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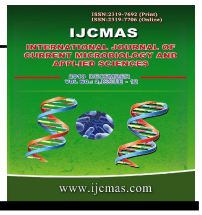


Original Research Article

Selection of Co-cultures for Production of Cellulase-free Xylanase using Agro-waste and Optimization of Cultural conditions by Response surface methodology and Box-Behnken design

B.Ramesh and O.V.S.Reddy*

Dept of Biochemistry, Sri Venkateswara University, Tirupati - 517 502, India
Corresponding author

K e y w o r d s

Box-Behnken design;
Co-cultures;
Optimization;
Xylanase;
Corn cob and Pineapple peel.

A B S T R A C T

Optimum cultivation conditions for production of cellulase-free xylanase, on corn cob with pineapple peel by selected co-cultures of *Trichoderma koeningi* and *Sclerotium rolfsii* was carried out by response surface methodology and Box-Behnekan design. The experiments established the optimum cultivation conditions of moisture (6 mL/g) of the substrate and incubation temperature of 35°C for solid state fermentation that led to the maximum production of cellulase-free xylanase at a level of 56.6 U/gds by the co-cultures. Good correlation was observed between the actual and predicted results indicated that the present model was applicable to production of xylanase. The crude xylanase successfully hydrolyzed the Birch-wood xylan that resulted in the release of sugar components including xylose.

Introduction

Solid state fermentation (SSF) offers numerous opportunities in processing of agro-industrial residues. This is partly because of lower energy requirements during SSF processes, and in turn they produce lesser waste water and are eco-friendly in nature as they resolve the problem of solid waste disposal (Muthuvelayudham and Viruthagiri, 2005). A comparison between the SSF and submerged fermentation (SmF), explains higher enzyme titers in the former than in the latter, when we use same fungal strain and fermentation broth (Viniegra-González *et al.,* 2003). Large amounts of agro - industria residues are generated every year from diverse economic activities. In the planet earth most of the freely available energy rich resources are agricultural residues, and if not used or discharged properly, they cause the environmental pollution (Frazier *et al*., 2008). Xylan, the main component of hemicellulose consists of a β-1,4-linked D-xylosyl residue backbone branched with other pentoses, hexoses and uronic acids (Gilbert *et al.,* 1993; Lynd *et al.,* 2002). From the business point of view, important group of carbohydrolases are xylanases, and have a worldwide market of around 200 million dollars. Xylanases have potential industrial applications including

biofuel production from hydrolysis of lignocelluloses to fermentable sugars, making of bread and clarification of juice and beer due to these commercial reasons (Rao *et al.,* 2002). Xylanases are also essential to improving the nutritional quality of animal feed (Gilbert *et al.,* 1993). Thermostable cellulase-free alkaline xylanases can be used in the prebleaching of kraft pulps, in order to replace up to 20–30% of the chlorine required to achieve a target pulp brightness (Viikari *et al.,* 1993) and consequently reduce by up to 50% the chloroorganics that are known to form toxic dioxins in the effluent. Many microorganisms including bacteria, yeast, actinomycetes and filamentous fungi produce xylanase (Eriksson *et al*., 1980). Filamentous fungi such as *Aspergillus spp.* and *Trichoderma spp*. are of particular interest, because they can excrete higher levels of xylanase than yeast and bacteria (Subramaniyan *et al.,* 2002). As an agricultural by-product, corncob is an ideal raw material for producing xylo-oligosaccharides (XOS) because its hemi-cellulose content is the highest among all of the agricultural byproducts (Yang *et al.,* 2004). Xylan (28%) and xylose (23%) is a rich source of structural component in the corncobs, exists in the form of xylan–lignin complex molecules. It has been shown that xylan is connected with lignin by covalent bonds (Dekker *et al*., 1976; Francis *et al.,* 2003) and might be connected with other polysaccharides such as pectin by certain chemical bonds (Dekker *et al.,* 1976; Sarra *et al.,* 1993).

Statistical interpretation techniques can be used to check the importance of individual factors, the aptness of this operative form and acuteness of the response to each factor (Mandels *et al.,* 1969). The classical method of 'one-variable-at-a-time' bioprocess design may be adequate in some directions, but if the combined effect of all involved factors is needed, it fails to consider (Selvendran *et al.,* 1985). Recently the bioprocess optimization was done in many statistical experimental design methods. Among them, RSM is one of the easy and suitable methods for identifying the effect of individual variables and for searching the optimum conditions for a multivariable system without difficulty. Factorial design optimization and response surface methodology fulfill this requirement. RSM is a group of statistical and mathematical techniques generally used to determine the effects of different variables and to optimize several biotechnological processes (Pandey *et al.,* 1999). This method has been successfully applied to optimize (time, pH, wheat bran) xylanase production (Cao *et al.,* 2008).

The aim of this work was to select co-cultures and optimize conditions for higher production of xylanase under SSF using statistical methods. The optimum parameters including moisture, time, temperature and inoculum as cultural variables in the medium were obtained by RSM. Probably this is the first report on co-culture fermentation and their optimization for production of xylanase.

Materials and Methods

Preparation of substrate

Corn cob and pineapple peel were collected from agro-fields and fruit market in around Tirupati (India) and processed to different particle sizes of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mm and washed with distilled water twice and dried at 70°C for 8 h in the oven. Processed substrates were collected in the polythene covers and stored at room temperature.

Microorganisms

Trichoderma koeningi and *Sclerotium rolfsii* were isolated from the sorghum dumping waste on potato dextrose medium (PDA). The isolates were identified on the basis of cultural characteristics and morphological features (Frazier *et al.*, 2008).

Screening for mutualism between the fungal strains

Isolated twenty fungal strains are used to test the mutualism between the fungal growth in Petri plates. Two types of common fungal culture media like Malt extract agar and PDA were used. Sterile Petri dishes were prepared with 15 mL of culture medium, and kept in a sterile laminar flow chamber under UV light until it is solidified. Whatmann filter paper discs having the inoculum from the initial cultures were transferred to Petri dishes and incubated in the B.O.D incubator for 7 days. Middle straight line was drawn on the bottom of each Petri dish in order to examine the crossing point of cultures that coincide with each other.

