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ORIGINAL PAPER

Xylanase production using agro-residue in solid-state fermentation from *Bacillus pumilus* ASH for biodelignification of wheat straw pulp

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Abstract Two stage statistical design was used to optimize xylanase production from *Bacillus pumilus* ASH under solid-state fermentation. Initially, Plackett–Burman designing (PB) was used for the selection of crucial production parameters. Peptone, yeast extract, incubation time, moisture level and pH were found to be the crucial factors for the xylanase production. Crucial variables were further processed through central composite designing (CCD) of response surface methodology (RSM) to maximize the xylanase yield. Each significant factor was investigated at five different levels to study their influence on enzyme production. Statistical approach resulted in 2.19-fold increase in xylanase yield over conventional strategy. The determination coefficient (R^2) as shown by analysis of variance (ANOVA) was 0.9992, which shows the adequate credibility of the model. Potential of cellulase-free xylanase was further investigated for biobleaching of wheat straw pulp. Xylanase aided bleaching through $XCDED_1D_2$ sequence resulted in 20 and 17% reduction in chlorine and chlorine dioxide consumption as compared to control. Significant increase in pulp brightness (%ISO), whiteness and improvement in various pulp properties was also observed.

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Introduction

Xylanases (1,4-β-D-xylan xylanohyrolase; EC 3.2.1.8) constitute one of the most important industrial enzyme that depolymerizes xylan molecule into xylose units and is produced by different microorganisms, including bacteria (Sanghi et al. 2008; Lakshmi et al. 2009; Garg et al. 2011; Nagar et al. 2010), fungi (Shirkolaee et al. 2008; Camassola and Dillon 2010; Sanghvi et al. 2010) and actinomycetes (Beg et al. 2000; Bajaj and Singh 2010). In recent years, interest in xylanases have been increased due to their potential use in the pulp and paper industry, textile industry, food processing and wine industry (Hang and Woodams 1997; Csiszar et al. 2006; Battan et al. 2007, 2008; Dhiman et al. 2008, 2009).

The cost of enzyme production is one of the main factors which determine the use of enzyme at industrial scale (Beg et al. 2001). Therefore, the use of agricultural waste by solid-state fermentation (SSF) using statistical design approach can reduce the cost of enzyme production. Utilization of agro-residues as substrate in SSF provides an alternative to these under or non-utilized residues. SSF is the process of cultivating microorganism in the absence of free flowing water. Most of the reports on xylanase production are through submerged fermentation, but

SSF is more economical and advantageous in term of improved product recovery, lower capital investment, less space requirement, reduced wastewater output and the use of abundant agriculture waste as a substrate for xylanase production. Generally traditional approach of 'one factor at a time' is employed for the optimization of various physical and nutritional factors for the enhanced enzyme production. These are time consuming and unable to detect the frequent interaction between two or more factors. These limitations can be overcome with application of appropriately designed experiments. Statistical designing includes Plackett–Burman (PB) (Plackett and Burman 1946), central composite design (CCD)–response surface methodology (RSM) in which minimum number of experiments are formed for a large number of factors (Liu et al. 2008; Cui et al. 2009; Fang et al. 2010).

Non-woody agro-residues act as an important source of raw material for pulp and paper industry due to the shortage of trees and easy availability of non-woody agro-residues. In conventional paper making process, bleaching of pulp requires large amount of chlorine based chemicals which generate highly toxic organochlorine pollutants, thereby, causing environmental pollution. Bleaching is the process of removal of lignin from pulp to produce the bright and completely white pulp. Xylanase treatment removes reprecipitated xylan on the surface of the fiber thereby making the fiber more permeable to lignin removal (Subramaniyan and Prema 2002) hence leads to significant reduction in these chlorine based chemicals for achieving the comparable level of pulp brightness (Dhiman et al. 2009). Xylanases are useful in paper and pulp industries only if the cellulosic fiber of the pulp is not affected so cellulase-free xylanases are preferred for biobleaching of paper pulp (Viikari et al. 1994). A large number of microorganism have been reported to produce xylanases but only few of them are either alkaliphilic and/or thermophilic. As the bleaching of the pulp is carried out at high pH and temperature so the xylanases with higher temperature and pH stabilities are required (Beg et al. 2001; Rifaat et al. 2005).

Various studies on pre-bleaching of pulp by xylanases showed that they can decrease the amount of chemicals required to attain same brightness in subsequent bleaching stages and hence decrease the environmental pollution (Dhiman et al. 2009; Jacobs-Young et al. 2000). A number of reports are available on the use of xylanase for biobleaching of kraft pulp but only a few reports are available on the application of bacterial xylanases in biobleaching of wheat straw pulp (Li et al. 2005; Choudhary et al. 2006; Garg et al. 2011).

In the present investigation, statistical tools were used to achieve high level of xylanase production under SSF using wheat bran as a cost effective substrate. Further, the xylanase produced was evaluated for its bleach boosting effect on wheat straw pulp.

Materials and methods

Microorganism

The bacterial strain used in the present investigation was isolated from sanitary landfill and identified as *Bacillus pumilus* ASH by the Institute of Microbial Technology (IMTECH), Chandigarh, India on the basis of its morphological, physiological and biochemical characterization. It has been given MTCC accession no. 7411. The culture was maintained and stored at 4°C on nutrient agar medium.

