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Application of Statistical Design to Optimize Culture Medium for Xylanase Production by *Bacillus pumilus* AB-1

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ABSTRACT

The objective of the study was to optimize the culture medium and conditions for the production of xylanase from Bacillus pumilis AB-1. Xylanase production was found to increase when wheat bran is used as a lignocellulosic substrate at pH 5.0 and at 37°C temperature when moisture ratio was kept 1:01. Further optimization was carried out using Plackett-Burman design and Central-Composite Design. The Plackett-Burman multifactorial design was first employed to screen the important nutrient sources in the medium for xylanase production by Bacillus pumilis AB-1 and subsequent use of the response surface methodology (RSM) was further optimized for xylanase production by Central Composite Design. Wheat bran, pH and Temperature showed greater value of the F-ratio then the F-table (F-ratio > 4.21001) and exerted positive influence on the Xylanase production. The Central Composite Design predicts that the xylanase production is located at the actual values of 1.86 gm% of wheat bran at pH 6.21. After the statistical optimization 5.58 fold increase in the xylanase production was observed over unoptimized medium.

Keywords: *Bacillus pumilis AB-1; xylanase; Plackett-Burman design; Central Composite Design, lignocellulosic substrate.*

INTRODUCTION

Xylans are hemicellulose and the second most abundant polysaccharide[1]. These compouds are present in the cell wall and the middle lamella of plant cells. Classes of hemicellulose are named according to the main sugar unit. Thus, when a polymer is hydrolysed and yields xylose, it is xylan;in the same way hemicellulose includes mannas, glucans, arabinas and galactans[2,3].

D-xylans are the most abundant noncellulosic polysaccharides in hardwood and annual plants, where they account for 20-35% of the total dry weight[4]. In softwoods they are found in lesser quantities, comprinsing approximately 8% of the dry weight. The basic structure of xylan is linear polymer of β-D-xylopyranosyl units linked by (1-4) glycosidic bonds.

Due to the structural heterogeneity of the xylans, xylan degrading enzyme systems include saveral hydrolytic enzymes. The best know of these are endo-β-1,4 xylanase which attack the main chain of xylans and β-xylosidase which hydrolyze xylooligosaccharides to D-xylose in addition to these enzymes several accessory enzyme activities are necessary for disbranching the substituted xylans[5].

Endo-1, 4-β-xylanase (Endo-β-1, 4-xylan, xylanohydrolase; EC. 3.2.1.8, commonly called xylanase) is an industrially important enzyme which degrades xylan randomly and produces xylooligosaccharides, xylobiose and xylose. It is mainly present in microbes and plants but not in animals. Xylanases from fungal and bacterial sources have been extensively studied and produced commercially. Its potential use in paper industries has been discussed which is directly related to reduction in environmental pollution. It has role in bio-bleaching paper pulp and increasing pulp brightness. Besides, it can be exploited for

ethanol production and as an additive in animal feedstock to improve its nutritional value. Endo-1, 4-β-xylanase can also be exploited in baking and fruit juice industries[6].

Two possible cultivation methods for microbial xylanase production are solid-state and submerged cultivation. In recent year, SSF has received more and more interest from researchers, since several studies for enzymes[7,8], flavours[9], colourants[10] and other substances of interest to the food industry have shown that SSF can give higher yields[11,12] or better product characteristics than submerged fermentation (SmF)[13]. In addition, costs are much lower due to the efficient utilization and value addition of wastes[14]. The detail economic analysis of the production of *Penicillium restrictum* lipase in both SmF and SSF was carried out and it was found that for a production scale of 100m^3 lipase concentrate per year, total capital investment needed for SmF was 78% higher than that needed for SSF. Also, SSF unitary product cost was 47% lower than the selling price. These studies pointed out that the great advantage of SSF processes is the extremely cheap raw material used as main substrate[15] Therefore, SSF is certainly a good way of utilizing nutrient rich solid wastes as a substrate. Both food and agriculture wastes are used in huge amounts and since they are rich in carbohydrates and other nutrients, they can serve as a substrate for the production, of bulk chemicals and enzymes using SSF technique.