Inoculum preparation

After completion of screening, the selected co-cultures were employed for xylanase production. For the preparation of inoculum, the fungi were grown on broth containing (g L^{-1}): Glucose, 10.0; KH_2PO_4, 2.0; $MgSo_4.7H_20$, 0.5; $CaCl_2$, 0.2; NH_4Cl, 0.10 and thiamine, 0.001. After inoculation with cultures, incubation was carried out at room temperature on a rotary shaker (120 rpm) for 4 days to get a fungal unit (pellets) suspension.

Xylanase production medium and SSF

Erlenmeyer conical flasks (250 mL) containing corn cob with pineapple peel to act as a carbon source (5 g) and basal medium (15 mL) of following composition (g/L): KH_2PO_4, 1.6; $(NH_4)_2SO_4$, 1.4; $CaCl_2 \cdot 2H_2O$, 0.4; $MgSO_4 \cdot 7H_2O$, 0.6; urea, 0.2; Proteose peptone, 0.25; yeast extract, 0.2; and trace metal solution, 1 mL [$FeSO_4 \cdot 7H_2O$, 3; $MnSO_4 \cdot 7H_2O$, 4.6 ; $ZnSO_4 \cdot 7H_2O$, 3.34; $CoCl_2 \cdot 2H_2O$, 1.5 mg/ L], Tween 80, 1 mL and p H adjusted to 5.6, were sterilized at 121ºC for 20 min, 1.25 mL of each of conidial suspension was transferred to sterilized flask. The moisture content (60-70%) in each flask was maintained and incubated at room temperature for 7 days. Thereafter, the enzyme was extracted by adding 100 mL of 50 mM citrate buffer pH 5.0 under shaking at 150 rpm on a rotary shaker and at 30°C for 30 min., for three times. The resultant slurry was filtered through a muslin cloth and centrifuged at 10,000 × g for 10 min, and the supernatant was stored at 4ºC as crude enzyme solution for further analysis.

Enzyme assays

X**y**lanase and cellulase activities were determined spectrophotometrically according to the procedures described by Mandels and Weber 1969; Tan *et al.*, 1987, respectively, at 50°C. Reducing sugars estimation was carried out by using 3, 5 dinitrosalicylic acid method (Miller 1959). One unit is defined as that amount of enzyme which liberates 1 µmole equivalent of glucose or xylose, as the case may be, per min. Enzyme activity was expressed as units per gram of the dry weight of substrate (gds).

Xylan degradation by co-culture xylanases

Xylan from Birch wood was used as a source substrate for confirmation of xylan

degradation process. Reaction mixture in the Eppendorf tubes contained as follows: sample of the xylan 10 mg per ml in 25 mM Tris-HCl buffer, pH 7.0 and crude xylanase sample 0.4 U/mL. Control tubes were prepared with xylan without crude xylanase and incubated at 70°C in a thermostatic water bath for 3 h. The hydrolysis reaction was terminated by cooling the reaction mixture on ice followed by centrifugation at 10000 rpm for 15 min at 4°C and the supernatants were qualitatively analyzed.

High - performance liquid chromatography (HPLC)

A HPLC system (LC-10A HPLC Series, Shimadzu) Equipped with a pump system, a refractive index detector (RID-10A) for sugar analysis, and a UV/Vis detector (SPD-20A) monitored at 210 nm, was used for the analysis of sugars simultaneously on an Aminex HPX-87H column (300×7.8 mm) (Bio-Rad) kept at 55°C. The analytical conditions used were as follows: flow 0.3 mL min^{-1}, eluent 0.045N H_2SO_4 with 6% acetonitrile (v/v). Xylose, xylobiose, xylotriose, xylotetratose, xylopentaose and xylohexaose were eluted their gradient and their retention times were compared with the standards.

Experimental design

The experimental design and statistical analysis were performed on Box-Behnken design with quadratic model was employed to study the combined effect of four independent variables namely Moisture (X_1, ml/L), Time (X_2, h), Temperature (X_3, °C) and Inoculum (X_4, %) for the dependent variable such as xylanase (U/gds) production using response surface analysis method using Design-expert 8.0.7.1 (Stat-Ease, USA) trial version software. The total number of experimental combinations is 2h+2h+n0, where h is the number of independent variables and n0 is the number of repetitions of the experiments at the centre point. This dependent variable was expressed individually as a function of the independent variables known as response function. Independent process parameters in quantitative form are represented by RSM (Box and Behnken 1960). Table 1 shows the actual levels correlative to the coded settings and the experimental design appropriately. This design is interpreted by a second-order polynomial regression model as follows:

$$Y = \beta_0 + \Sigma \beta_i X_i \Sigma \beta_{ii} X_i^2 + \Sigma \beta_{ij} X_i X_j \quad (1)$$

Where Y is the predicted response, β_0 is the response function; X_i and X_j are experimental coded variables, (Table 3, 4), respectively. These coded variables are related to uncoded variables using the following relationship equation.

$$X_i = \frac{2(a_i - b_i)}{\delta_i} \quad (2)$$

Where a_i is the variable value in actual units of the *i*th observations, b_i is the mean of highest and the lowest variables, value of a_i and d_i is the difference between the highest and lowest variable value of a_i. The variable based on the above relationship Eq. (2) is given in Table 1. A regression model containing 4 linear (X_i), 4 quadratic (X_i^2), 6 interaction (X_iX_j) and β_0 block term used. The overall second order polynomial mathematical relationship of the response Y_1 and four variables, i.e. Moistur, Time, Temperature and Inoculum can be approximated by the quadratic Eq. (3).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta$$

$$_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{44}X_4^2 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{15}X_1X_5 + \beta_{23}X_2X_3 + \beta_{24}X_2X_4 \quad (3)$$

Where Y is the predicted response variable, X_1, X_2, X_3 and X_4 are independent variables, b_0 is the offset term, b_1, b_2, b_3, b_4 and b_5 are linear effects, b_{11}, b_{22}, b_{33} and b_{44} are squared effects, and b_{12}, b_{13}, b_{14}, b_{15}, b_{23} and b_{24} are interaction terms. The significance of all terms in the polynomial functions were assessed statistically using F-value at a probability (*P*) of 0.001, 0.01 or 0.05. The regression coefficients were then used to generate response surface graphs from the regression models. The response surface graphs were generated by keeping one variable constant at the center point and varying the other variables within the experimental range.