Medium

Erlenmeyer flask (250 ml) containing 50 ml of nutrient broth (pH 8.0) was used for the inoculum production at 37°C under agitated conditions (200 ± 4 rpm). For the optimization of production medium, experiments were performed in 250 ml Erlenmeyer flask containing 10 g of wheat bran and moistening agent supplemented with different nutrients. Different nutritional and growth conditions were adjusted according to the design as suggested by the statistical model. The flasks were autoclaved at 121°C for 30 min, cooled and inoculated with 1.5% (w/v) inoculum (18 h old, $\sim 3.6 \times 10^6$ counts/ml).

Screening of significant factor using PB designing

PB designing was employed to evaluate the important factors affecting the xylanase production. Ten physical and nutritional variables, i.e. peptone, yeast extract, KNO_3, $MgSO_4 \cdot 7H_2O$, K_2HO_4, incubation time, temperature, inoculum size, moisture level and pH were

Table 1 Plackett–Burman design with experimental and predicted values of xylanase production

Run	Variables											Xylanase activity IU/g	
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*J*	*K*	*L*	Experimental	Predicted
1	1	6	1	0.1	0.1	144	50	20	1.5	9	1	103.17 ± 15.25	77.09
2	6	1	6	1	0.1	144	30	5	1.5	9	1	3606.60 ± 102.12	3580.52
3	6	1	6	0.1	0.1	24	50	20	3.5	6	1	2902.75 ± 104.50	2876.67
4	6	6	1	1	0.1	24	30	20	3.5	9	−1	4257.46 ± 146.32	4283.54
5	1	1	6	1	1.5	24	50	20	1.5	9	−1	2303.05 ± 98.82	2329.13
6	1	1	1	0.1	0.1	24	30	5	1.5	6	−1	450.75 ± 40.56	476.83
7	6	1	1	0.1	1.5	144	50	5	3.5	9	−1	3004.62 ± 60.00	3030.70
8	6	6	1	1	1.5	24	50	5	1.5	6	1	454.82 ± 70.32	428.74
9	1	6	6	0.1	1.5	24	30	5	3.5	9	1	3311.46 ± 107.26	3285.38
10	1	6	6	1	0.1	144	50	5	3.5	6	−1	31.69 ± 5.62	57.77
11	6	6	6	0.1	1.5	144	30	20	1.5	6	−1	43.77 ± 6.36	69.85
12	1	1	1	1	1.5	144	30	20	3.5	6	1	25.48 ± 5.41	−0.60

A peptone% (w/w), *B* yeast extract % (w/w), *C* KNO_3% (w/w), *D* $MgSO_4{\cdot}7H_2O$ (g/L), *E* K_2HPO_4 (g/L), *F* incubation time (h), *G* temperature (°C), *H* inoculum % (v/w), *J* moisture level (mL/g), *K* pH, *L* dummy −1

selected to study their effect on xylanase production. Each variable was studied at two extreme levels, i.e. higher (+1) and lower (−1) for their screening. In PB design, the total no of experiments performed was $n + 1$ where n is the no of variables studied. Experimental design with experimental and predicted xylanase activity is given in Table 1. Variables having *P* value less than 0.0500 are significant. Significance of the model was determined through '*F*' and '*P*' values and the proportion of variance by R^2 (multiple coefficient of variance) of the model.

Optimization of concentration of significant variables through CCD

Significant factors (peptone, yeast extract, incubation time, moisture level and pH) determined through PB strategy were further evaluated through CCD–RSM for obtaining the enhanced xylanase production under SSF. Each factor was studied at five different levels, that is, −2.37 (lowest), −1 (low), 0 (medium), +1 (high), +2.37 (highest) in coded form (Table 2). A 2^5 factorial central composite experimental design with five factors and eight replicates at the centre point, leading to a set of 50 experiments were used to optimize the factors for the production of xylanase. The full experimental plan with experimental and predicted response of xylanase yield is given in Table 3.

Table 2 Range and levels of five variables in terms of actual and coded factors for central composite design

Variables	Levels				
	−2.37	−1	0	+1	+2.37
X_1 Peptone % (w/w)	−2.45	1	3.5	6	9.44
X_2 Yeast extract % (w/w)	−2.45	1	3.5	6	9.44
X_3 Incubation time (h)	−58.70	24	84	144	226.71
X_4 Moisture level (ml/g)	0.12	1.5	2.5	3.5	4.88
X_5 pH	3.93	6	7.5	9	11.07

The analysis of variance (ANOVA) was performed and the proportion of variance explained by the polynomial model was given by multiple coefficients. The significance of the model can be checked through various criteria. In present case R^2, *adj.* R^2 and *pred.* R^2 were taken into consideration. Experimental design was generated by using the statistical software package 'Design Expert® 6.0' Stat-Ease Inc., Minneapolis, MN, USA. Statistical analysis of experimental data was also performed using this software.

