Statistical Optimization of Nutritional and Physical Parameters for Xylanase Production from Newly Isolated *Aspergillus oryzae* LC1 and Its Application in the Hydrolysis of Lignocellulosic Agro-Residues

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Xylanase is a key enzyme in the conversion of lignocellulosic biomass into various oligosaccharides and simpler monomeric units through the hydrolysis of hemicellulose. Rice straw is readily available around the world and is a rich source of hemicellulose. Recently, there has been growing interest in the exploitation of rice straw as a low-cost substrate for the production of hemicellulolytic enzyme, *i.e.*, xylanase. This study aimed to optimize the nutritional components (rice straw, magnesium sulphate, and calcium chloride concentrations) and physical parameters (temperature and pH) for xylanase production with a newly isolated *Aspergillus oryzae* LC1 under submerged fermentation using central composite design based response surface methodology. The optimum media constituents were 1% rice straw (w/v), 1.0 g/L calcium chloride, and 0.3 g/L magnesium sulphate, and the optimum physical parameters were pH 5 and 25 °C. The statistical design showed increased xylanase production with a maximum activity of 935 ± 2.3 IU/mL. The enzyme production was 3.8-fold higher than for the un-optimized Mendel's Stenberg Basal Salt medium (245 ± 1.9 IU/mL). The enzyme was stable over wide ranges of pH (3 to 10) and temperature (25 to 60 °C). The partially purified xylanase enzyme was used for the enzymatic hydrolysis of different lignocellulosic agro-residues.

INTRODUCTION

Biofuel from lignocellulosic biomass is a good alternative to conventional fossil fuels. Biofuel is considered carbon neutral (Nagraj *et al.* 2014) and a renewable energy source (Kaur *et al.* 2016) because the CO_2 produced by combustion can be utilized in the next cycle of plant growth. Lignocellulosic biomass is generated worldwide as agricultural and forest residues, and its disposal is a major concern (Dashtban *et al.* 2009; Knob *et al.* 2014). Lignocellulosic biomass consists of polymeric components, *i.e.*, lignin, cellulose, and hemicellulose. Hemicellulose is an integral part of lignocellulosic biomass and the second most abundant polymer in nature. Xylanases (endo-1,4-β-D-xylanohydrolase; EC3.2.1.8) are enzymes that play an important role in the conversion of lignocellulosic polysaccharides into various oligosaccharides and simpler monomeric sugars (Dutta *et al.* 2014). These monomeric sugars can be further converted to biofuels and value-added

products by microbial fermentation. Thus, the utilization of agricultural residues as substrates for xylanase production is useful and cost-effective, and it helps in lignocellulosic waste disposal (Singhvi and Gokhale 2013).

Various microorganisms have been utilized for xylanase production, including *Bacillus* sp. (Irfan *et al*. 2016), *Bacillus mojavensis* UEB-F (Kallel *et al*. 2016), *Actinomycetes*, and *Streptomyces* sp. (Kumar *et al*. 2012; Fatokun *et al*. 2016), as well as the fungal species *Aspergillus niger* (Pal and Khanum 2011), *Aspergillus oryzae* (Kimura *et al.* 2000; He *et al.* 2015) *Rhizopus stolonifer* (Zhang *et al.* 2013), and *Penicillium citrinum* (Saha and Ghosh 2014)*.* Filamentous fungi show promising results for commercial xylanase production because of their high production level of extracellular xylanase in a medium and their ease of cultivation (Knob *et al.* 2014; Uday *et al.* 2016; Zahari *et al.* 2016). Filamentous fungi can efficiently utilize various agro-residues for enhanced xylanase production (Knob *et al.* 2014).

Rice is a staple crop worldwide, with the result that a large amount of agricultural waste is generated every year. According to the United States Department of Agriculture (USDA 2017), the estimated global rice production was 481.14 million tonnes for the year 2016 to 2017. India contributes approximately 22.15% of that production, *i.e.*, 106.60 million tonnes. Rice straw is a readily available agro-residue with a rich hemicellulosic content (24% of dry weight) (Knob *et al.* 2014). Therefore, it can be utilized as an inexpensive alternate to xylan as a substrate for xylanase production.

The production of xylanase enzymes by different microbial strains is regulated by the physiological, nutritional, and biochemical nature of the microbes used. The xylanase yield is regulated by various factors, such as pH, temperature, carbon source, metal ions, and incubation time. Therefore, the medium composition and the physical parameters must be optimized to enhance xylanase production.

In the present study, a novel fungal strain of *Aspergillus oryzae* LC1 was isolated from a decomposed leaf sample of *Lantana camera* from a forest in the Cachar district of Assam, India. It was identified and characterized by morphological and molecular methods. The effective nutrients component of the Mendel's Stenberg Basal salt (MSBS) medium and selected agro-wastes were determined using a one factor at a time approach. Finally, the selected nutritional (rice straw, magnesium sulphate, and calcium chloride concentrations) and physical parameters (temperature and pH) were further optimized for xylanase production under submerged fermentation (SmF) with a central composite design (CCD) based response surface methodology (RSM).

EXPERIMENTAL

Materials

The chemicals used were of analytical grade and purchased from Hi-Media, Mumbai, Maharastra, India or Merck, Mumbai, Maharastra, India. Agro-residues were collected from local farms in Rajasthan, India. They were rinsed twice with distilled water and oven-dried (50 °C). They were ground in an electric grinder, sieved to a 2 to 5 mm mesh size, and stored in air tight poly bags until use.

Strain Isolation and Identification

The fungus was isolated from a decomposed leaf sample of *Lantana camera* collected from a forest in the Cachar district of Assam, India. The isolated strains were

transferred onto a PDA plate, incubated at 28 °C for 5 days, and stored in a refrigerator at 4 °C. The isolated strains were subjected to screening for xylanase production by plate assay as suggested by Meddeb-Mouelhi *et al.* (2014). The isolated fungal strains were grown on Czapek's dox agar medium containing 1% xylan for 5 days at 28 °C. The plates were flooded with 0.1% iodine solution. After 10 min the iodine solution were drained off followed by 1M sodium chloride washing. The fungi with clear zone is considered as positive for the production of xylanase.

The genomic DNA of the selected strain was extracted by using the protocol of Prabha *et al*. (2013). PCR amplification and DNA sequencing were performed on the partial rRNA region, as described by Schoch *et al*. (2012). The partial 18S rRNA SSU-ITS1-5.8SrRNA-ITS2- partial LSU rRNA region was amplified with the primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3'); ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3').

The PCR product was sequenced at Genomics Corp, Xceleris labs (Ahmedabad, Gujarat, India). The strain was identified with an ITS sequence analysis using BLASTn search tools (www.ncbi.nlm.nih.gov). To construct the phylogenetic tree, the related sequences from the blast output were downloaded for alignment and compiled in a multifasta format. These sequences were aligned using default parameters of the MUSCLE alignment tool with MEGA-5 software (Tamura *et al*. 2011). The alignments were manually edited with the MEGA editor, and the edited alignment file was used to infer the phylogenetic relatedness of the sequences. To establish the taxonomic rank of the isolate, the neighbour joining and maximum likelihood phylogenetic trees were made in MEGA by the following parameters: statistical method (neighbour joining), number of bootstrap replication (1000 replicates), substitution type (nucleotide), mode (jukes and cantor model), rates among sites (uniform rates), and gap missing deletion treatment (complete deletion).