The experimental design for the variables, i.e. Moisture (2-10 mL/L), Time (48-192 h), Temperature (20-50°C) and Inoculum (2-6%) were taken for optimization and for measuring the enzymatic activities. The design was applied for selection range of each variable (minimum and maximum) as shown in Table 2. Total 29 experiments were designed by the model and performed (Table 3) in triplicate. The obtained results in the performed assays, executed in terms of xylanase production on corncob with pineapple peel have been recorded.

Result and Discussion

Screening of mutualism in fungal strains

Among the twenty fungal isolates screened, only four strains (*Trichoderma koeningi* and *Sclerotium rolfsii; Aspergillus foetidus* and *Fusarium oxysporum*) were to grow effectively on the PDA than the MEA plates. Screened fungal strains were grown in the plates sympathetically did not overlap or suppress the co-strains indicating that these strains are having capacity to grow in mixed cultures. The Table 1 shows that certain monocultures and their combinations could produce higher amounts of cellulose-free xylanase than the other co-cultures and monocultures. Guiterrez-Correa *et al*., (1999) used *Trichoderma reesei* and *Aspergillus niger* as mixed cultures for production of cellulases.

Optimization of cultural variables for the enhanced production of xylanase by co-culture is designed to provide in-depth information about a few variables identified, during the screening as having the greatest impact on performance of statistical methods. Bocchini *et al.,* (2002) have stated that xylanase production from *Bacillus circulans* D1, independent variables of cultivation time, xylan concentration and their interactions have significant effects. Table 2 shows the maximum and minimum levels of independent variables chosen for trials in Box-Behnken design. RSM based on the Box-Wilson, which was used to optimize cultivation conditions for xylanase production, 29 experimental runs with different combinations of four factors and four levels were carried out (Table 3). The variables such as moisture, time, temperature and inoculum that influence xylanase production in co-culture fermentation were investigated using RSM. Table 2 and 3 represent the summarized results. The effect of each factor and their interaction were analyzed using the analysis of variance (ANOVA) and X^2 test as appropriate to the experimental design being used. The calculated regression equation for the optimization of co-culture cultivation conditions for the production of xylanase

(Y_1, U/gds) is a function of the Moisture (X_1, mL/L), Time (X_2, h), Temperature (X_3, °C) and Inoculum (X_4, %). By applying multiple regression analysis on the experimental data, the following second order polynomial equation was found to explain the xylanase production shown as below:

Co-culture xylanase (U/gds)

$Y_{1=}$ 37.50 + $1.65X_1$ -$1.61X_2$ + $4.39X_3$ + $3.55X_4$ -$3.63X_1^2$ -$3.27X_2^2$ +$0.75X_3^2$– $1.58X_4^2$+$15.37X_1X_2$-$7.85X_1X_3$-$5.87X_1X_4$-$1597X_1X_5$-$10.63X_2X_3$+$1X_2X_.$ (4).

The predicted levels of xylanase production in corn cob with pineapple peel substrate using the above equations are given in Table 4 along with experimental data. Several indicators were used to evaluate the adequacy of the fitted model. The R^2 value for the response variables was higher than 0.90, indicating that regression model explained the reaction well. The quadratic regression model illustrate that Equation 4 are highly significant statistical models for co-culture xylanase production responses in the corn cob with pineapple, as it was evident from the Fisher's F-test with a very low probability value [(P model>F) =0.0001].

Co-culture xylanase production

The economics of a biocatalytic process clearly indicates that the cost of enzymes is one of the main factors that determine the feasibility of the process, and it can be reduced by finding optimum conditions for their maximum production (Kulkarni *et al.,* 1999). In order to minimize the enzymes production cost, perceivable progress has been made in strain development, optimization of culture conditions, mode of cultivation (Miller *et al.,* 1959; Muthuvelayudham *et al.,* 2003) and archetypal the fermentation process. From the regression model (Y_1) of xylanase production, the value of determination of coefficient (R^2=0.9203) indicates that only 8.97% of the total variations were not explained by the model. Haaland (1989) tells generally a regression model having an R^2 value higher than 0.9 is considered to have a very high correlation. Among the model terms X_1 and X_3 were significant with a probability 99% and X_4 is significant with a probability of 95% (Table 3). In the interaction also X_1 and X_3 had significant influence on xylanase production on corncob with pineapple peel.

Xylan degrading products

Identification of released degradation products from xylan by xylanase was obtained by the HPLC (Figure 1). Xylose and other six sugars were detected in the reaction mixture treated by crude xylanase. Xylotriose was the main sugar in the mixture from Birchwood xylan. Figure 1 clearly indicates that crude xylanase having the capacity to degrade the xylan substance. In the control reaction mixture tubes, xylan degradation products were not obtained as the peaks of the degradation products did not appear in the HPLC chromatogram.