Enzyme extraction

The enzyme from each flask was extracted twice with 10 mM sodium phosphate buffer pH 7.0 (100 ml for 10 g of wheat bran) by keeping the flasks in the

Table 3 Central composite design with experimental and predicted value of xylanase production

Run	Variables					Xylanase activity IU/g	
	X_1	X_2	X_3	X_4	X_5	Experimental	Predicted
1	−1	1	1	−1	1	10363 ± 60.00	10264.20
2	1	−1	−1	1	1	7460 ± 51.26	7610.93
3	−1	−1	−1	−1	−1	4215 ± 45.80	4466.52
4	0	0	0	0	0	20619 ± 352.32	20593.65
5	0	0	0	0	−2.37	25103 ± 532.00	24782.97
6	1	−1	−1	1	−1	8961 ± 80.38	9136.12
7	1	1	−1	−1	1	6749 ± 150.34	6797.53
8	1	−1	1	1	−1	11903 ± 108.23	12008.25
9	1	−1	1	1	1	2595 ± 204.56	2440.67
10	0	0	−2.37	0	0	18 ± 8.02	−576.77
11	−1	−1	1	1	1	2178 ± 75.36	2206.10
12	−1	1	−1	−1	1	5479 ± 54.41	5480.08
13	0	−2.37	0	0	0	4698 ± 45.82	4585.07
14	−1	1	−1	1	−1	10506 ± 250.32	10622.30
15	0	0	0	0	0	20619 ± 325.65	20593.65
16	−2.37	0	0	0	0	7066 ± 125.36	6921.36
17	1	1	1	1	1	2537 ± 98.65	2564.30
18	1	1	1	1	−1	10895 ± 250.56	11028.50
19	1	1	−1	−1	−1	4512 ± 154.36	4604.22
20	1	−1	−1	−1	1	8735 ± 198.68	8882.40
21	0	0	2.37	0	0	8242 ± 174.58	8528.08
22	0	0	0	2.37	0	436 ± 26.00	−76.45
23	0	0	0	0	0	20619 ± 352.45	20593.65
24	2.37	0	0	0	0	7913 ± 45.95	7748.93
25	1	1	1	−1	1	9928 ± 256.57	9817.27
26	0	0	0	0	0	20619 ± 460.56	20593.65
27	0	0	0	0	2.37	14984 ± 365.58	14995.33
28	1	−1	1	−1	−1	19452 ± 378.65	19598.72
29	1	−1	1	−1	1	12740 ± 230.68	12646.28
30	0	0	0	0	0	20619 ± 463.92	20593.65
31	−1	1	−1	1	1	9229 ± 129.26	9344.35
32	−1	1	−1	−1	−1	3943 ± 136.58	4142.89
33	1	1	1	−1	−1	15796 ± 289.53	15666.35
34	0	0	0	0	0	20619 ± 253.56	20593.65
35	0	2.37	0	0	0	4543 ± 246.69	4347.23
36	1	−1	−1	−1	−1	7871 ± 362.23	7792.47
37	−1	1	1	1	1	5124 ± 150.56	5194.35
38	−1	−1	−1	−1	1	4828 ± 220.00	4700.32
39	−1	−1	−1	1	−1	7793 ± 360.56	7993.30
40	1	1	−1	1	−1	8700 ± 430.24	8900.50
41	−1	−1	1	−1	−1	18369 ± 250.59	18037.14
42	0	0	0	0	0	20619 ± 15.50	20593.65
43	−1	1	1	1	−1	14502 ± 309.12	14514.67

Table 3 continued

Run	Variables					Xylanase activity IU/g	
	X_1	X_2	X_3	X_4	X_5	Experimental	Predicted
44	0	0	0	−2.37	0	4151 ± 120.36	4354.75
45	−1	−1	−1	1	1	5420 ± 192.65	5611.98
46	1	1	−1	1	1	8243 ± 196.62	8478.68
47	0	0	0	0	0	20619 ± 423.05	20593.65
48	−1	1	1	−1	−1	16912 ± 368.56	16969.40
49	−1	−1	1	1	−1	12514 ± 80.38	12629.80
50	−1	−1	1	−1	1	10181 ± 145.80	10228.58

X_1 peptone % (w/w), X_2 yeast extract % (w/w), X_3 incubation time (h), X_4 moisture level (ml/g), X_5 pH

incubator shaker at 37°C, 200 rpm for 30 min and the contents were squeezed through a wet muslin cloth. The enzyme extract was centrifuged at 10,000*g* for 30 min at 4°C and the clear supernatant was used as the enzyme source.

Xylanase assay

Xylanase activity was assayed by using 1% birchwood xylan (Sigma-Aldrich, USA) dissolved in 0.1 M sodium phosphate buffer (pH 7.0). The enzymatic reaction was carried out at 60°C for 10 min. The enzyme activity was determined by estimating the amount of reducing sugars released during the enzyme substrate reaction using Miller's method (1959). One unit (IU) of xylanase activity was defined as the amount of enzyme that catalyzes the release of 1 µmol of reducing sugar equivalent to xylose per min under the specified assay conditions. Similarly assay for cellulase was carried out using CM—cellulose as substrate.

Optimization of various parameters for pretreatment of pulp

Different independent variables viz. pH, enzyme dose, temperature and retention time were optimized at 10% pulp consistency in transparent plastic bags. Experiments were conducted over a broad range of pH (7–10) and enzyme dose (5.0–12.5 IU/g oven dried pulp) to study their effect on pulp. Optimization of retention time and temperature was carried out by treating the moistened unbleached pulp over a broad range of temperatures (55–70°C) at varying retention time (90–210 min). To determine the efficiency of each treatment process, the pulp was washed with distilled water and was made into hand sheets according to Technical Association of Pulp and Paper Industry (TAPPI) standard method (TAPPI 2000). Effect of different concentration of independent variables was studied by observing various dependent variables such as brightness, whiteness, yellowness, kappa number and permanganate number.

