1
13	2	2	1	1	2	2	1	1	2,106.2±26.8
14	2	2	1	1	2	2	1	2	1,965.42±24.6
15	2	2	1	2	1	1	2	1	2,075.3±24.15
16	2	2	1	2	1	1	2	2	1,954.2±12.5

* Indicates that all above results were taken in triplicate.

A: ferrous sulphate, B: calcium chloride, C: potassium dihydrogen phosphate, D: fructose, E: peptone, F: tween 80, G: ammonium sulphate, H: magnesium sulphate.

size of the effect by taking the difference between the highest and lowest characteristics average for a factor. A highest delta value indicates a greater effect of that component. Rank orders the factors from the greatest effect (on the basis of delta values) to the least effect on the response characteristics. The order in which the individual components selected in the present study affected the production of xylanase by SSF were, ammonium sulphate > potassium dihydrogen phosphate > calcium chloride > fructose > tween 80 > magnesium sulphate > peptone > ferrous sulphate. This indicates that ammonium sulphate has the maximum and ferrous sulphate has the least effect on xylanase production. Figure 2 shows the main effect plots for the system, and how each factor affects the characteristics differently. MINITAB generates the main effect plot by plotting the characteristic average for each factor level. In the present study, calcium chloride, fructose, potassium dihydrogen phosphate and magnesium sulphate showed maximum production at level 1, whereas ferrous sulphate, peptone, tween 80 and ammonium sulphate showed effect at level 2. These levels also represented the optimum concentrations of individual components in the medium. The predicted optimum concentrations were validated and a maximum production of 2,143.5 U/gds was obtained.

Response surface methodology (RSM) was used to optimize fermentation parameters for enhancing xylanase production. RSM has four steps: procedures to move into the optimum region, behavior of the response in the optimum region, estimation of the optimal condition and verification. A central composite design was employed in this study. For statistical calculation, the experimental variables X_i have been coded as x_i according to the following transformation equation: $x_i = (X_i - X_0)/\Delta X_i$, where x_i is the dimensionless coded value of the variable X_i , X_0 is the value of X_i

Table 2. Response table for signal to noise ratios (S/N).

	A	B	C	D	E	F	G	H
Level	(Ferrous sulphate)	(Calcium chloride)	(Potassium dihydrogen phosphate)	(Fructose)	(Peptone)	(Tween 80)	(Ammonium sulphate)	(Magnesium sulphate)
1	70.3888	71.0420	71.0902	70.9111	70.2556	70.2006	69.3323	70.7058
2	70.5571	69.9039	69.8557	70.0348	70.6903	70.7453	71.6136	70.2401
Delta	0.1682	1.1381	1.2344	0.8764	0.4347	0.5446	2.2813	0.4657
Rank	8	3	2	4	7	5	1	6

Fig. 2. Main effect plot for S/N ratios.

at the center point, and ΔX_i is the step change. In this study, the central composite design with three factors and five levels, including six replicates at the center point, was used for fitting a second order response surface. Xylanase production was analyzed by multiple regression through the least squares method to fit the following equation: $Y = A_0 + A_i X_i + A_{ii} X_i^2 + A_{ij} X_i X_j$ where Y is the predicted response variable, A_0 , A_i , A_{ii} , A_{ij} are constant regression coefficients of the model, and X_i , X_j ($i = 1, 3$; $j = 1, 3$; $i \neq j$) represent the independent variables (medium composition) in the form of coded values. The accuracy and general ability of the above polynomial model could be evaluated by the coefficient of determination R^2 . Each experimental design was carried out in triplicate, and the mean values were calculated. Most significant variables obtained from the orthogonal array experiment were selected for optimization at a higher level, in which the various concentrations of the selected media components were optimized and interactions among them were investigated by RSM. The three most significant factors for the xylanase production were $(\text{NH}_4)_2\text{SO}_4$, KH_2PO_4 , and CaCl_2 . The experimental design and results of RSM for studying the effects of four independent variables are presented in Table 3. Based on a regression analysis of the data from the xylanase experiments, the effects of the three independent variables on xylanase production were predicted by a least squares equation:

$$\begin{aligned} \text{Response 1} = & +2,285.27330 - 28.5867 \\ & \times (\text{NH}_4)_2\text{SO}_4 - 13.98537 \times \text{KH}_2\text{PO}_4 + 16.97802 \\ & \times \text{CaCl}_2 + 4.00731 \times (\text{NH}_4)_2\text{SO}_4^2 - 128.28000 \\ & \times \text{KH}_2\text{PO}_4^2 - 159.8788 \times \text{CaCl}_2^2 + 32.23125 \\ & \times (\text{NH}_4)_2\text{SO}_4 \times \text{KH}_2\text{PO}_4 - 117.4337 \times (\text{NH}_4)_2\text{SO}_4 \\ & \times \text{CaCl}_2 - 90.2312 \times \text{KH}_2\text{PO}_4 \times \text{CaCl}_2 \end{aligned}$$