Enzyme Production by Submerged Fermentation

Aspergillus oryzae LC1 was subjected to SmF for the quantitative assay of xylanase production. A spores suspension (4.35×10^7 spore/mL) of seven-day old culture of *A. oryzae*, was inoculated in a 250-mL Erlenmeyer flask that contained 100 mL of modified MSBS medium (1% Xylan w/v; 0.1% v/v Tween 80; 3.5 g/L $(NH4)_2SO_4$; 2.0 g/L KH_2PO_4; 0.3 g/L Urea; 0.2 g/L $CaCl_2$; 0.3 g/L $MgSO_4$; 5 mg/L $FeSO_4$; 1.6 mg/L $MnSO_4$; 1.4 mg/L $ZnSO_4 \cdot 7H_20$; 2.0 mg/L $CoCl_2$; 1 g Peptone; pH 5), which was modified according to Shah and Madamwar (2005). The selection of agro-wastes for xylanase production was performed by replacing 1% xylan with 1% rice straw (RS), rice husk (RH), wheat straw (WS), wheat bran (WB), and sugarcane bagasse (SB). The inoculated medium was kept under static conditions at 28 ± 1 °C. Samples were periodically drawn after intervals of 24 h. The culture broth was centrifuged at RCF of 9390 g for 10 min at room temperature, and the supernatant obtained was used for the enzyme assay. The un-inoculated sample was used as a control in each experiment.

Quantitative Assay of the Xylanase

The xylanase activity was assayed according to the method of Miller (1959). The total amount of reducing sugar liberated was measured. Enzyme extract (0.5 mL) was mixed to 0.5 mL of xylan solution. Xylan solution was prepared by adding 1% (w/v) birch wood xylan (4-O-methylglucuronoxylan) in acetate buffer (0.05 M, pH 4.8). The reaction mixture composed of xylan solution and enzyme extract was incubated at 50 °C for 30 min. The reaction was stopped by adding 1 mL of 3,5-dinitrosalicylic acid and incubating the

solution at 100 °C for 10 min. After cooling, the absorbance value at 540 nm was read using UV-VIS Double Beam spectrophotometer (Halo DB-30, Dynamica Asia Ltd, Kwai chung, Hong Kong). The amount of reducing sugar was measured by using xylose as a standard, as was suggested by Ghose and Bisaria (1987). One international unit (IU) of enzyme activity is defined as the number of μmol of reducing sugar (Xylose) produced per min of hydrolysis per mL of enzyme under assay conditions.

Experimental Design for the Optimization of Xylanase Production

A CCD based RSM was used to find the optimum concentrations of the nutritional components, along with the optimum physical factors for enhancing the production of xylanase enzyme by *A. oryzae* under SmF. In the present study, an experimental design protocol for RSM was developed using Design Expert V9 Software (Version 9.0.6, Stat-Ease Corporation, Minneapolis, MN, USA). The CCD was set up to study the interaction of five significant independent variables or factors for enhanced xylanase production, which were the percentage of rice straw (A), concentration of magnesium sulphate ($MgSO_4$) (B), concentration of calcium chloride ($CaCl_2$) (C), temperature (D), and pH (E). For the design, a 2^5 full factorial CCD with three coded levels (-1, 0, +1) was used, which led to a total number of 26 experimental runs (N) where $N = 2k - 1 + 2K + n_0$; where k is 5, which is the number of factors/variables to be tested). The minimum and maximum values for each variable were chosen on the basis of preliminary experimental data (data not shown). Table 1 represents the actual levels that corresponded to each factor. The complete experimental design is shown in Table 2 with the values of different variables in coded forms, along with the values of the dependent variable (response).

Table 1. Experimental Range and Three Levels of the Independent Variables for the CCD based RSM Experiment

Factor	Name	Min	Max	Coded	Values
A	Rice straw (%)	0.5	1	-1.0 = 0.5	1.0 = 1.5
B	Magnesium Sulphate (g/L)	0.3	0.7	-1.0 = 0.3	1.0 = 0.7
C	Calcium chloride (g/L)	0.5	1	-1.0 = 0.5	1.0 = 1
D	Temperature (°C)	25	45	-1.0 = 25	1.0 = 45
E	pH	3	7	-1.0 = 3	1.0 = 7

The results were expressed as the mean of triplicate values. The results obtained from the RSM were subjected to an analysis of variance (ANOVA). The response, *i.e.* measured xylanase activity (Y), was fitted using a second order polynomial equation (Eq. 1) and a multiple regression analysis, which was carried out to fit the data,

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_5 E + \beta_1\beta_2 AB + \beta_1\beta_3 AC + \beta_1\beta_4 AD + \beta_1\beta_5 AE + \beta_2\beta_3 BC + \beta_2\beta_4 BD + \beta_2\beta_5 BE + \beta_3\beta_4 CD + \beta_3\beta_5 CE + \beta_4\beta_5 DE + \beta_1^2 A^2 + \beta_2^2 B^2 + \beta_3^2 C^2 + \beta_4^2 D^2 + \beta_5^2 E^2 \tag{1}$$

where *Y* is the response variable, β_0 is the intercept, β_1, β_2, β_3, β_4, and β_5 are the linear coefficients, $\beta_1\beta_2$, $\beta_1\beta_3$, $\beta_1\beta_4$, $\beta_1\beta_5$, $\beta_2\beta_3$, $\beta_2\beta_4$, $\beta_2\beta_5$, $\beta_3\beta_4$, $\beta_3\beta_5$, and $\beta_4\beta_5$ are the interaction coefficients, and *A*, *B*, *C*, *D*, *E*, *AB*, *AC*, *AD*, *AE*, *BC*, *BD*, *BE*, *CD*, *CE*, and *DE* are the levels of the independent variables. This fitted polynomial equation was expressed as three-dimensional response surface contour plots that showed the relationship/interactions among the five independent factors, while the other parameters were kept constant. The determination of the statistical significance of the model was done by Fisher's test value

(f-test) and Student's t-test, and the quality of the fit was estimated by the coefficient of determination (R^2) and adjusted R^2 values.

Characterization of the Xylanase from *Aspergillus oryzae*

A calculated amount of solid ammonium sulphate was added to the culture supernatant from *A. oryzae* LC1 with constant stirring to achieve 60% saturation. After centrifugation at RCF of 6010 g at 4 °C for 20 min, supernatant was discarded and the precipitate was dissolved in small amount of same buffer used for enzyme assay. The enzyme solution was subjected to dialysis for overnight at 4˚C against 50 mM acetate buffer (pH 5.0), and 100 ppm sodium azide was added to avoid microbial contamination, with three alternating changes of the buffer, xylanase activity, and protein estimation was carried out.

This partially purified xylanase was characterized with respect to the temperature and pH optima. The optimum pH was determined by measuring the enzyme activity over a pH range of 3 to 10 in different buffers, *i.e.*, acetate (pH 3 to 6), phosphate (pH 7 to 8), and glycine-sodium hydroxide (pH 9 to 10). The optimum temperature for xylanase activity was determined by incubating the enzyme over a temperature range of 25 to 70 °C. The thermal stability and pH stability of the partially purified xylanase enzyme was studied based on the residual activity of the enzyme by incubating the enzyme at different pH values and temperatures for up to 24 h. At regular 2 h intervals, samples were withdrawn to determine the residual enzyme activity.

SDS – PAGE of Partially Purified Xylanase Enzyme

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli 1970, using 12% polyacrylamide. The gel was analysed by Coomassie brilliant blue R-250 staining method.