Sequence for biobleaching of wheat straw pulp

Extensively washed oven dried wheat straw pulp was filled in dry plastic bags. Further, the pulp was pretreated with xylanase under optimized conditions. Then the pulp was subjected to the bleaching sequence ($CDED_1D_2$). In chlorine, chlorine dioxide treatment (CD) step enzyme pretreated pulp (3% consistency) was subjected to 2.486% chlorine: chlorine dioxide in ratio of 90:10 at pH 2.0 for 45 min at ambient temperature. In alkali treatment (E) step the pulp (3% consistency) was treated with 1.8% sodium hydroxide pH 12 at 70°C for 120 min. The alkali-treated pulp (10% consistency) was than treated with 0.9 and 0.4% chlorine dioxide twice to remove the lignin at 70°C (D_1 D_2). After each treatment, the pulp was filtered and washed. A portion of pulp after thorough washing in distilled water was made into hand sheets to determine the quality of pulp (TAPPI Test Method 2000) and remaining pulp was used for next step.

Analysis of biobleached pulp

Bio-bleached wheat straw pulp was thoroughly washed and hand sheets were prepared under standardized condition according to TAPPI standard

methods (TAPPI Test Method 2000). Different pulp properties, viz. kappa number (T 236 om-99), brightness (T 452 om-98), P. no. (permanganate no.), yellowness and whiteness (T 1216), viscosity (TAPPI method T 230 om-99), burst factor (T 403 om-97), breaking length (T 404 cm-92), Double fold (T 423 cm-98), Gurley porosity (T460 om-96), tear factor, tearness (T 414 om-98) and total chlorine dioxide consumed of the biobleached effluent were determined according to the standard protocols of TAPPI (TAPPI Test Method 2000).

Results and discussion

Plackett–Burman design

Plackett–Burman designing was carried out using 12 run to identify the significant factors for xylanase production. Ten variables (peptone, yeast extract, KNO_3, $MgSO_4{\cdot}7H_2O$, K_2HO_4, incubation time, temperature, inoculum size, moisture level, pH) and one dummy variable were analyzed for their effect on xylanase production using wheat bran as substrate. Experimental design and the predicted xylanase yields are given in Table 1. The xylanase activity varied considerably between 25.48 and 4257.46 IU/g with in tested conditions as given by PB design. Further, the second-order polynomial equation which was used to explain the xylanase production as:

$$\begin{aligned} Y = {} & 10247.83 + 4022.21\,A - 2045.43\,B \\ & + 1951.51\,C + 431.29\,D - 1104.61\,E \\ & - 3432.48\,F - 1447.72\,G - 612.13\,H \\ & + 3285.65\,J + 6338.55\,K \end{aligned} \quad (1)$$

where Y is xylanase activity, A, B, C, D, E, F, G, H, J, K represents peptone, yeast extract, KNO_3, $MgSO_4{\cdot}7H_2O$, K_2HO_4, incubation time, temperature, inoculum size, moisture level and pH.

The adequacy of the model was tested using the Fisher's test for ANOVA. ANOVA showed that model is significant as the F value for the model is 370.86. There is only a 4.04% chance that a 'Model F-Value' could occur due to noise. The P-value of the model is significant (0.0404), generally P values less than 0.05 are desirable. Further, the P values of A, B, F, J and K variables were 0.0248, 0.0486, 0.0290, 0.0303 and 0.0157, respectively, were consider to have a significant effect on xylanase production and were selected for further studies (Table 4). These results are in reasonable agreement as obtained by Liu et al. (2008) and Fang et al. (2010).

Table 4 Results of analysis of variance (ANOVA) of Plackett–Burman design

Source	Sum of squares	DF	Mean square	F value	P value (Prob > F)
Model	1.09	10	1.09	370.86	0.0404
A	1.94	1	1.94	660.76	0.0248
B	5.021	1	5.021	170.88	0.0486
C	4.570	1	4.570	155.54	0.0509
D	2.232	1	2.232	7.60	0.2216
E	1.464	1	1.464	49.83	0.0896
F	1.41	1	1.41	481.21	0.0290
G	2.515	1	2.515	85.60	0.0685
H	4.496	1	4.496	15.30	0.1593
J	1.29	1	1.3	440.92	0.0303
K	4.821	1	4.82	1640.94	0.0157
Residual	2.938	1	2.938		
Cor Total	1.090	11			

CCD

In order to determine the optimal concentration of crucial factors and their interaction with each other, CCD was carried out using the outcome of PB designing. An experimental design of 50 experiments was developed from five significant factors (Table 3). In all 50 runs, the xylanase activity varied from 18 to 25103 IU/g. Further, the second-order polynomial Eq. 2 was used to explain the xylanase production and interaction among the factors (in coded form) as:

$$\begin{aligned} Y = {} & 1.277 + 1078.31\,X_1 - 310.34\,X_2 \\ & + 11866.99\,X_3 - 5775.67\,X_4 \\ & - 12757.05\,X_5 - 14531.60\,X_1^2 \\ & - 17676.19\,X_2^2 - 18213.68\,X_3^2 \\ & - 20226.19\,X_4^2 - 771.92\,X_5^2 \\ & - 4439.94\,X_1X_2 - 2734.88\,X_1X_3 \\ & - 3383.69\,X_1X_4 + 1326.69\,X_1X_5 \\ & - 1153.38\,X_2X_3 + 4576.31\,X_2X_4 \\ & + 1710.19\,X_2X_5 - 13847.63\,X_3X_4 \\ & - 12465.88\,X_3X_5 - 4053.56\,X_4X_5 \end{aligned} \quad (2)$$

where Y presents the xylanase activity; X_1, X_2, X_3, X_4, X_5 are peptone, yeast extract, incubation time, moisture level and pH, respectively.