In order to produce xylanase at an industrial scale for its variety of biotechnological applications, optimization of fermentation conditions and screening of important nutritional factors are of essential importance to determine the optimal parameters for efficient production. As a result, the production cost for xylanase should be significantly reduced. Conventional single dimensional search involves changing one independent variable at a time while fixing the others at a constant level, which gives unreliable results, inaccurate conclusion, and even frequent interactions of two or more factors. Statistical experimental designs including Plackett-Burman

and response surface methodologies (RSM) can collectively eliminate these limitations of a single factor optimization process. Plackett-Burman design[16] is a powerful statistical technique for screening medium components in a shake flask and has been widely used in fermentation optimization [17-20].This technique cannot determine the exact quantity but can provide indication and tendency regarding the necessity of each factor in relatively few experiments. The following response surface methodology (RSM) can provide mathematical models showing the dependence of the enzyme activity on independent variables (the concentration of the separate components of the nutrient medium or operating parameters), and even give predictive results of responses and the possible levels of related independent variables[21]. The objective of the present study is to optimize cultivation conditions and medium components for xylanase production from *Bacillus pumilus* AB-1.

MATERIALS AND METHODS

Organism and growth conditions

The bacterial strain used in the present investigation was isolated from soil, and identified as *Bacillus pumilus* AB-1 by 16s rDNA sequencing by an xplorigen technologies pvt. Ltd., New Delhi, India and EMBL has given accession no. FR852574. Culture was maintained on nutrient agar media (g/l): peptone, 10.0; NaCl, 5; beef extract, 3; and agar-agar, 20 at 4^0C.

Media preparation and inoculation

The basal salt solution (g/l): $MgSO_4$, 0.2; K_2HPO_4, 0.4; yeast extract pH, 7.0 was used to moisten the support material (10 g of wheat bran in 20 ml salt solution) in 250 ml Erlenmeyer flask. The requisite volume of media constituents were pipetted out from their stock solution of higher concentration and were mixed together before sterilization. Wheat bran was separately sterilized at 15 psi for 30 min and mixed aseptically with the medium before inoculation. The pH of the medium was adjusted to 7.0 and sterilized by autoclaving at 15 psi for 15 min. Each flask was inoculated with 10% (w/v) of 24 hours old inoculums and incubated under static condition at 28 ± 2 °C for 48 hours.

Enzyme assay

The Xylanase activity was determined according to Bailey et al[22]. One milliliter reaction mixture containing 900 □l of 1% Birch-wood xylan (obtained from HIMEDIA, CAS No :9014-63-5 for molecular biology) prepared in 0.1 M sodium phosphate buffer (pH7.0) as the substrate and 100 □l of appropriately diluted enzyme was incubated at 50°C for 10 min. The reducing sugars released were determined by the

dinitrosalicylic acid method[23]. One unit of the enzyme (U) was defined as the amount of enzyme that produced 1µmol of xylose per minute. Cellulase activity was determined by replacing xylan with 1% of low viscosity carboxymethyl cellulose as substrate in the reaction mixture.

Protein estimation

The soluble protein was determined by Folin's method using bovine serum albumin as standard [24].

Optimization Procedure

Screening of important nutrient components

The Plackett-Burman design was used for screening of the factors (media components) that significantly influenced xylanase production. The design considers the main effect of these variables but not their interaction effects. It can be represented by the first order polynomial equation:

$$Y = \beta_\circ + \sum_{i=1}^{n} \beta_i x_i \quad \ldots\ldots\ldots (1)$$

Where Y represents the response, βo is the model coefficient, βi is the linear coefficient, xi is the variables, and n is the number of the parameters (variables). Each variable was represented in two levels, i.e. high (+), and low (-). The effect of each variable was determined by the following equation:

$$E_{(x_i)} = \frac{\sum M_{i+} - \sum M_{i-}}{N} \quad \ldots.(2)$$

Optimization of the screened components

Response surface methodology was used to optimize the screened components for enhanced production of Xylanase and to attain specific yield using central composite design (CCD). The behavior of the system was demonstrated by the following quadratic equation.