Application of Xylanase in the Hydrolysis of Agro-Residues

Different agro-residues, including RS, RH, WB, WS, SB, groundnut shell (NS), barley husk (BH), and pearl millet husk (PMH), were washed, dried, and sieved to a 2 to 5 mm particle size. The agro-residues were suspended in sodium acetate buffer (50 mM , pH 4.5) at 2% (w/v) and supplemented with xylanase enzyme (100 IU/g of substrate). The reaction mixture was then incubated at 50 °C. The total reaction volume was 10 mL. Every 2 h, the reaction mixture was withdrawn, centrifuged at RCF of 2350 g at 4 °C for 15 min, and the supernatants were used for the quantification of the reducing sugar content by the 3,5-dinitrosalicylic acid method using xylose as a standard. The saccharification percentage was calculated by the equation given by Baig *et al*. (2004), which is shown below (Eq. 2):

$$Saccharification\ (\%) = \frac{Xylose\ (\frac{mg}{mL})}{Substrate\ (\frac{mg}{mL})} * 100 \qquad (2)$$

The reducing sugar in the saccharification mixture was determined for 2 to 12 h, and the time profiles for the saccharification of the agro-residues were plotted.

Determination of Hydrolysis Products by Thin Layer Chromatography

The hydrolyzed products generated by the hydrolysis of xylan and other agro-residues by xylanase were identified by thin layer chromatography (TLC) by Chapla *et al.* (2012). The supernatants obtained after 24-h of the hydrolysis experiments were subjected

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to TLC on silica gel plates using 1-butanol/ethanol/water (5:3:2 v/v/v) as the mobile phase. The plates were air-dried at room temperature. The chromatogram was developed with an aniline diphenylamine reagent and placed in an oven at 100 °C for 10 min. The D-xylose was run as control. Formation of dark brown spots was confirmation of the presence of xylose.

RESULTS AND DISCUSSION

Isolation and Identification of the Strain

A total of 20 fungal cultures were isolated. The strains were subjected to plate and quantitative assays using xylan as the sole carbon source. A strain designated as LC1 exhibited the maximum xylanase activity as per the plate and quantitative assays.

A 619 base pair fragment of the 18s rDNA of the strain was amplified and sequenced. The size of the PCR product was confirmed by gel electrophoresis. The 18s rDNA gene sequence was identified by ITS sequence analysis. The sequence was compared with the BLASTn library, and the homology between LC1 and the reference strain YNCA1113 was approximately 99%. Based on the morphological characteristics and phylogenetic tree, the isolated strain was identified as *A. oryzae*.
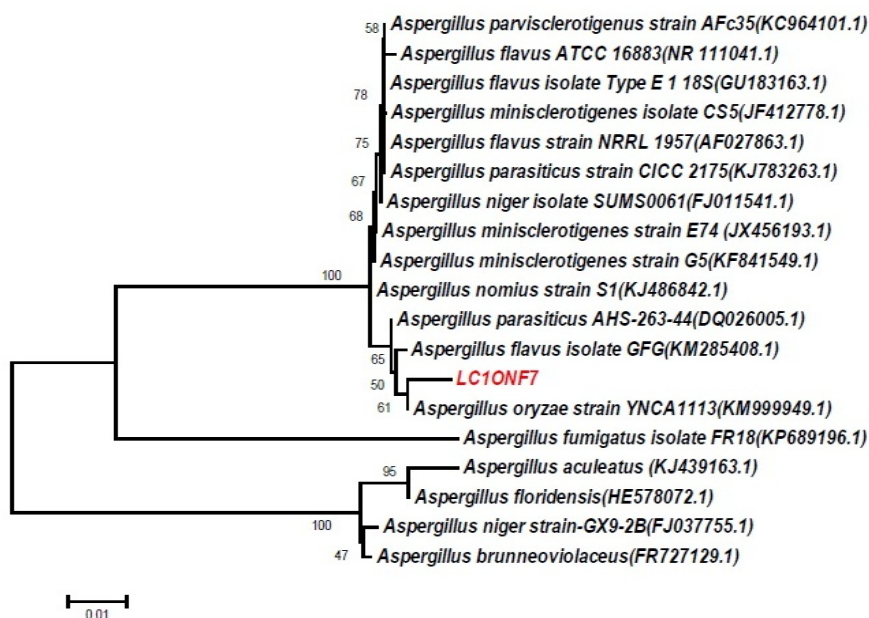

Fig. 1. Phylogenetic tree based on the 18s rDNA gene sequence of *A. oryzae* LC1 indicating the position of isolate among the sequences of closest phylogenetic neighbours obtained from NCBI BLAST analysis. Numbers in parenthesis indicate the accession numbers of corresponding sequences. Scale bar indicates evolutionary distances

Kinetics of xylanase Production by *A. oryzae* Using Modified MSBS Media

A. oryzae LC1 was subjected to SmF in a modified MSBS medium at a pH of 5 and temperature of 28 °C under static conditions. The maximum xylanase activity of 135 IU/mL was obtained after 14 d of incubation using 1% xylan as the sole carbon source under static conditions (Fig. 2.).
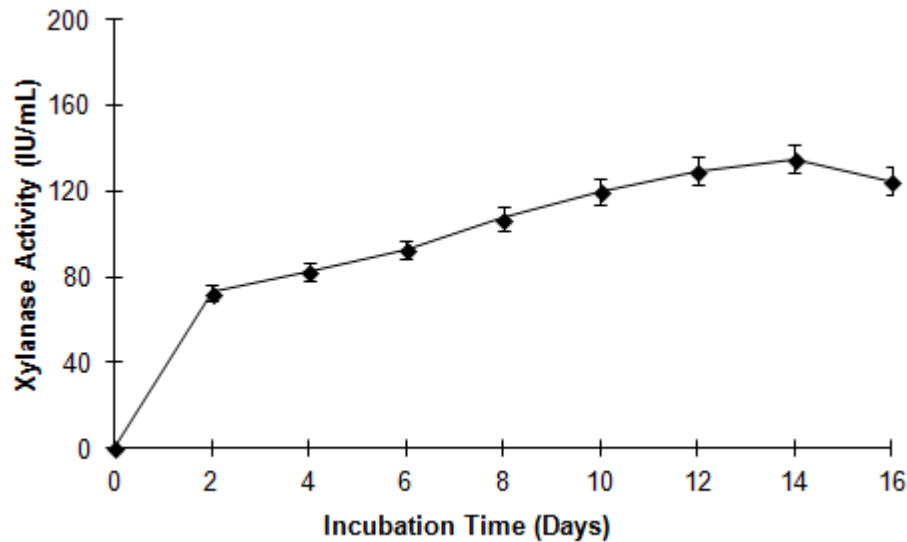
Fig. 2. Xylanase production by *A. oryzae* using modified MSBS media (1% xylan as carbon source)
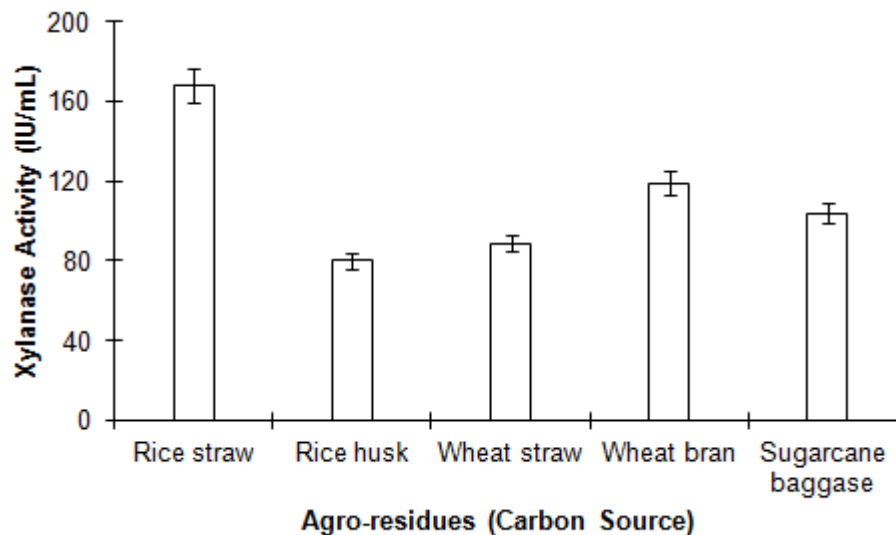