Optimization

The response surface plots showed the effect of moisture, time, temperature and inoculum on xylanase in corncob with pineapple peel substrate. The results represent that xylanase production reached

Table.1 Selection of co-cultures for production of xylanase on corncob and pineapple waste

Organism	**Xylanase (U/gds)**	**Cellulase (U/gds)**
Aspergillus foetidus (A)	23.8	ND
Trichoderma koeningi (B)	27.3	ND
Fusarium oxysporium (C)	13.4	ND
Sclerotium rolfsii (D)	24.9	ND
A+B	19.2	ND
A+C	39.5	ND
A+D	28.2	ND
B+C	16.7	ND
B+D	**56.6**	**ND**
C+D	25.4	ND

ND=Not detected

Table.2 Coded and actual values of the factors in Box-Behnken design

Factor	**Name**	**Units**	**Minimum**	**Maximum**	**Low**	**High**	**Mean**	**Std. Dev.**
A	Moisture	ml/L	2	10	–1	1	6	2.57
B	Time	hr	48	192	–1	1	120	46.32
C	Temperature	°C	20	50	–1	1	35	9.65
D	Inoculum	%	2	6	–1	1	8	1.29
Response	Name	Units	Obs*	Minimum	Maximum	Mean	Std. Dev.	Ratio
Y_1	Xylanase	U/gds	29	12.7	56.6	34.3	12.88	4.45

Obs* = Observed run values

its maximum point in the moisture and temperature with time optima. Response surface models have the capability to indicating the direction in which to change the optimum cultural variables in order to maximize the xylanase production in co-cultures. For xylanase, comparison of the predicted values and actual values clearly indicated that these data are in reasonable agreement (Figure 2). The response surface curves are plotted to explain the interaction of the variables and to determine the optimum level of each variable for maximum response. The resulting response surface plots show (Figure 3, 4 and 5) the interactive effect of moisture with time, temperature and inoculum that influence the xylanase production.

Figure 6 and 7 show the interactive effect of time with temperature and inoculum. Figure 8 shows the interactive effect of temperature and inoculum. The highest xylanase production was achieved at moisture level 6 mL/g of the substrate and incubation temperature of 35°C, is presented in Figure 3 which shows that there is significant interaction between these two factors and high xylanase activity occurs when these two factor are at their highest optimum levels. The minimum production of xylanase obtained under the optimization of cultural

Table.3 Box-Behnken design used to optimize the medium consequents for xylanase production

Std	A	B	C	D	Xylanase (U/gds)
1	-1	-1	0	0	48.7 (45.9)
2	1	-1	0	0	22.5 (18.48)
3	-1	1	0	0	14.3 (11.9)
4	1	1	0	0	49.6 (46.0)
5	0	0	-1	-1	33.4 (29.7)
6	0	0	1	-1	41.3 (36.5)
7	0	0	-1	1	36.4 (45.6)
8	0	0	1	1	48.3 (45.6)
9	-1	0	0	-1	15.3 (11.2)
10	1	0	0	-1	39.6 (46.2)
11	-1	0	0	1	53.8 (50.0)
12	1	0	0	1	14.6 (21.6)
13	0	-1	-1	0	12.7 (16.2)
14	0	1	-1	0	42.4 (44.9)
15	1	0	1	0	56.6(56.9)
16	0	1	1	0	22.4 (21.7)
17	-1	0	-1	0	16.4 (20.7)
18	1	0	-1	0	44.9 (39.7)
19	-1	0	1	0	36.6 (45.2)
20	1	0	1	0	33.7 (32.8)
21	0	-1	0	0	17.4 (20.0)
22	0	1	0	-1	34.9 (38.1)
23	0	-1	0	1	48.2(48.4)
24	0	1	0	1	23.2(23.9)
25[a]	0	0	0	0	37.5 (37.5)
26[a]	0	0	0	0	37.5 (37.5)
27[a]	0	0	0	0	37.5 (37.5)
28[a]	0	0	0	0	37.5 (37.5)
29[a]	0	0	0	0	37.5 (37.5)

Std: Standard run order; [a] = Central value.

Table.4 Analysis of variance for xylanase (Partial sum of squares).

Source	DF	Mean Square	F Value	p-value
		Y_1	Y_1	Y_1
Model	14	305.78	11.55	<0.0001*
A-Moisture(ml/L)	1	32.67	1.23	<0.0001*
B-Time (h)	1	31.04	1.17	0.2971#
C-Temperature (°C)	1	231.44	8.75	<0.0001*
D-Inoculum (%)	1	151.23	5.71	0.0314#
AB	1	945.56	35.73	<0.0001*
AC	1	246.49	9.31	0.0086#
AD	1	1008.06	38.09	<0.0001*
BC	1	1020.80	38.57	<0.0001*
BD	1	451.56	17.06	0.0010#
CD	1	4	0.15	0.7033#
A^2	1	85.63	3.24	0.0936#
B^2	1	69.39	2.62	0.1277#
C^2	1	3.69	0.14	0.7145#
D^2	1	16.26	0.61	0.4462#
Residual	14	26.46		
Lack of Fit	10	37.05		
Pure Error	4	0		
Cor Total	28			

Y_1 = Xylanase, * $p< 0.05$-Significant at 5% level, # $p< 0.001$- Significant at 1% level

conditions of moisture (6 mL/g), time (48 h), temperature (20°C) and inoculum (4%) was 12.7 (U/gds). The result from three replications was coincident with the predicted value and the model was proven adequate.The maximum response prediction of xylanase from the model was 56.6 (U/gds), and the experimental results clearly indicated the increase in xylanase production by 3.6 fold.

The xylanase production by microorganisms is strongly influenced by many factors, such as carbon source, nitrogen source, growth factors, inorganic salts and cultivation conditions. Therefore, it is crucial to search for the key influencing factors from the many related ones. To perform such a task, it is extremely difficult and time-consuming. The current method such as RSM has been employed in the present study for searching desirable responses needed for optimum conditions of factors, designing an experiment, evaluating the effects of factors and building models.