$$Y = \beta_0 + \sum \beta ixi + \sum \beta ijxixj + \sum \beta iixi^2$$

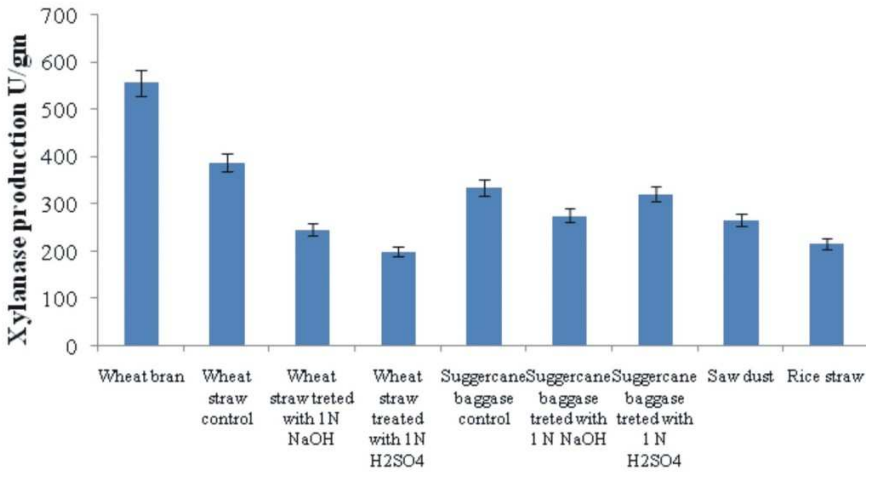
where Y is predicted response, β_0 is a constant, βi is the linear coefficient, βii is squared coefficient, βij is the cross product coefficient, x_i is the dimensionless coded value of (X_i). The above equation was solved by using the software Design- Expert (Version 7.1.3, State ease inc., USA). A 2^5 factorial design with five replicates at the center point with a total number of 13 trials were employed.

RESULTS AND DISCUSSION

Production of xylanase on various lignocellulosic substrates.

Selection of an appropriate substrate is a crucial step for fermentation, various substrates have been used by different researchers as per the need and availability of substrate for the xylanase production under solid substrate fermentation. Some of the substrates like wheat bran, sugar cane bagasse, saw dust, wheat straw, etc. have been used for xylanase production. Wheat bran, which is inexpensive and abundantly available substrate showed highest xylanase production (557.0U/g) followed by untreated and treated wheat straw with acid and alkali respectively Sugarcane baggase, treated with alkali, and acid, saw dust, rice straw showed (389U/g, 246.7U/g, 201.0U/g, 335.3U/g, 276.2U/g, 321.3U/g, 266.7U/g, 216.7U/g) of xylanase production respectively after 24 hrs of incubation at 37°C (Fig-1).

Fig. 1: Effect of different substrates on xylanase production by *Bacillus pumilus* AB-1 at 37°C with a significance level of 95%