The ANOVA of model demonstrated that the Eq. 2 is highly significant, as was evident from the model F value 1894.94 and a P value ($P > F$) (<0.0001) (Table 5). Similarly, P value of 0.0001 was reported in *Bacillus pumilus* SV-85S for xylanase production (Nagar et al. 2010).

Determination coefficient R^2 value of 0.9992 indicated that only 1% of the total variations were not explained from the model. This implies that the prediction of experimental data is quite satisfactory. The value of adjusted determination coefficient '*Adjusted* R^2' 0.9987 was also very high which supported the significance of the model. The '*Predicted* R^2' value of 0.9966 is in reasonable agreement with the '*Adjusted* R^2' 0.9987. '*Adequate precision*' is a measure of the signal to noise ratio and a value generally greater than 4 is desirable so the '*Adequate Precision*' value 165.768 indicates the adequate signal.

CCD was used for five variables to evaluate their effect on xylanase production. To investigate the interaction between two factors on xylanase production, RSM was used and three-dimensional plots were drawn between two factors and xylanase activity at a time, keeping all other factors at fixed level. The response surface plots between yeast extract and peptone, peptone and incubation time, yeast extract and pH are shown in Figs. 1, 2, and 3. The circular shape of the curve indicates no interaction while elliptical shape indicates good variation of two variables.

Table 5 Results of analysis of variance (ANOVA) of central composite design

Source	Sum of squares	DF	Mean square	F value	P value (Prob > F)
Model	8.12	20	4.059	1894.94	<0.0001
A	5.036	1	5.036	23.51	<0.0001
B	4.172	1	4.172	1.95	0.1735
C	6.100	1	6.100	2847.60	<0.0001
D	1.445	1	1.445	674.53	<0.0001
E	7.049	1	7.049	3290.77	<0.0001
A2	1.173	1	1.173	5478.11	<0.0001
B2	1.736	1	1.736	8105.53	<0.0001
C2	1.843	1	1.843	8605.96	<0.0001
D2	2.273	1	2.273	10612.86	<0.0001
E2	3.311	1	3.311	15.46	0.0005
AB	6.308	1	6.308	294.49	<0.0001
AC	2.393	1	2.393	111.74	<0.0001
AD	3.664	1	3.664	171.04	<0.0001
AE	5.632	1	5.632	26.29	<0.0001
BC	4.257	1	4.257	19.87	0.0001
BD	6.702	1	6.702	312.86	<0.0001
BE	9.359	1	9.359	43.69	<0.0001
CD	6.136	1	6.136	2864.66	<0.0001
CE	4.973	1	4.973	2321.49	<0.0001
DE	5.268	1	5.258	245.47	<0.0001
Residual	6.212	29	2.142		
Lack of fit	6.212	22	2.824		
Pure error	0	7	0		
Cor total	8.12E + 10	49			

Validation of experimental model

Results and regression equation of experimental model were confirmed by producing the xylanase under optimized medium conditions. Value of predicted yield of xylanase (24782.97 IU/g) is comparable to the actual yield (25103 IU/g), which confirmed the validity and predictiveness of the model. Statistical approach results in 2.19-fold increase in xylanase activity as compare to one variable at a time strategy (11465 IU/g).

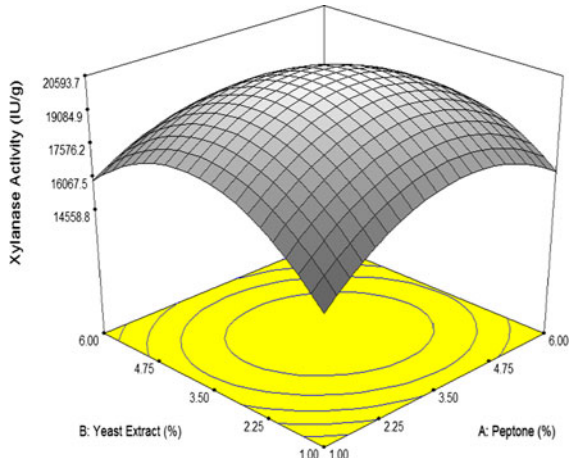

Fig. 1 Response surface plot showing the effect of yeast extract and peptone and their mutual interaction on xylanase activity (IU/g)