Fig. 3. Effect of different agro-residues on the xylanase activity

Selection of Agro-Residue for Xylanase Production

RS, RH, WS, WB, and SB were substituted as the sole carbon source in the modified MSBS media. Figure 3 shows that *A. oryzae* LC1 exhibited a maximum xylanase activity of 167.9 IU/mL after 6 d of incubation with RS as the sole carbon source, which was much higher than the other agro-residues. The xylanase activity obtained when RH, WS, WB, and SB were used as substrates were 80, 88.9, 119, and 103.9 IU/mL, respectively.

Statistical Optimization of the Nutritional and Physical Parameters for Xylanase Production from *A. oryzae* LC1

In the present work, RSM was employed using CCD to obtain a quadratic model. The independent variables were percentage of rice straw (A), concentration of magnesium sulphate (B), concentration of calcium chloride (C), temperature (D), and pH (E). The

experimental design was generated by the Design Expert software. Table 2 summarizes the central composite experimental plan, as well as the predicted and observed responses for each experiment.

Table 2. Experimental Design and Results of the CCD based RSM

Run	X1	X2	X3	X4	X5	Xylanase Activity (IU/mL)	
			Coded Values			Experimental	Predicted
1	-1	-1	1	-1	-1	368.74	382.12
2	-1	-1	-1	1	1	34.46	34.88
3	0	0	1	0	0	425.03	438.19
4	-1	-1	1	1	1	65.48	66.96
5	0	0	0	1	1	102.24	102.77
6	0	-1	-1	1	-1	13.78	-0.15
7	**0**	**-1**	**1**	**-1**	**0**	**535.3**	**514.55**
8	-1	1	1	1	-1	113.72	115.6
9	1	-1	0	0	-1	272.25	275.04
10	-1	1	0	-1	0	294.07	290.74
11	1	0	-1	-1	1	390.56	376.86
12	-1	-1	1	1	-1	96.49	95.1
13	0	-1	-1	-1	1	336.57	361.04
14	0	-1	1	1	1	99.94	96.23
15	-1	-1	1	-1	1	305.56	296.57
16	-1	1	1	-1	-1	281.44	277.03
17	0	-1	-1	-1	-1	400.9	403.93
18	0	1	1	1	-1	145.89	127.6
19	0	-1	1	1	-1	40.21	52.38
20	1	1	-1	1	0	33.31	44.23
21	0	1	-1	-1	-1	369.89	374.2
22	-1	-1	-1	-1	-1	372.18	363.22
23	-1	1	1	1	1	64.33	64.83
24	0	1	1	-1	1	371.04	386
25	0	1	-1	0	1	336.57	320.61
26	-1	1	-1	1	-1	124.06	133.48

The regression equation was obtained after the ANOVA provided an estimate of the level of xylanase production as a function of the independent variables. The production of xylanase enzyme incorporating the interactions of the different levels of each variable may be predicted best by the following polynomial mathematical model (Eq. 3),

$$Y = (414.283)(31.1701 \times A) + (-7.96657 \times B) + (20.0236 \times C) + (-127.022 \times D) + (-23.4161 \times E) + (13.678 \times AB) + (12.4415 \times AC) + (-33.2967 \times AD) + (17.9938 \times AE) + (-5.16226 \times BC) + (31.3982 \times BD) + (-5.66029 \times BE) + (-4.03226 \times CD) + (7.329 \times CE) + (14.3548 \times DE) + (-11.7998 \times A^2) + (3.63433 \times B^2) + (-27.9246 \times C^2) + (-122.479 \times D^2) + (-48.7459 \times E^2)$$ (3)

where Y is the xylanase enzyme production (IU/mL), A is the rice straw percentage (%), B is the magnesium sulphate concentration (g/L), C is the calcium chloride concentration (g/L), D is the temperature (°C), and E is the initial pH of the medium.

Statistical analysis was employed with Fisher's f-test and Student's t-test. Table 3 shows the summary of the ANOVA for the response surface quadratic model. The model F-value of 46.82 (p = 0.0002) implied that the model had significance, *i.e.*, there was only a 0.02% chance that noise could occur with such a high "Modal F-value". A "Prob > F" value, or p-value, less than 0.0500 indicates that the model terms are significant. In this case, A, C, D, E, AD, AE, BD, A^2, D^2, and E^2 were significant model terms.

Table 3. ANOVA for the Quadratic Polynomial Model of the Xylanase Production

Source	Sum of Squares	df	Mean Square	F-Value	p-value Prob > F	
Model	5.960E + 005	20	29800.91	46.82	0.0002	**Significant**
A - Rice straw	12667.47	1	12667.47	19.90	0.0066	
B - Magnesium sulphate	861.79	1	861.79	1.35	0.2971	
C - Calcium chloride	5652.16	1	5652.16	8.88	0.0308	
D – temperature	2.420E + 005	1	2.420E + 005	380.31	< 0.0001	
E – Ph	6982.63	1	6982.63	10.97	0.0212	
AB	3146.53	1	3146.53	4.94	0.0768	
AC	2559.96	1	2559.96	4.02	0.1012	
AD	19783.00	1	19783.00	31.08	0.0026	
AE	4376.87	1	4376.87	6.88	0.0470	
BC	343.14	1	343.14	0.54	0.4958	
BD	17526.45	1	17526.45	27.54	0.0033	
BE	429.06	1	429.06	0.67	0.4490	
CD	247.84	1	247.84	0.39	0.5600	
CE	680.32	1	680.32	1.07	0.3486	
DE	3065.15	1	3065.15	4.82	0.0796	
A^2	6268.74	1	6268.74	9.85	0.0257	
B^2	14.22	1	14.22	0.022	0.8870	
C^2	705.88	1	705.88	1.11	0.3405	
D^2	21502.97	1	21502.97	33.79	0.0021	
E^2	5257.03	1	5257.03	8.26	0.0348	
Residual	3182.19	5	636.44			
Core Total	5.992E + 005	25				

The optimum levels of the selected variables were obtained by analyzing the response surface contour and surface plots, along with solving Eq. 3 (Abdelhay *et al*. 2008; Singh *et al*. 2009).

The significance of the regression coefficient of the variables was determined using the Student's t-test. Similarly, the significance of the test was estimated by the p-value. The p-value of a test parameter below 0.05 indicates that the test was significant (Mohana *et al.* 2008). In general, a higher t and smaller p value result in a more significant corresponding coefficient term (Harris 1987; Mohana *et al.* 2008). In the present work, the fit of the model was evaluated by taking into consideration the R^2, predicted R^2, adjusted R^2, precision, and 'Lack of Fit'. The R^2 value of 0.9947 indicated that the model could explain 99.47% of the variability in the response (Tables 5). Therefore, Eq. 3 can be used for the response prediction for any combination of the five variables in the experimental design by substituting the corresponding coded values in the equation. The predicted R^2 value of 0.6093 was not as close to the adjusted R^2 value of 0.9734, *i.e.* the predicted R^2 was much lower than the regular R^2. This may have been because there were too many terms in the given model and the sample size was not very large (Rao and Satyanarayana 2007).