Figure.1 HPLC chromatogram for xylan degradation by xylanase at 75^{0}C by xylanase from coculture. t=8.0, xylose; t=4.8, xylotetrose; t=4.5, xylobiose; t=3.9, xylotriose; t=4.4, xylohexose; t10.2, xylopentose; t=12.6, maltotriose

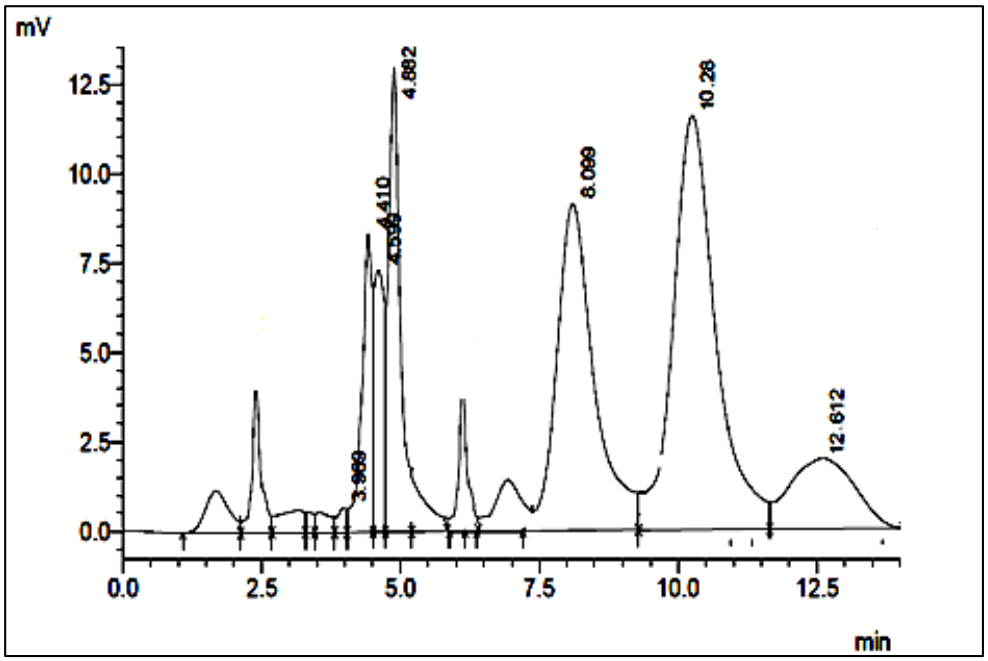


Figure.2 Predicted vs. actual observation run values for co-culture xylanase production

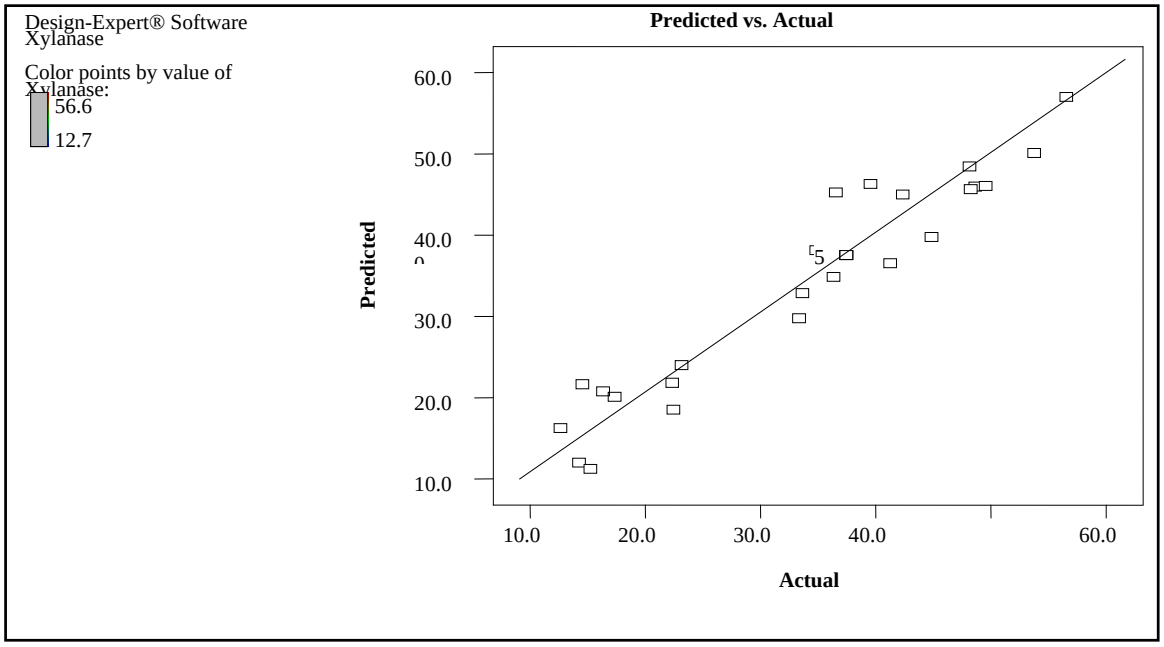


Figure.3 Response surface plot shows the interactive effect of moisture and time on co-culture xylanase production