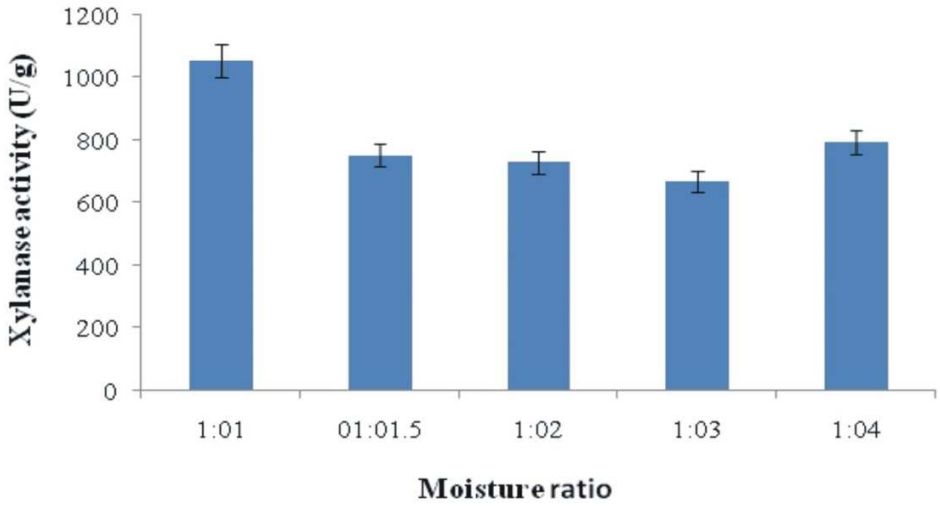


All other substrates showed poor yield compare to that obtained with wheat bran. Higher xylanase production on wheat bran may be possibility due to the low lignin content and more amount of protein as compare to the other substrates. Moreover, wheat bran is considered as the universal substrate among various substrates because it act as a complete nutrient feed for the microorganism having all the ingredients and remains loose even under moist condition providing a large surface area[25.]The biochemical composition of wheat bran indicates that it contains various soluble sugars like glucose, xylose, arabinose, galactose, etc. which are helpful for the initiation of growth of microorganism[26].

Effect of moisture level

The enzyme production was maximum (1055.08U/g) when wheat bran was used in the ratio of 1:1 (Fig-2). If the moisture content is above or below the optimum value, the metabolic activities of the organism be affected and consequently the enzyme production could be affected when moisture level is increased the xylanase yield decreased[27]. Increasing the moisture level is believed to reduce the porosity of the wheat bran, thus limiting oxygen transfer[28]. Low moisture content causes reduction in the solubility of nutrients of the substrate and low degree of swelling and higher water tension [29].

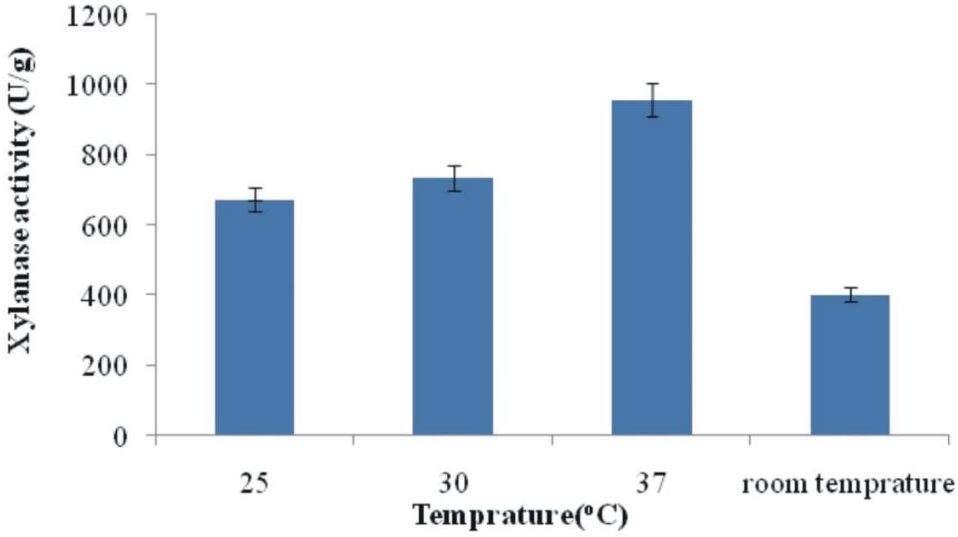
Fig. 2: Effect of different moisture level on xylanase production by *Bacillus pumilus* AB-1 at 37°C with a significance level of 95%



Effect of temperature

Highest xylanase activity was obtained at 37°C (956.7U/g) (Fig- 3.). The temperature allows to obtain a good cellular viability, best enzymatic production and extracellular protein synthesis, less loss of fresh weight[30].