Table 4. Estimated Regression Coefficients for Xylanase

Coefficient Factor	Coef.	SE coef.	t-value		
			Low	High	p-value
Intercept	414.28	31.62	333.01	495.56	
A - Rice straw	31.17	6.99	13.21	49.13	0.0002
B - Magnesium sulphate	-7.97	6.85	-25.57	9.63	0.0066
C - Calcium chloride	20.02	6.72	2.75	37.3	0.2971
D - Temperature	-127.02	6.51	-143.77	-110.28	0.0308
E - pH	-23.42	7.07	-41.59	-5.24	< 0.0001
AB	13.68	6.15	-2.14	29.49	0.0212
AC	12.44	6.2	-3.51	28.39	0.0768
AD	-33.3	5.97	-48.65	-17.94	0.1012
AE	17.99	6.86	0.36	35.63	0.0026
BC	-5.16	7.03	-23.23	12.91	0.047
BD	31.4	5.98	16.02	46.78	0.4958
BE	-5.66	6.89	-23.38	12.06	0.0033
CD	-4.03	6.46	-20.64	12.58	0.449
CE	7.33	7.09	-10.89	25.55	0.56
DE	14.35	6.54	-2.46	31.17	0.3486
A^2	-11.8	3.76	-21.46	-2.13	0.0796
B^2	3.63	24.32	-58.87	66.14	0.0257
C^2	-27.92	26.52	-96.08	40.24	0.887
D^2	-122.48	21.07	-176.64	-68.31	0.3405
E^2	-48.75	16.96	-92.35	-5.15	0.0021

Table 5. Coefficient for Determination (R^2)

Std. Dev.	25.23	R^2	0.9947
Mean	230.54	Adjusted R^2	0.9734
C.V. %	10.94	Predicted R^2	0.6093
PRESS	2.341E + 005	Adequate Precision	22.701

A ratio of more than 4 is desirable and indicates an adequate signal. In the present work, the ratio of 22.7 was an adequate signal. The degree of freedom for "Lack of fit" was 5, which clearly implied that the lack of fit was not significant relative to the pure error. Therefore, the results from running the experimental design of the CCD fit per Eq. 3, which can be used effectively to navigate the design space.

Interactions Among the Independent Variables

The comparative effect of the interactions between the independent variables on the xylanase activity was described by response surface plots (3D) and their contour plots (2D), which were generated and plotted using the second order polynomial (Eq. 3). The response surface and contour plot can be utilized for predicting the optimum values for all of the independent variables for enhanced enzyme production. These plots showed interactions between rice straw and temperature, pH and rice straw, and temperature and magnesium sulphate, which were the most likely interaction combinations determined from the response evaluation.

Rice straw vs. temperature

The response surface curves and contour plot in Fig. 4A showed how the xylanase production was a function of the RS and temperature when keeping the levels of $MgSO_4$, $CaCl_2$, and pH at 0.5 g/L, 0.89 g/L, and 5, respectively. From Fig. 4A, it is evident that the xylanase production varied significantly with the temperature, and a higher activity was seen for a temperature range of 25 to 30 °C. The xylanase production was not much affected by the RS, as its activity remained almost unaltered over the concentration range of 0.5% to 1.0%. However, a high level of interaction was observed between the two variables.

The xylanase production improved significantly when the temperature decreased from 45 to 25 °C and the RS concentration was constant at 1%. Similarly, keeping the temperature constant at 25 °C and increasing the concentration of RS from 0.5% to 1% increased the xylanase production. The interactions between the two variables were also reflected in the ANOVA, where AD was a significant model term.

Rice straw vs. pH

The response surface curves and contour plot in Fig. 4B showed that the xylanase production was a function of the RS and pH when keeping the levels of $MgSO_4$, $CaCl_2$, and temperature at 0.5 g/L, 0.89 g/L, and 35 °C, respectively. From Fig. 4B, it was evident that the xylanase production varied significantly with pH. A higher activity was seen for the pH range of 3 to 5. However, at a pH above 5, a decrease in the xylanase activity was observed. The xylanase production was greatly increased as the RS concentration increased from 0.5% to 1.0%, while the pH was constant at 5. The interactions between the two variables were also reflected in the ANOVA, where AE was a significant model term.
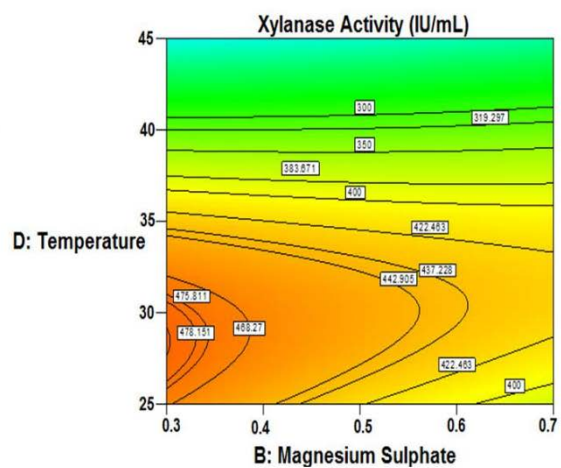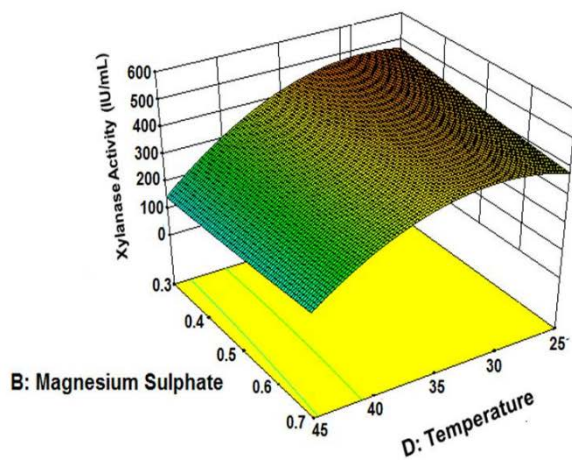
Magnesium sulphate vs. temperature