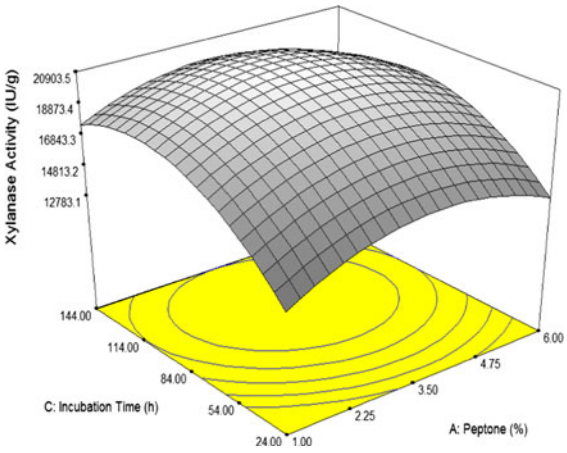

Fig. 2 Response surface plot showing the effect of peptone and incubation time and their mutual interaction on xylanase activity (IU/g)

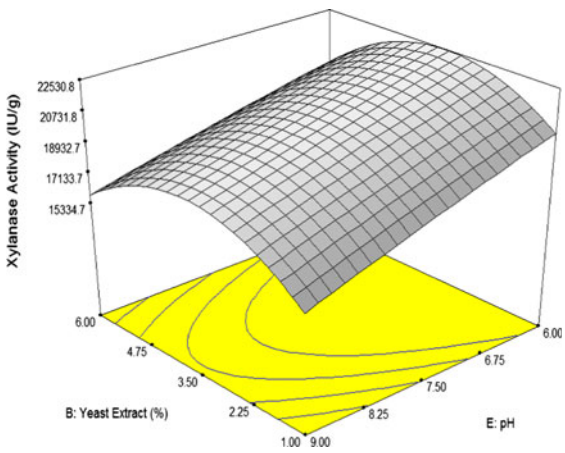

Fig. 3 Response surface plot showing the effect of yeast extract and pH and their mutual interaction on xylanase activity (IU/g)

Optimization of enzyme treatment conditions

Various parameters such as pH, enzyme dose, incubation temperature and retention time were optimized. Enzyme dose of 5 IU/g of oven dried pulp and a pH of 8 were found to be the optimum for the pretreatment of wheat straw pulp (Table 6). Incubation temperature of 60 and 65°C (Table 6) gave the best results. To make the process cost effective, temperature of 60°C was used to optimize the retention time. A retention time of 180 min (Table 6) was found to be optimum for biobleaching of wheat straw pulp with xylanase. Similarly, xylanase produced by *Staphylococcus* sp. SG-13 found to be effective at pH 9.5–10.0 when incubated at 50°C for 4 h in biobleaching of kraft pulp (Gupta et al. 2000). Optimized conditions resulted in 7 and 6% reduction in permanganate number and kappa number, respectively (Table 7), which indicates the removal of lignin and xylan attached to lignin in wheat straw pulp. Reduction in kappa number indicates reduction in chlorine required for subsequent bleaching (Gupta et al., 2000). Commercially available Xylanase HS when used for biobleaching of bagasse and wheat straw pulp decreased kappa number by greater than one (Shirkolaee et al. 2008). An increase of 5.3 and 14.5% in brightness and whiteness, respectively, and decrease of 9.7% in yellowness was obtained in enzyme-treated unbleached wheat straw pulp (Table 7). Similarly, Xyn B from *Thermotoga maritima* enhanced the wheat straw pulp brightness by 5.5% (Jiang et al. 2006). An increase of 1.0, 1.5 and 3.0% in brightness was reported when xylanase isolated from *Bacillus* sp. Ag12, Ag20 and Ag32, respectively, acted upon kraft pulp (Azeri et al. 2010).

Biobleaching of wheat straw pulp

The biobleaching effect of enzyme treatment was observed on unbleached wheat straw pulp at each stage of chemical bleaching. The enzyme pretreated pulp when subjected to $CDED_1D_2$ process resulted in remarkable increase in brightness and whiteness as compared to control after each stage of processing. The final increase of 1.5%ISO and 1.1% of brightness and whiteness, respectively, was obtained after biobleaching of wheat straw pulp (Table 7). Li and coworkers reported that wheat straw pulp pre-treated with cellulase-free xylanase isolated from fungus *Thermomyces lanuginosus* brightened the pulp by 1.8%ISO over the control (Li et al. 2005). Following the bleaching sequence after enzyme treatment $CDED_1D_2$, D1 stage showed the maximum gain in brightness which suggests that addition of ClO_2 is an important step in bleaching sequence. Addition of ClO_2 to alkali-treated pulp changes the pulp color to pale yellow, but after washing with water its colour changes to bright white which shows the effectiveness of ClO_2 on pulp. An incubation period of 3 h is required for the proper ClO_2 treatment to occur.