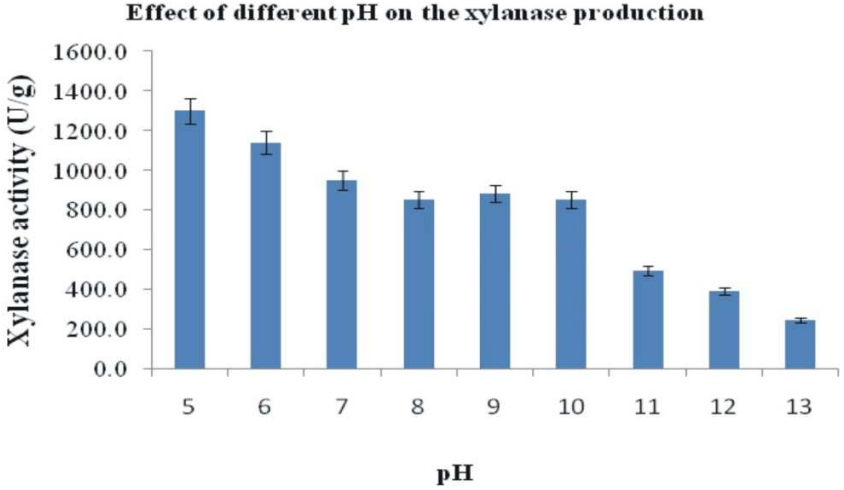
Fig. 3: Effect of different Temprature on xylanase production by *Bacillus pumilus* AB-1 with significance level 95%



Effect of pH

pH is an important environment parameter that determines growth rates of microorganisms and significantly affects the level of xylanases produced. The influence of pH culture on xylanase production during *Bacillus pumilus* AB-1 cultivation is shown in Fig-3. Xylanase activity was detected in all pH evaluated. The effect of pH on the production of xylanase was studied by varying the pH range. Highest activity was found in pH 5.0 (1299.7U/g).

Fig. 4: Effect of different pH on xylanase production by *Bacillus pumilus* AB-1 at 37°C with a significance level of 95%



Screening of important medium components using Plackett-Burman design

To enhance the production of xylanase, Plackett-Burman Design was employed as statistical approach for the screening of suitable medium components. Table- 1 represents the independent variables and their respective high and low concentrations used in the optimization study.

Table 1: Variables showing fermentation parameters used in Plackett-Burman design

Variable	Medium components	H (+) **g/l**	L (-) **g/l**
X_1	Wheat bran	15	5
X_2	$MgSO_4 \cdot 7H_2O$	0.5	0.1
X_3	K_2HPO_4	0.1	0.01
X_4	Yeast extract	2	0.2
X_5	pH	9	5
X_6	Inoculum	15%	5%
X_7	Temperature	37	30
X_8	Time (hrs.)	48	24

Table-2 shows the Plackett-Burman experimental design for 12 trials. The X_1-X_8 represents the experimental variables, where as D_1–D_3 represents the dummy variables.

Table 2: Plackett-burman design matrix of eight process variables and three dummy variables (D_1-D_3) for the xylanase production

Trial	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	D1	D2	D3	Xylanase activity U/g
1	+	+	-	+	+	+	-	-	-	+	-	148.7 ± 22.6
2	-	+	+	-	+	+	+	-	-	-	+	723.4 ± 36.9
3	+	-	+	+	-	+	+	+	-	-	-	189.9 ± 39.1
4	-	+	-	+	+	-	+	+	+	-	-	803.3 ± 43.2
5	-	-	+	-	+	+	-	+	+	+	-	680.4 ± 31.2
6	-	-	-	+	-	+	+	-	+	+	+	624.6 ± 34.9
7	+	-	-	-	+	-	+	+	-	+	+	108.8 ± 17.8
8	+	+	-	-	-	+	-	+	+	-	+	155.2 ± 18.9
9	+	+	+	-	-	-	+	-	+	+	-	202.1 ± 17.2
10	-	+	+	+	-	-	-	+	-	+	+	519.5 ± 40.1
11	+	-	+	+	+	-	-	-	+	-	+	123.5 ± 22.1
12	-	-	-	-	-	-	-	-	-	-	-	553.2 ± 47.9
C^{*1}	0	0	0	0	0	0	0	0	0	0	0	182.2 ± 15.8
C^{*2}	0	0	0	0	0	0	0	0	0	0	0	219.0 ± 18.5

Table -3 represents the ANOVA results for Placket-Burman effect. If the value of the F-ratio was greater than the F-table, those variables were considered as the significant variables. The F-ratio of $MgSO_4 \cdot 7H_2O$, K_2HPO_4, yeast extract, inoculum size, and fermentation hours were less than the F-table (F-ratio < 4.21001) for the xylanase production and considered as insignificant variables. During the present investigation, wheat bran, pH and Temperature showed greater value of the F-ratio then the F-table (F-ratio > 4.21001) and exerted positive influence on the Xylanase production.