The response surface curves and contour plot in Fig. 4C showed that the xylanase production was a function of the magnesium sulphate concentration and temperature by keeping the levels of RS, $CaCl_2$, and pH at 0.75%, 0.89 g/L, and 5, respectively. From Fig. 4C, it was evident that the xylanase production varied significantly with the temperature, and a higher activity was found for the temperature range of 25 to 30 °C. The xylanase production was slightly affected by the magnesium sulphate concentration for the range of 0.3 to 0.7 g/L. However, a high level of interaction was observed between the two variables. The xylanase production was significantly improved when the temperature decreased from 45 to 25 °C and the magnesium sulphate concentration was 0.3 to 0.4 g/L. The interactions between the two variables were also reflected in the ANOVA, where BD was a significant model term.
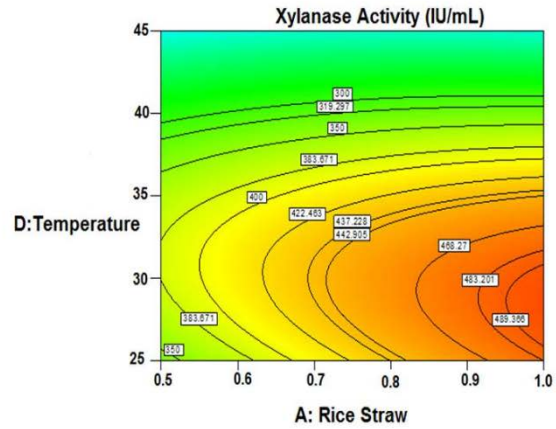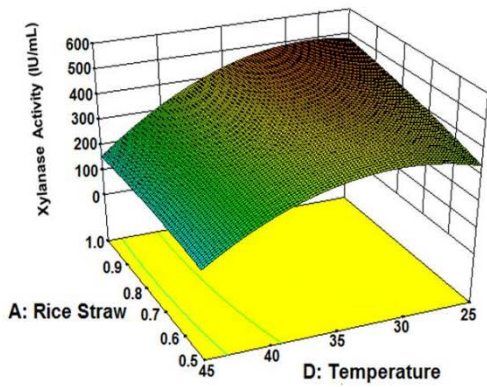
4 (A)
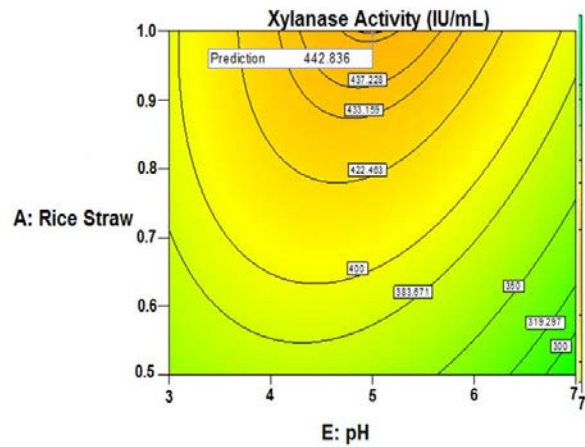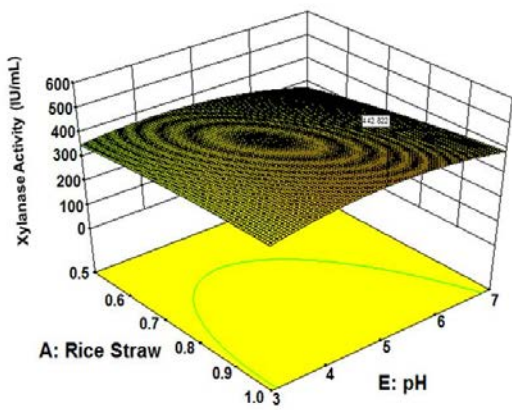
4(B)

4(C)

Fig. 4. Response surface curves and contour plots of xylanase production from *A. oryzae* showing the interaction between (A) RS and temperature, (B) RS and pH, and (C) magnesium sulphate and temperature

The optimum xylanase production was analyzed by the cube plot (Fig. 5.). The results indicated that the optimum predicted xylanase activity of 515.46 IU/mL was obtained at point D-, B-, and A+, which corresponded to a decreasing level of temperature and magnesium sulphate, and increasing level of RS at a pH of 5.
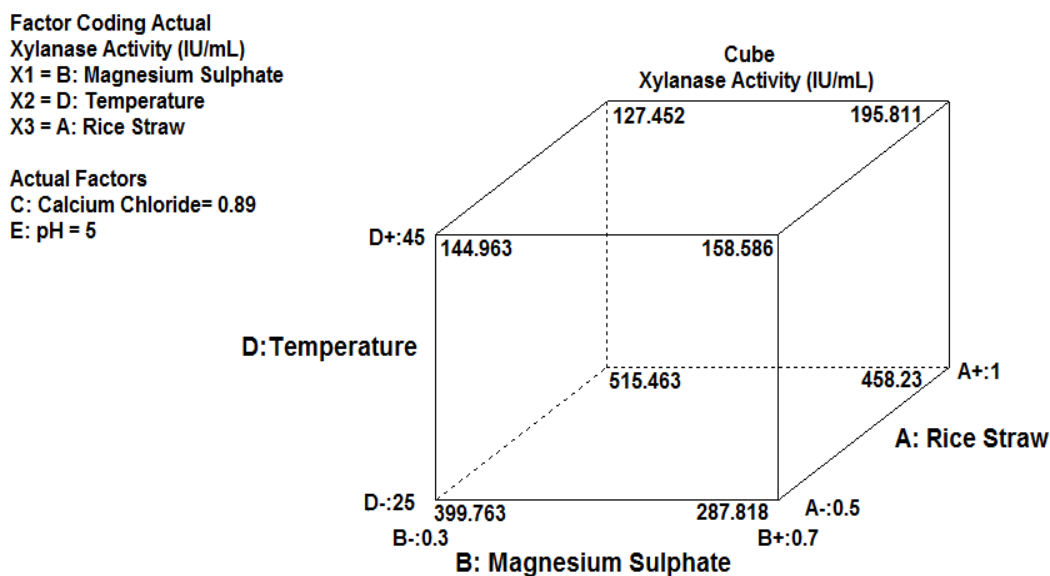
Fig. 5. Cube plot for analysis of the optimal points for xylanase production

Validation of the Model

The optimum values for the three most effective variables per the cube plot were determined to be 0.3 g/L magnesium sulphate, 1% (w/v) RS, and a temperature of 25 °C. The calcium chloride concentration was 0.89 to 1.0 g/L and the initial pH of the medium was set to 5.

The validity of the model was confirmed by using the optimized values for all of the variables in the MSBS medium for production of the xylanase enzyme. The validation experiments suggested that 1% RS, 0.3 g/L $MgSO_4$, 1.0 g/L $CaCl_2$, 25 °C, and a pH of 5 were the optimum conditions for xylanase production. These conditions led to a xylanase production of 535.3 IU/mL, which was comparable to the model predicted value of 514.46 IU/mL. Hence, the CCD based RSM models were considered to be accurate and reliable for optimizing the xylanase production.

Xylanase Enzyme Production Under Shaking Conditions

Xylanase production using the un-optimized and optimized parameters under shaking conditions (100 RPM) was conducted in order to assess the effect of shaking on the enzyme production. The maximum xylanase activity of 935 ± 2.3 IU/mL was obtained with the optimized parameters after 3 d of incubation. For the un-optimized parameters, the maximum xylanase yield obtained was 245 ± 1.9 IU/mL after 6 d of incubation. Therefore, using the optimized parameters, the xylanase yield was enhanced 3.8-fold and the time required for enzyme production was reduced by half, *i.e.* 3 d compared with 6 d for the un-optimized parameters under shaking conditions. The maximum xylanase activity observed for *A. oryzae* LC1 was higher than the maximum xylanase activity obtained by several other *Aspergillus* spp. The maximum xylanase activity for *A. fumigatus*