Table 6 Effect of different pretreatment conditions on pulp properties

Enzyme dose (IU/g)	pH	Temperature (°C)	Incubation time (min)	B[a] (%ISO)	P. no.[b]	K. no.[c]	b[d]	W/CIE[e]
Nil	8.0	60	180	43.1 ± 0.15	7.1 ± 0.10	11.5 ± 0.32	15.66 ± 0.15	−24.6 ± 0.20
5.0	8.0	60	180	45.7 ± 0.10	6.6 ± 0.15	10.9 ± 0.26	14.71 ± 0.13	−18.4 ± 0.32
7.5	8.0	60	180	45.4 ± 0.35	6.6 ± 0.20	10.9 ± 0.17	14.99 ± 0.09	−19.9 ± 0.26
10	8.0	60	180	45.2 ± 0.45	6.6 ± 0.21	11.0 ± 0.20	14.97 ± 0.14	−19.8 ± 0.15
12.5	8.0	60	180	45.4 ± 0.32	6.6 ± 0.10	11.0 ± 0.35	14.85 ± 0.10	−19.6 ± 0.10
5.0	7.0	60	180	45.4 ± 0.68	6.9 ± 0.17	11.3 ± 0.15	14.17 ± 0.15	−20.2 ± 0.15
5.0	7.5	60	180	45.6 ± 0.35	6.6 ± 0.10	11.0 ± 0.26	14.12 ± 0.13	−19.6 ± 0.20
5.0	8.0	60	180	45.9 ± 0.32	6.5 ± 0.21	10.9 ± 0.21	13.95 ± 0.17	−18.5 ± 0.36
5.0	8.5	60	180	45.7 ± 0.20	6.5 ± 0.10	10.9 ± 0.32	14.21 ± 0.23	−19.3 ± 0.26
5.0	9.0	60	180	44.3 ± 0.26	6.5 ± 0.20	11.2 ± 0.20	14.48 ± 0.26	−19.5 ± 0.45
5.0	10.0	60	180	44.2 ± 0.45	6.6 ± 0.17	11.4 ± 0.10	14.51 ± 0.14	−19.9 ± 0.21
5.0	8.0	55	180	44.9 ± 0.30	6.7 ± 0.10	11.0 ± 0.32	14.42 ± 0.23	−20.0 ± 0.30
5.0	8.0	60	180	45.8 ± 0.10	6.6 ± 0.17	10.8 ± 0.35	14.35 ± 0.15	−19.5 ± 0.15
5.0	8.0	65	180	45.8 ± 0.26	6.6 ± 0.20	10.8 ± 0.26	14.38 ± 0.14	−19.6 ± 0.26
5.0	8.0	70	180	44.8 ± 0.68	6.7 ± 0.15	10.9 ± 0.20	14.76 ± 0.10	−19.9 ± 0.20
5.0	8.0	60	90	42.8 ± 0.36	7.0 ± 0.17	11.3 ± 0.30	15.30 ± 0.26	−25.7 ± 0.35
5.0	8.0	60	120	43.9 ± 0.32	6.9 ± 0.21	11.2 ± 0.36	15.08 ± 0.13	−22.8 ± 0.32
5.0	8.0	60	150	44.9 ± 0.35	6.7 ± 0.36	10.9 ± 0.21	14.98 ± 0.17	−19.7 ± 0.17
5.0	8.0	60	180	45.1 ± 0.21	6.5 ± 0.20	10.8 ± 0.30	14.68 ± 0.15	−19.3 ± 0.21
5.0	8.0	60	210	44.9 ± 0.35	6.6 ± 0.10	10.9 ± 0.20	14.78 ± 0.14	−19.5 ± 0.26

All the experiments were performed at 10% pulp consistency

[a] Brightness of hand sheet in %ISO

[b] Permanganate number of unbleached pulp

[c] Kappa number of unbleached pulp

[d] Yellowness of unbleached pulp

[e] Whiteness of unbleached pulp

Pretreatment of pulp with xylanase and its subsequent treatment with $CDED_1D_2$ stage resulted in 20% reduction in chlorine consumption and up to 17% reduction in chlorine dioxide to obtain the same %ISO brightness (Table 7). Chlorine reduction makes the process economical as well as eco friendly. Xylanase from *Bacillus stearothermophilus* SDX reduced the chlorine consumption up to 15% in kraft wood pulp (Dhiman et al. 2009). Whereas 20 and 10% saving in chlorine and chlorine dioxide consumption, respectively, using xylanase from *Bacillus pumilus* ASH were reported in kraft pulp biobleaching (Battan et al. 2007). Further, a reduction of 25 and 19% in chlorine and chlorine dioxide consumption by using pectinase and xylanase from *Bacillus subtilis* and *Bacillus pumilus*, respectively, had been reported (Ahlawat et al. 2007). Xylanase enzyme isolated from *Thermomyces lanuginosus* SSBP reduces chlorine dioxide consumption by 2.5 kg/t in case of wheat straw pulp (Shirkolaee et al. 2008). Pretreatment of pulp with xylanase from *Streptomyces cyaneus* SN 32 reduced the kappa number by 8.7%, enhanced the brightness index by 3.56% in case wheat straw-rich soda pulp (Ninawe and Kuhad 2006). Kaur and coworkers reported 25% less chlorine consumption in kraft pulp with the use of xylanase and pectinase isolated from the same bacteria *Bacillus pumilus* (Kaur et al. 2010).