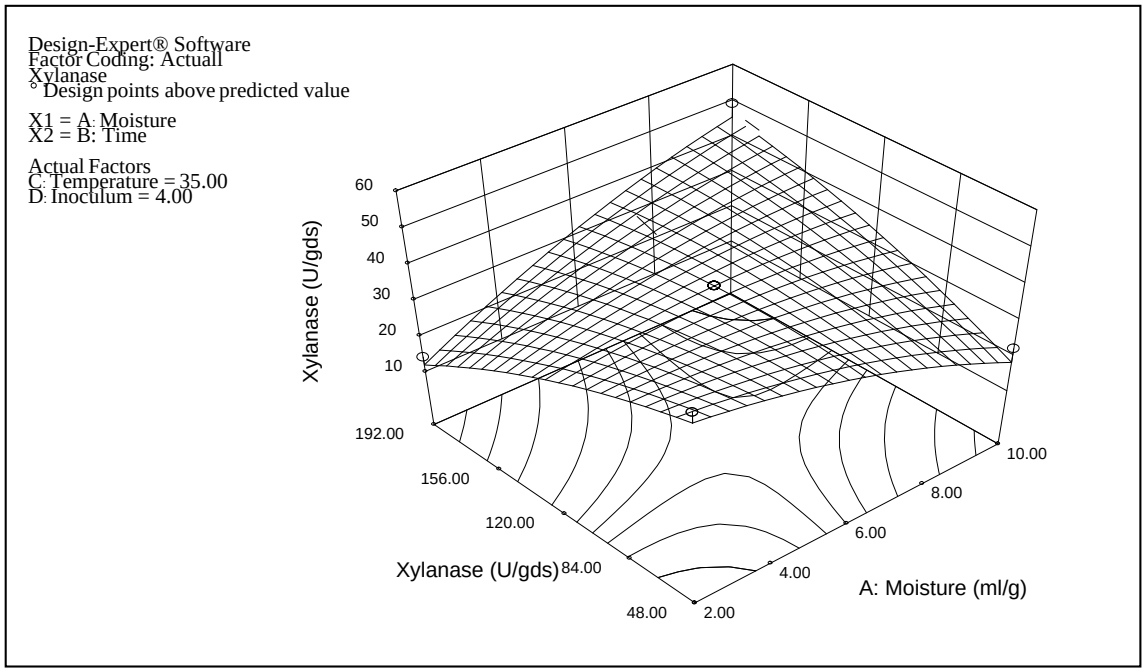


Figure.4 Response surface plot shows the interactive effect of moisture and temperature on co-culture xylanase production

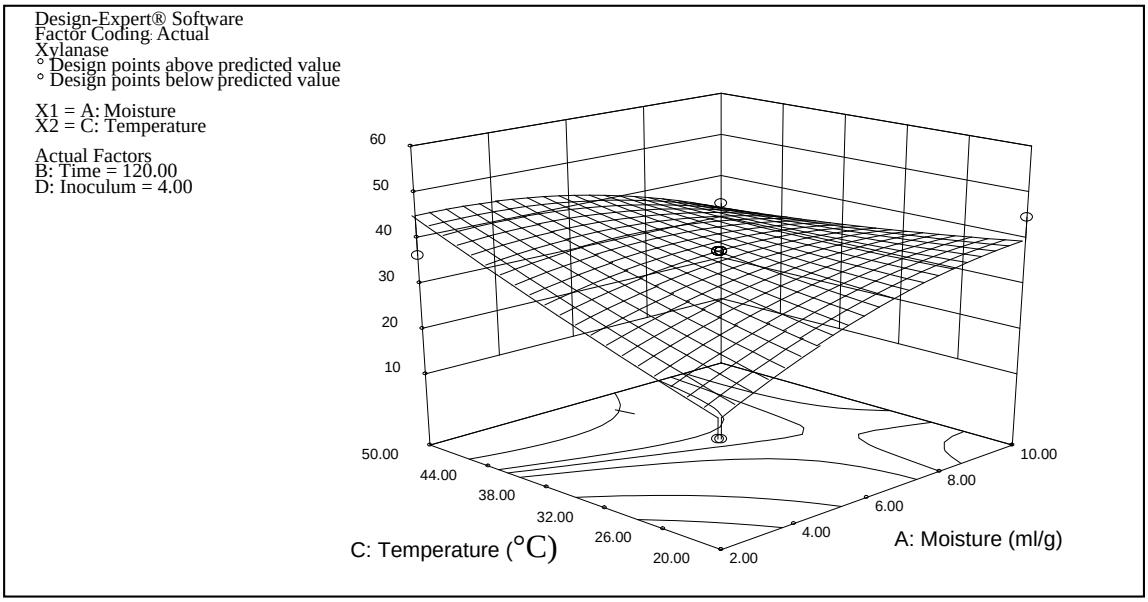


Figure.5 Response surface plot shows the interactive effect of moisture and inoculum on co-culture xylanase production

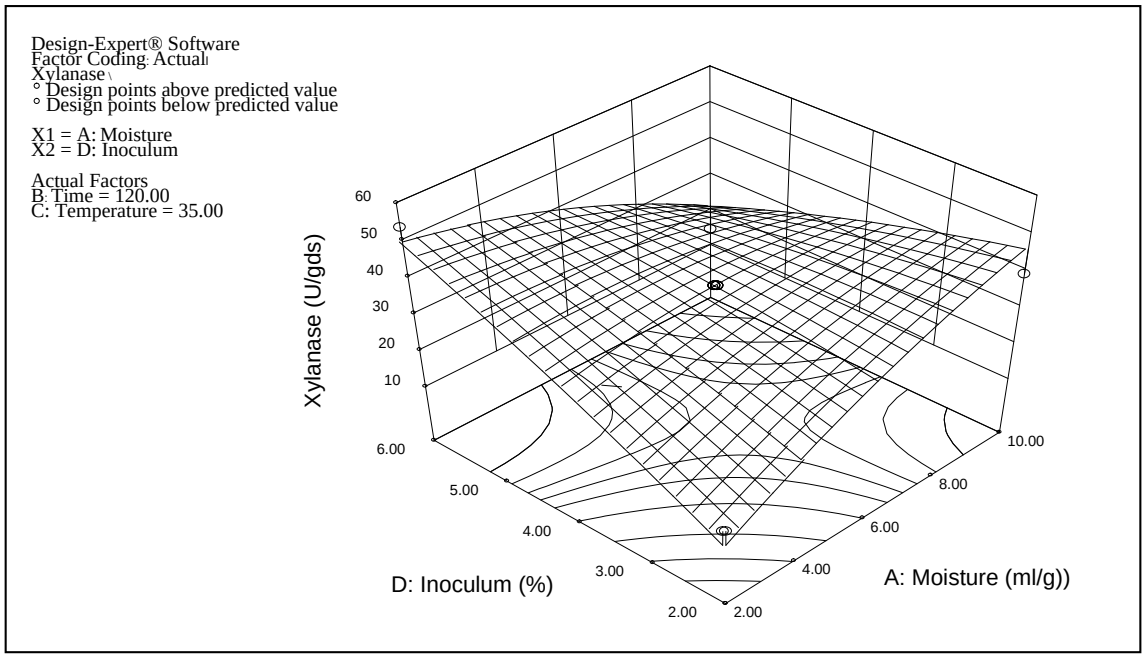


Figure.6 Response surface plot shows the interactive effect of time and temperature on co-culture xylanase production