The coefficient of determination (R^2) for xylanase

production, was 1.00. It provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions and is always between 0 and 1. The closer the value of R^2 to 1.00, the stronger the model and the better it predicts the response. R^2 is interpreted as the percent variability in the response explained by the statistical model. It implied that the sample variation of 99.9% for xylanase production could be attributed to the independent variables and only 0.1% of the total variation was not explained by the model. This ensures a satisfactory adjustment of the quadratic model to the experimental data. The Predicted R^2 value of 0.9997 is in reasonable agreement with the Adjusted R^2 value of 0.9999. A ratio greater than 4 is desirable. A ratio of 449.129 indicates an adequate signal. The Model F -value of 28,983.46 implies the model is significant. There is only a 0.01% chance that a Model F -value this large could occur due to noise. Values of Prob $> F$ less than 0.0500 indicate model terms are significant. In this case A, B, C, A^2 , B^2 , C^2 , AB, AC, and BC are significant model terms. The 3D response surface and the 2D contour plots described by the regression model are drawn to illustrate the effects of the independent variables, and interactive effects of each independent variable on the response variable. The shape of the corresponding contour plots indicates whether the mutual interactions between the independent variables are significant or not. Each contour curve represents an infinite number of combinations of two test variables with the other two maintained at their respective zero levels. The elliptical nature of the contour in the 3D-response surface graphs (Fig. 3a, b, and c) depicted the mutual interactions of all the variables. There was a relatively significant interaction between every two variables, and there was a maximum predicted

Table 3. RSM design for optimization of xylanase production.

Run	Ammonium sulphate (mg/100 ml)	Potassium dihydrogen phosphate (mg/100 ml)	Calcium chloride (mg/100 ml)	Actual xylanase* (U/gds)	Predicted xylanase* (U/gds)
1	150	300	150	1,850.2±12.3	1,851.28
2	250	300	150	1,965.98±15.2	1,964.51
3	150	500	150	1,937.55±17.6	2,181.47
4	250	500	150	2,180.72±21.4	2,300.57
5	150	300	250	2,300.74±22.0	1,944.06
6	250	300	250	1,945.25±19.8	2,027.67
7	150	500	250	2,025.63±16.54	1,800.09
8	250	500	250	1,800.6±13.11	2,344.68
9	66	400	200	2,347.21±21.0	2,248.53
10	284	400	200	2,246.82±21.4	1,945.96
11	200	232	66	1,944.65±15.4	1,945.96
12	200	568	200	1,901.05±13.54	1,898.92
13	200	400	66	1,805.5±12.7	1,804.51
14	200	400	284	1,861.45±21.0	1,861.62
15	200	400	200	2,285.25±15.8	2,285.27
16	200	400	200	2,285.25±15.8	2,285.27
17	200	400	200	2,285.25±15.8	2,285.27
18	200	400	200	2,285.25±15.8	2,285.27
19	200	400	200	2,285.25±15.8	2,285.27
20	200	400	200	2,285.25±15.8	2,285.27

* Indicates that all above results were taken in triplicate.

Fig. 3. 3D-response surface plots for xylanase production.

(a) Effect of interaction of calcium chloride and potassium dihydrogen phosphate when other variable is held at zero level. (b) Effect of interaction of calcium chloride and ammonium sulphate when other variable is held at zero level. (c) Effect of interaction of potassium dihydrogen phosphate and ammonium sulphate when other variable is held at zero level.

yield as indicated by the surface confined in the smallest ellipse in the contour diagrams. The model predicted maximum xylanase production up to 2,351.5 U/gds and could be achieved using the medium (mg/100 ml), ammonium sulphate 250, KH_2PO_4 185.5 and calcium chloride 216.5. It shows that ammonium sulphate has inhibitory effects on xylanase production by

increasing its concentration, whereas calcium chloride and KH_2PO_4 gave maximum interactions with each other and other media components on increasing their concentrations. Hence it can be concluded that calcium chloride and KH_2PO_4 are the key nutrients for xylanase production.

There is a growing acceptance of the use of statisti-

cal experimental designs in biotechnology. A statistical optimization method for the fermentation process could overcome the limitations of classic empirical methods and it has been proved to be a powerful tool for the optimization of xylanase production by *Aspergillus foetidus* MTCC 4898. Taguchi Orthogonal array gives the most significant factors of the media while RSM gives the optimum concentration of these significant factors; thus Taguchi L_{16} followed up by RSM was used for media optimization. According to Taguchi L_{16} the order of most significant factors affecting xylanase production were found to be ammonium sulphate > KH_2PO_4 > calcium chloride. Statistical methods of media optimization increased xylanase yield from 1,800 U/gds to 2,350 U/gds. Validation experiments were also carried out to verify the availability and the accuracy of the models, and the results showed that the predicted values agreed with the experimental values.

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