Table 3: ANOVA for 12 run Plackett-Burman Design

Desired Confidence = 0.95						
Column	Variable Name	SS	DF	Variance	F-Ratio	F-Table
1	Wheat bran	1909429	1	1909429	170.444	4.21001
2	$MgSO_4 \cdot 7H_2O$	870.354	1	870.354	0.07769	4.21001
3	K_2HPO_4	7032.17	1	7032.17	0.62772	4.21001
4	Yeast extract	12799.5	1	12799.5	1.14254	4.21001
5	pH	77413	1	77413	6.9102	4.21001
6	Inoculum	45047.6	1	45047.6	4.02114	4.21001
7	Temperature	117035	1	117035	10.447	4.21001
8	time hrs	4305.05	1	4305.05	0.38429	4.21001

The above results indicated that the Placket-Burman design considered to be a powerful tool for identifying factors which had significant influence on the xylanase production. The optimal concentration of the individual factor was further determined by the subsequent central composite design experiments.

Optimization of screened medium components using Central Composite Design

The experimental design performed by the RSM method is based on mathematical techniques that enable us to investigate the interactions between variables of the medium components. The Central Composite Design was used to determine the optimal concentration (level) of the medium components. A total of 13 experiments with two variables (components of the medium) and five coded levels (five different concentrations) were performed. Based on the results obtained from the Plackett-Burman Design, we selected two variables namely wheat bran, pH. Whereas, wheat bran and pH have negative influence on xylanase production hence lower concentration of wheat bran and pH result in higher xylanase production. The other components of the production medium namely, $MgSO_4 \cdot 7H_2O$, K_2HPO_4, yeast extract, inoculum size, temperature and fermentation hours were found to be insignificant, so their concentrations were set at their middle level in Central Composite Design. The other culture conditions were as follows: $MgSO_4 \cdot 7H_2O$ 0.5%, K_2HPO_4 0.01%, yeast extract 0.2%, inoculum size 15%, and temperature 37^oC and fermentation hours 24hrs.

The coded and actual values of the variables used in the experimental design with respect to two variables wheat bran and pH were shown in Table- 4.

Table 4: Coded and actual values of the variables used in central composite design

Independent variables	Level				
	-α	-1	0	1	A
Wheat bran	1.625	0.5	2.75	5	3.875
pH	6	5	7	9	8

The central composite design matrix matrix along with the experimental results of predicted responses for the xylanase production is shown in Table- 5.

Table 5: Central Composite Design (CCD) matrix of independent variables and the corresponding experimental and predicted values for the xylanase production

Std	Run	Wheat bran (gm%)	pH	Response 1 xylanase activity (U/gm)
5	1	1.63	7	323.78
11	2	2.75	7	502.16
3	3	1.86	7.79	350.06
10	4	2.75	7	502.16
13	5	2.75	7	436.36
9	6	2.75	7	493.51
4	7	3.64	7.79	487.73
1	8	1.86	6.21	473.30
6	9	3.88	7	788.53
12	10	2.75	7	843.56
2	11	3.64	6.21	824.37
8	12	2.75	8	338.73
7	13	2.75	6	627.27

The experimental values for the regression coefficient were obtained by quadratic polynomial equation, where only significant coefficients ($P < 0.05$) were considered. The smaller *P*-values indicate the higher significance of the corresponding coefficient. The insignificant coefficients were not omitted from the equations, since it was a hierarchical model. The predicted responses Y for the xylanase production were obtained as follows:

Y = 537.8112- 218.93 * A -7.09254 * B

Y= 1278.827-246.584* A- 8.98698* B

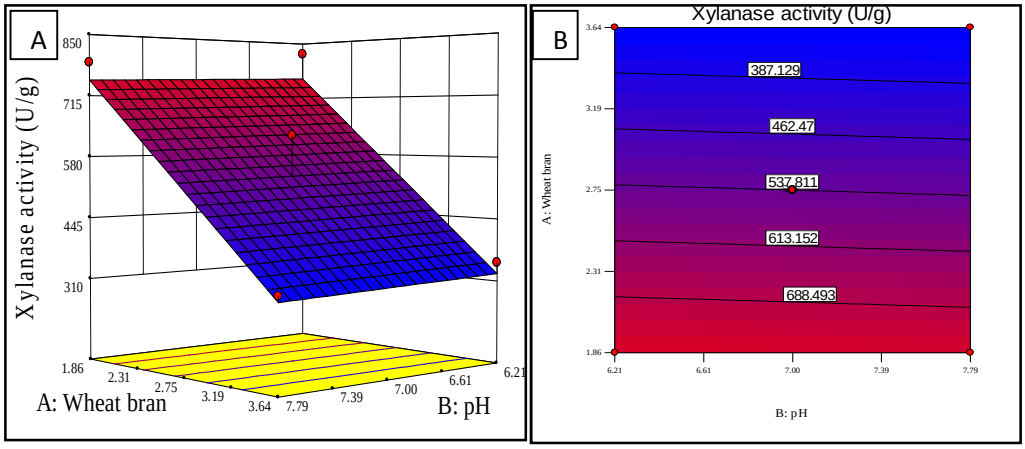
Where the Y is the xylanase production (U/g), A and B are coded values for the independent Variables (wheat bran and pH respectively). The statistical significance of the quadratic model For the experimental responses was evaluated by the analysis of variance (ANOVA). According To the ANOVA results (Table-6), the model was significant with an *F*-test of a very low Probability value ($P > F$) < 0.0001. The goodness of fit for the model was expressed by the Coefficient of determination R^2 and the values were found to be 0.890318. The values of R^2 Indicate that the experimental values were significantly in agreement with the predicted values and also suggested that the model is suitable and practicable. The lack of fit *F*-values 0.6754 for the xylanase productions were not significant relative to pure error. These large values could Occur due to noise. The purpose of statistical analysis is to determine which experimental factors generate signals, which are large in comparison to noise. The adequate precision value measures signal to noise ratio and ratio greater than 4.0 is desirable. In the present study, the value of this Ratio was higher than desire value for production and suggested that the polynomial quadratic model can be used to navigate the design space and further optimization. The 3-D surface plots and their respective contour plots illustrates the response over a region of interesting factor levels, the relationship between the response and experimental levels of each variable and the type of interactions between the test variables in order to deduce the optimal composition of the culture medium. In contrast to the circular shape contour plots, the elliptical nature of the curves indicates significant mutual interactions between variables.

Table 6:Analysis of variance (ANOVA) for the experimental results of the CCD

Desired Confidence = 0.95						
Column	Variable Name	SS	DF	Variance	F-Ratio	F-Table
1	Wheat bran	1909429	1	1909429	170.444	4.21001
2	MgSO4·7H2O	870.354	1	870.354	0.07769	4.21001
3	K2HPO4	7032.17	1	7032.17	0.62772	4.21001
4	Yeast extract	12799.5	1	12799.5	1.14254	4.21001
5	pH	77413	1	77413	6.9102	4.21001
6	Inoculum	45047.6	1	45047.6	4.02114	4.21001
7	Temperature	117035	1	117035	10.447	4.21001
8	Fer time hrs	4305.05	1	4305.05	0.38429	4.21001

The 3-D surface plots and contour plots illustrate the effect of wheat bran and pH on xylanase production (Fig-5A and B). It was obvious from the 3-D curve that xylanase production was increased with decrease in wheat bran and pH and yielded maximum at lower values. At higher value of wheat bran, decrease in concentration of pH result in increased xylanase production. But there was no significant change observed in xylanase production at higher value of pH with decrease in wheat bran concentration. The data obtained from the 3-D surface plots and contour plots and the equations obtained from the multiple regression analysis, we can determine the optimal concentration of the medium components.