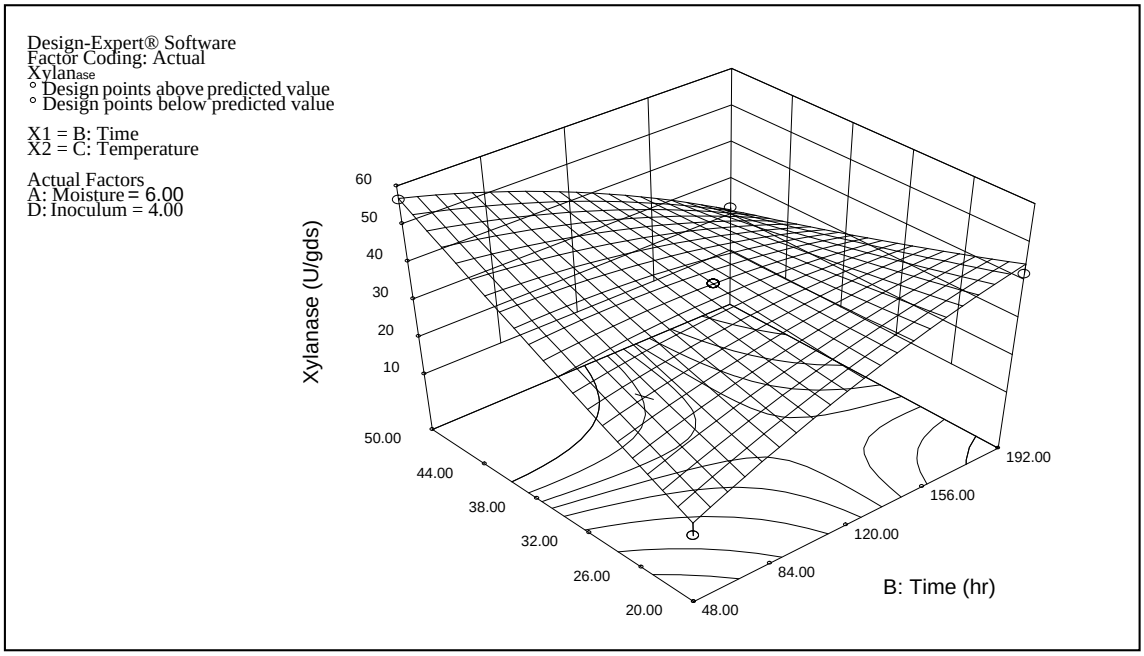


Figure.7 Response surface plot shows the interactive effect of time and inoculum on co-culture xylanase production

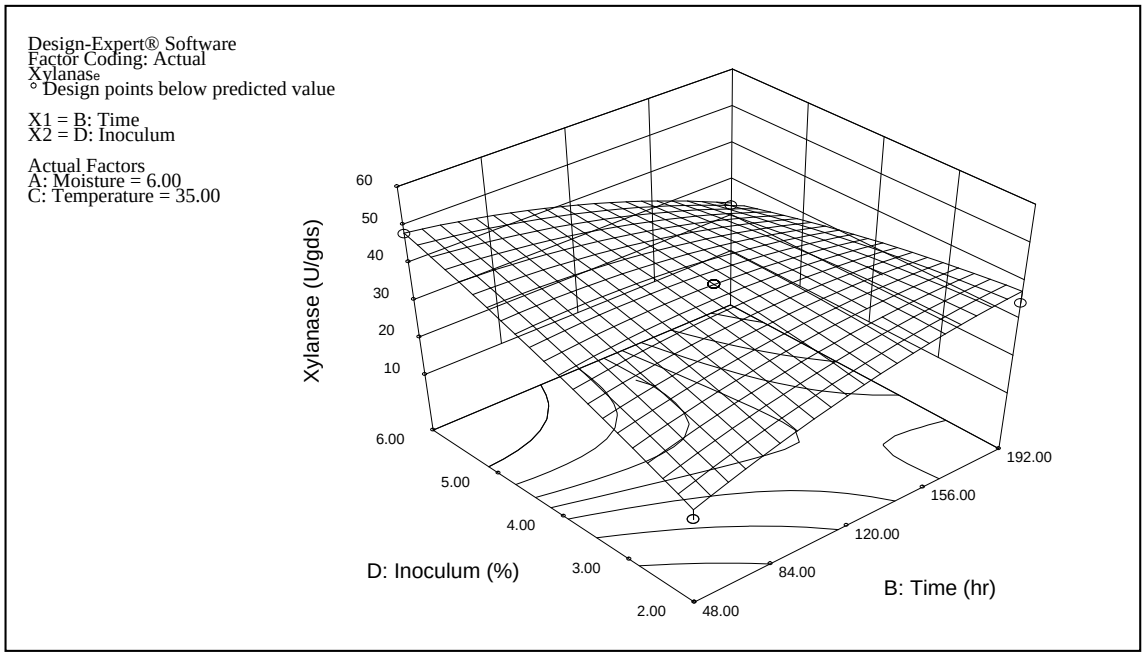
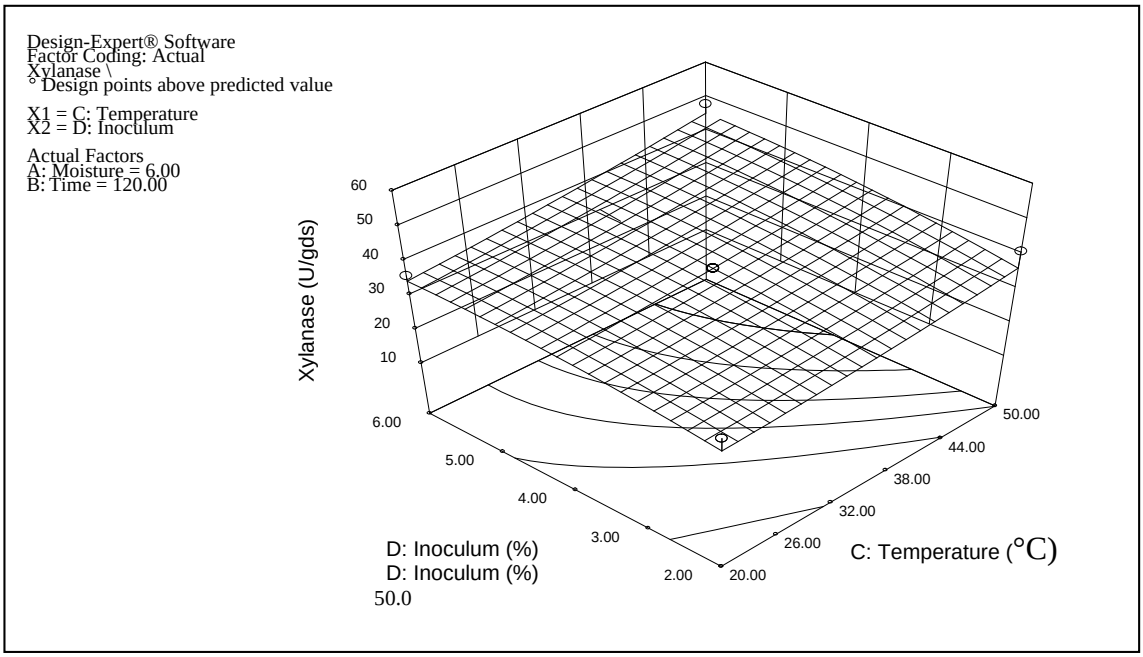