NITDGPKA3, *Aspergillus fumigates* AR1 and *Aspergillus niger* was 235.9, 135, and 65.8 U/mL using alkali pretreated rice straw, xylose and H_2O_2 + sodium hydroxide pretreated bagasse (Muhammad *et al.* 2010; Sarkar and Aikat 2012; Sarkar and Aikat 2014).
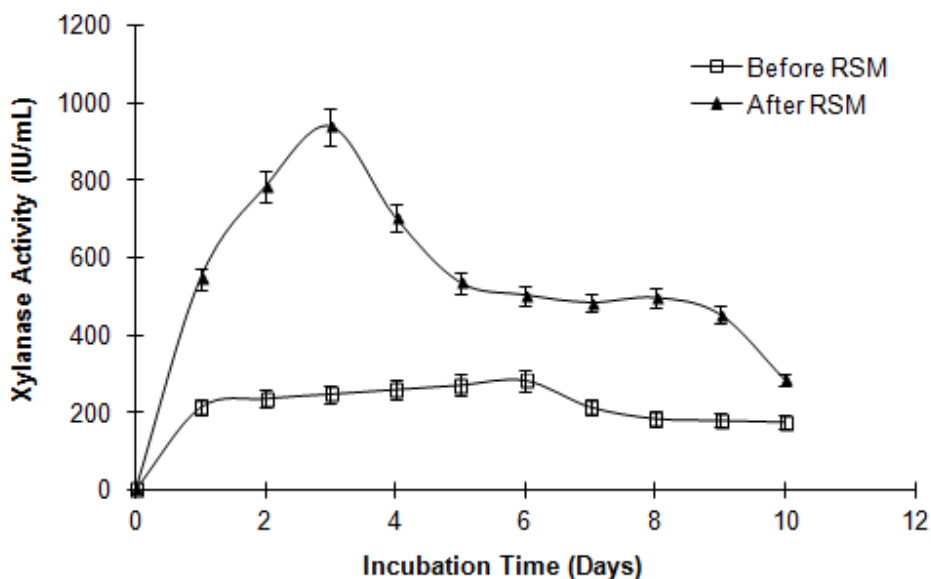

Fig. 6. Xylanase production by *A. oryzae* under shaking conditions using the un-optimized (before RSM) and optimized (after RSM) conditions

Characterization of the Xylanase from *A. oryzae*

The partially purified xylanase enzyme was characterized in order to find the optimum pH and temperature. The pH stability and thermal stability of the enzyme was determined by incubating the enzyme at the requisite pH and temperature, and the xylanase activity was then evaluated. The pH and temperature optima were found to be 5 and 25 °C, respectively (Figs. 7A and 7B). The enzyme was active over a wide pH range, *i.e.* 3 to 10, with only a 37% loss of activity at a pH of 10 when compared with the maximum activity of 4183.7 IU/mL at a pH of 5. The enzyme showed stability when the pH was alkaline and acidic both. As can be seen in Fig. 7D, it was clear that the enzyme retained 63% of activity in the pH range of 3 to 10 after 8 h of incubation. The enzyme showed thermal stability at temperature as high as 60 °C, where it retained 50% of activity after 6 h of incubation (Fig. 7C).

Previous studies by He *et al.* (2015) have also showed similar results where the xylanase enzyme obtained from *Aspergillus oryzae* HML 366 remained active within the pH range 4 to 10 and temperatures below 70 °C. Therefore as xylanase from *A. oryzae* LC1 is stable within the pH range of 3 to 10 and a temperature range of 25 to 60°C (Fig 7). It follows that the enzyme may be very useful for its application in paper, pulp and biofuel industries.

SDS – PAGE of Partially Purified Xylanase Enzyme

The partially purified enzyme was obtained on SDS PAGE. The molecular mass of the purified xylanase were estimated to be around 35 kDa (Fig. 8). This result is in accordance with the result obtained by He *et al.* (2015), where the molecular weight of xylanase enzyme XynF1 obtained from *Aspergillus oryzae* HML366 was 35.402 kDa.
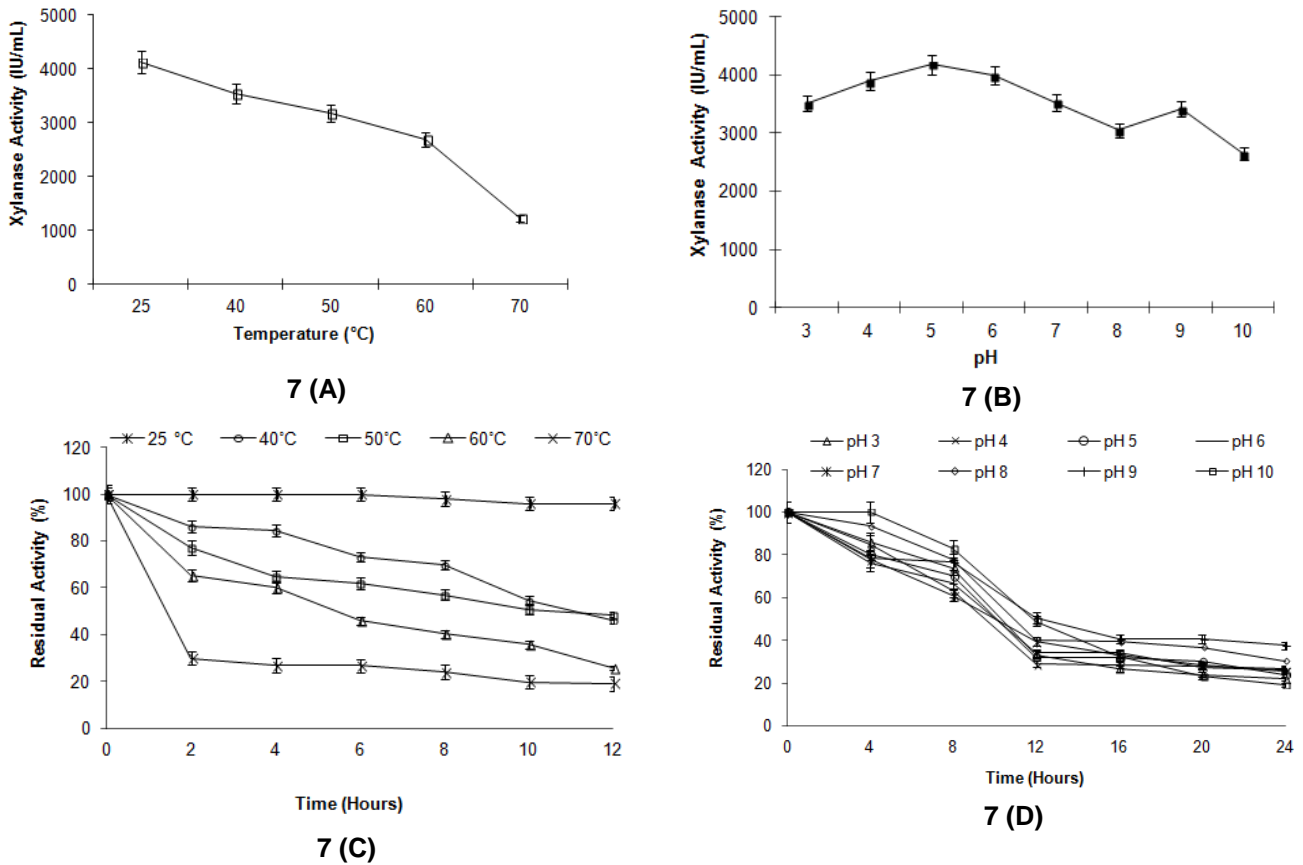
Fig. 7. Effect of different temperatures and pH values on the xylanase activity produced by *A. oryzae*; A: temperature optima of the xylanase enzyme; B: pH optima of the xylanase enzyme; C: thermal stability of the xylanase enzyme; and D: pH stability of the xylanase enzyme
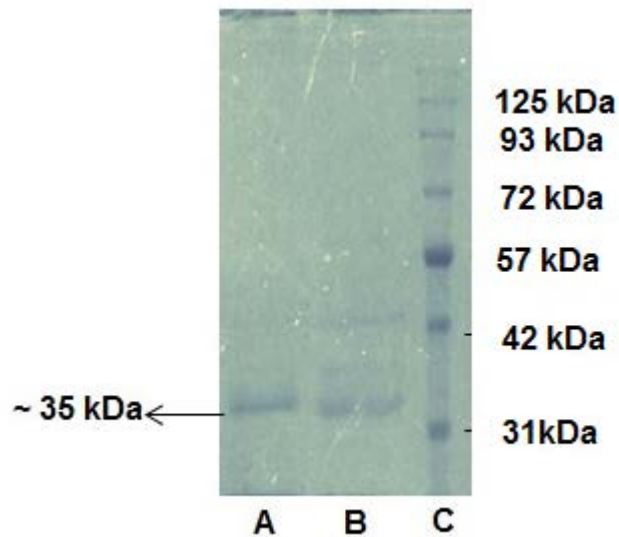