Enzyme-treated pulp when subjected to $CDED_1D_2$ bleaching sequence resulted not only in less chlorine consumption but also improved various physical pulp properties. Bio-bleaching resulted in significant

Table 7 Effect of enzyme treatment on pulp biobleaching

Parameters	Control	Enzyme treated			
Enzyme treatment stage[a] (E)					
Brightness (%ISO)	43.6 ± 1.08	45.9 ± 0.79			
Yellowness	15.97 ± 0.14	14.42 ± 0.32			
Whiteness	−23.5 ± 1.25	−20.1 ± 1.54			
Permanganate no.	7.1 ± 0.26	6.6 ± 0.36			
Kappa no.	11.5 ± 1.37	10.8 ± 0.82			
Chlorine, chlorine dioxide treatment stage[b] (CD)					
Chlorine added (%)	100%	100%	90%	80%	70%
Total Cl_2 added (%)	2.508	2.508	2.257	2.006	1.756
Cl_2:ClO_2	90:10	90:10	90:10	90:10	90:10
Cl_2 Consumed (%)	2.485	2.439	2.211	1.954	1.730
Brightness (%ISO)	53.6 ± 1.38	54.6 ± 1.71	54.4 ± 1.87	53.9 ± 1.85	53.2 ± 1.7
Yellowness	11.60 ± 0.26	10.69 ± 0.15	10.95 ± 0.10	11.27 ± 0.17	11.83 ± 0.20
Whiteness	8.0 ± 0.10	8.4 ± 0.15	8.2 ± 0.10	8.1 ± 0.17	7.8 ± 0.20
Alkali stage[c] (E)					
Brightness (%ISO)	55.4 ± 1.87	56.8 ± 1.25	56.6 ± 1.08	55.5 ± 1.15	54.9 ± 1.21
Yellowness	10.15 ± 0.78	9.85 ± 68	9.95 ± 0.74	10.07 ± 0.49	10.92 ± 0.35
Whiteness	22.5 ± 1.21	23.8 ± 1.08	23.3 ± 1.05	22.4 ± 1.25	21.8 ± 1.05
D-1 Stage[d]					
ClO_2 added (%)	0.9%	0.9%	0.81%	0.72%	0.63%
ClO_2 consumed (%)	0.806	0.803	0.738	0.663	0.607
Brightness (%ISO)	81.5 ± 1.25	82.5 ± 1.18	81.8 ± 1.31	81.6 ± 1.25	81.1 ± 1.08
Yellowness	2.95 ± 0.10	2.24 ± 0.17	2.85 ± 0.14	2.97 ± 0.15	3.12 ± 0.10
Whiteness	71.52 ± 1.41	75.9 ± 1.71	73.91 ± 1.25	71.61 ± 1.18	70.92 ± 1.21
D-2 Stage[d]					
ClO_2 added (%)	0.4%	0.4%	0.36%	0.32%	0.28%
ClO_2 consumed (%)	0.317	0.313	0.291	0.263	0.251
Brightness (%ISO)	85.5 ± 1.35	86.8 ± 1.17	86.2 ± 1.41	85.6 ± 1.18	85.3 ± 1.35
Yellowness	1.42 ± 0.07	1.27 ± 0.10	1.37 ± 0.05	1.40 ± 0.12	1.51 ± 0.6
Whiteness	81.6 ± 1.71	82.5 ± 1.41	82.0 ± 1.25	81.8 ± 1.35	81.4 ± 1.08

[a] Enzyme treatment was performed at an enzyme dose of 5 IU/g of oven dried pulp, pH 8, at 60°C, and 10% consistency for 180 min

[b] Treatment was carried out at room temperature and at pH 2.5 for 45 min

[c] Pulp was mixed with 1.8% alkali at pH 12 and treated at 70°C for 120 min

[d] Treatment was carried out at 70°C and pH 3.5 for 180 min

increase in burst, burst factor, tear, tear factor, double fold, tensile strength, breaking length, gurley porosity and viscosity, respectively (Table 8). Xylanase from *Staphylococcus* sp. SG-13 has been shown to give 30% reduction in kappa number and 11, 10, 1.8 and 17% increase in brightness, tensile strength, viscosity and burst factor, respectively (Gupta et al. 2000).

The action of enzyme on the pulp is still being elucidated. Xylanase might act on lignin carbohydrate complex thus facilitating the removal of lignin or by removing precipitated xylan from the surface of cellulose fibers resulted in increased permeability of bleaching chemicals in the pulp fibers (Techapun et al. 2003; Viikari et al. 1996).

Table 8 Physical properties of biobleached pulp

Pulp properties	Control	Enzyme treated	Gain %
Burst (lb/in^2)	38.0 ± 1.25	39.5 ± 0.92	3.94
Burst factor	41.42 ± 1.10	43.73 ± 1.44	5.57
Tear (mN)	150 ± 4.58	160 ± 4.57	6.66
Tear factor	47.44 ± 1.41	51.40 ± 1.30	8.34
Double fold (no.)	65 ± 1.53	68 ± 1.00	4.61
Tensile strength (N)	55.3 ± 0.79	56.8 ± 0.65	2.71
Breaking length (mr)	5830 ± 9.16	6082 ± 10.58	4.32
Gurley porosity (s)	86.4 ± 0.50	87.6 ± 0.36	1.38
Viscosity (cps)	19.80 ± 0.73	20.89 ± 0.56	5.50

Conclusions

Optimization of fermentation process under SSF through statistical approach could overcome the limitation of classical method. Statistical approach resulted in 2.19-fold increase in xylanase activity over conventional process. Cellulase-free xylanase enzyme produced from *Bacillus pumilus* ASH has wide range of thermal and pH activity which can be explored in various industry xylanase aided bleaching leads to reduction in chlorine and chlorine dioxide consumption by 20 and 17%, respectively, for gaining the same %ISO brightness as compared to control. Enzymatic treatment also resulted in improvement of various pulp properties like burst, burst factor, tear, tear factor, double fold, tensile strength, breaking length, gurley porosity and viscosity. This environment friendly technology can be used in paper pulp industry.

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