Figure.8 Response surface plot shows the interactive effect of temperature and inoculum on co-culture xylanase production



Experiments were carried out to optimize the effects of various cultural condition variables such as moisture, time, temperature and inoculum for xylanase production by Box-cox design. RSM is considered as a useful method for analyzing several independent parameters on response to expected dependent variables because it integrates mathematical and statistical techniques (Box and Benhnken, 1960). Xylanases are produced by excessive number of fungi and bacteria (Kambourova *et al.,* 2003). Many microorganisms are known to produce different types of xylanases; the nature of the enzymes varies depending upon the genetic make-up of the organisms. Among them, the enzymes from fungi such as the *Trichoderma* spp. deserve the most attention. *Trichoderma* spp. including *T. reesei, T. harzianum, T. viride,* and *T. koeningi are* well known as excellent producers of both cellulolytic and xylanolytic enzymes (Wong *et al.,* 1992). The benefit of co-culture is more evident in SSF conditions because the substrate may be utilized better in symbiotic association, as each culture may have the specificity to grow and degrade the substrate. In the present study, corncob with pineapple medium used for the growth of co-culture, *T. koeningi* and *S. rolfsii* for xylanase production with optimized culture conditions. Efficient and complete hydrolysis of xylan requires the synergistic action of the main- and side-chain-cleaving enzymes with different specificities (Das *et al.,* 1984).

For the co-fermentation of producing xylanase, treated with xylan substrate and degradation products were XOS and xylose. Optimization of media and process conditions are the most important factors to reduce the production cost. In preliminary study, optimization of xylanase production was done using conventional method, which involved varying one variable at a time while keeping the other variables constant. This method is lengthy and often does not produce the effect of interaction of different variables. To overcome this difficulty, RSM was used to optimize the media composition and few process variables (Selvendran *et al.,* 1985; Bocchini *et al.,* 2002; Techapun *et al.,* 2002).

The highest xylanase production in corn cob with pineapple (56.6 U/gds) was observed when the cultural conditions of moisture (6 mL/g) of the substrate and temperature (35°C) of incubation were optimized. Among the four factors, only two factors (moisture and temperature) were goodness of correlation, inoculum is 95% and time was 78% goodness fit of model. Investigations carried out by Subramaniyan *et al.,* (2002) show that initial moisture content of bagasse mass was the most important factor affecting xylanase production under the optimized conditions (81% moisture content and 17 g bagasse), which yielded 2700 U/gds, whereas its value predicted by a polynomial model was 2400 U/gds (Selvendran *et al.,* 1985). Experimental design techniques are very useful tools for this purpose, as they can provide statistical models which help in understanding the interactions among the nutrients at varying concentrations and in calculating the optimal concentration of each nutrient for a given maximal enzyme production (Royer *et al.,* 1989). In the employment of mixed cultures, SSF offers an option that cannot be achieved by SmF. This is due to the fact that during growth, fungal consortia secrete a broad spectrum of enzymes (Yang *et al.,* 2004; Holker *et al.,* 2005). During co-cultivation of different

fungi, some of the individual enzyme activities show synergistic increase whereas others remained unchanged (Guiterrez-Correa *et al.*, 1999). In the present studies, it was clearly indicated that co-cultures produce xylanase on different cultivation conditions, each one having its own specificity to cultivation conditions. Statistical analysis of the experimental data showed that all four independent variables significantly influenced the xylanase production from co-culture on the corn cob with pineapple peel.

Conclusion

This study clearly indicated that co-culture of *Trichoderma koeningi* and *Sclerotium rolfsii* produced high amount of xylanase compared to other co-cultures using cheaply available corncob and pineapple waste. RSM is adopted for optimization of cultivation conditions to increase the xylanase production to a yield of 56.6 U/gds. The profile of xylan degradation products indicated that co-culture crude xylanase is capable of degrading Birchwood xylan to various sugars, and has the prospects of use in various fermentation industries.

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