Fig. 8. SDS-PAGE showing purification of xylanase from *A. oryzae* LC1. Lanes: C molecular weight marker; A & B: Partially purified xylanase enzyme (Replicate)

The obtained xylanase enzyme had a high molecular weight. The protein fall in xylanase enzyme family glycosyl hydrolases (GH) 10 which are considered as high molecular weights (>30 kDa) with acidic pI values (Juturu and Wu 2012; He *et al*. 2015; Kocabacs *et al*. 2015).

Application of Xylanase in the Hydrolysis of Agro-Residues

The xylanase produced by *A. oryzae* was used for the hydrolysis of different agro-residues. The maximum saccharification percentage for the different agro-residues were 7.8% for RS, 34.5% for RH, 16.7% for WS, 8.9% for WB, 15.6% for SB, 28.1% for NS, 26.2% for BH, and 19.3% for PMH (Fig. 9).
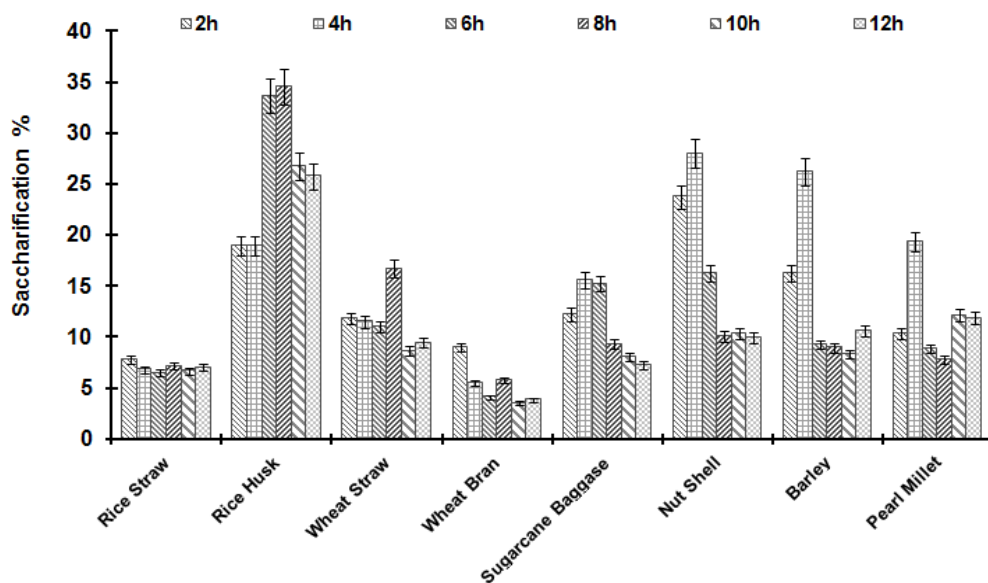

Fig. 9. Time profile of saccharification of agro-residues with xylanase from *A. oryzae*
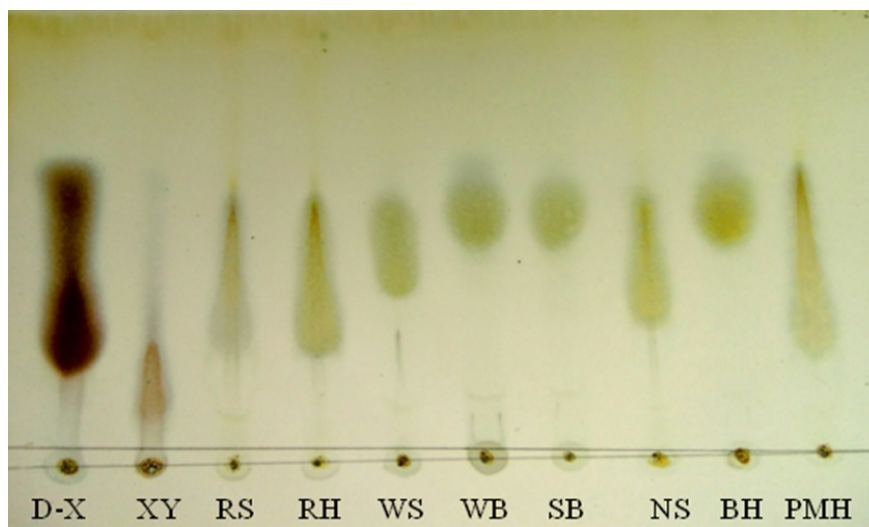

Fig. 10. TLC for the hydrolyzed product of xylan and agro-residues using D-xylose as a standard. From left to right, lanes in the gel represent: D-X: D-xylose standard; XY-1: Xylan hydrolysis after 2 h; XY-2: Xylan hydrolysis after 8 h; RS: rice straw; RH: rice husk; WS: wheat straw; WB: wheat bran; SB: sugarcane bagasse; NS: groundnut shell; BH: barley husk; PMH: pearl millet husk

Thus, the time course study of enzymatic saccharification revealed that the sugar yield increased until 8 h, and thereafter a slight decrease was observed. The saccharification yield can be further improved with various pretreatment methods (Chaturvedi and Verma 2013). The hydrolyzed products were further determined by TLC.

Determination of the Hydrolysis Products by TLC

The determination of the hydrolyzed product of the agro-residues by xylanase was performed by TLC. Spots were observed on the silica gel plates, which indicated that xylose was generated as a hydrolysis product of xylan (XY), RS, RH, WS, WB, SB, NS, BH, and PMH after 8 h of incubation when compared with the D-xylose standard (Fig. 10).

CONCLUSIONS

1. The statistical optimization of the nutritional and physical parameters for xylanase production under SmF of _A. oryzae_ LC1 was performed using a CCD based RSM approach. Various interactions between the independent variables were observed. The optimum conditions for enhanced production of xylanase for the nutritional components were determined to be 1% w/v RS, 0.3 g/L urea, 1.0 g/L $CaCl_2$, and 0.3 g/L $MgSO_4$, and the optimum physical parameters were a pH of 5 and incubation temperature of 25 °C. The amount of xylanase enzyme obtained with the optimized parameters was 935 ± 2.3 IU/mL after 3 d of incubation, which was 3.8-fold higher compared with the amount produced from the un-optimized conditions. Under the optimized conditions, the time required for xylanase production was reduced by half, _i.e._ 3 d compared with 6 d for the un-optimized conditions.

2. The enzyme showed stability over a wide pH range of 3 to 10 and thermal stability over the temperature range of 25 to 60 °C.

3. The partially purified xylanase enzyme was used for the hydrolysis of different lignocellulosic agro-residues. Among the different agro-residues, RH with xylanase had the maximum saccharification of 34.5% after 8 h of incubation at 50 °C. The monomeric sugars obtained after hydrolysis can be used for bioethanol (biofuel) production.

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