Fig. 7 (A) 3D surface plot and (B) Contour plot for the effect of wheat bran and pH on xylanase production



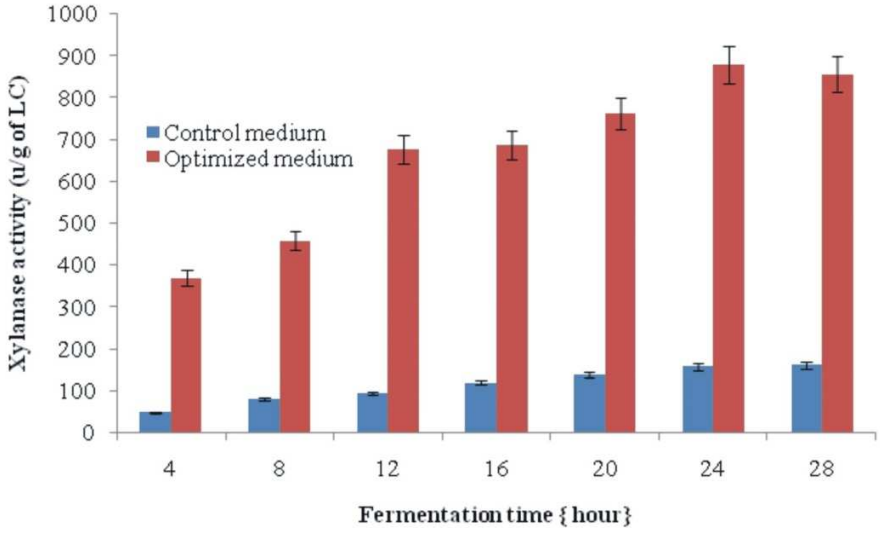
The model predicts that the xylanase production is located at the actual values: wheat bran 1.86 gm%, pH 6.21. The predicted values of the responses obtained and their corresponding concentration of medium components varies accordingly. Thus, graphical optimization of the overall desirability function was performed to determine the best possible combination for each response simultaneously. There are several reports on the optimization of medium composition for the production of xylanase using statistical approaches as it was found to be a reliable methodology to obtain reproducible results[31-33]. Using this statistical experimental design, the xylanase production under optimal condition reached 46.50 U/mL and an increase in xylanase activity of 1.34-fold was obtained compared with the original medium for fermentation carried out in a 30-L bioreactor from Penicillium sp.WX-Z1[21]. The production of β-glucosidase, β-xylosidase and xylanase by *Colletotrichum graminicola* was optimized using Response Surface Methodology (RSM). Maximal production occurred in wheat bran[34].

Validation of the experimental method.

In order to confirm the predicted results of the models, experiments were carried out under optimized conditions. The experimental values of xylanase production (878.34U/g) were even Higher and in good agreement with the predicted values of the models. As a result, the models

Developed by Design Expert software are considered to be reliable and accurate for predicting the xylanase production. The typical time courses using unoptimized and statistically optimized medium under the solid state conditions for 28 hrs were compared. The changes of xylanase production are shown in figure-8. The Xylanase production was increase up to 24 hrs then it decreases. The maximum xylanase production (157.14U/g) was achieved at 24 hrs. Thereafter, the production of Xylanase decreased. Conversely, when the statistically optimized medium was used, the maximum xylanase production (878.34U/g) was achieved at 24 hrs then the xylanase production was decreased. The above results indicate that using statistical optimization method the xyalanase production was increased with 5.58 fold over unoptimized medium.

Fig. 8: Xylanase production profile with optimized and unoptimized medium, with significance level of 95%



CONCLUSION

In the present investigation, enhanced production of Xylanase without supplementation of any additives was achieved. It could be grown easily at room temperature. The fermentation system was easy to handle and less expensive. According to the results obtained during the present study, it can be concluded that the one factor at a time method combined with the Plackett-Burman design and Response Surface Methodology were helpful to screen the suitable fermentation parameters and their optimal values. The statistical optimization method for fermentation was found to be scientific, credible and worth applying. Validation experiments were also carried out to verify the accuracy of the models and reproducibility of the results. Furthermore, the enzyme extract obtained from the fermentation was highly concentrated and resulting Xylanase activity was extracellular. Under the optimized conditions xylanase production was obtained up to (878.34U/g) which was 5.58 times higher than the initial production (157.14U/g). The results were even higher and in good agreement with the predicted values of the models.

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