Enhanced production of bacterial xylanase and its utility in saccharification of sugarcane bagasse

Alokika[1] · Bijender Singh[1,2]

Abstract
An investigation was carried out using sugarcane bagasse as the agricultural residue to study the optimization of xylanase production by solid-state fermentation. Maximum xylanase production (20.35 U/g substrate) was achieved by *Bacillus substilis* subsp. *subtilis* JJBS250 using 'one variable at a time approach' at pH 7.0, 40 °C after 48 h. After statistical optimization by response surface methodology (RSM) there was 4.82-fold improvement in xylanase production (98.16 U/g substrate). Further optimization of untreated and sodium carbonate pretreated sugarcane bagasse enzymatic hydrolysis was carried out using both bacterial (*Bacillus substilis* subsp. *subtilis* JJBS250) and fungal (*Myceliophthora thermophila* BJTLRMDU3) xylanases that showed high amount of reducing sugar liberation from untreated sugarcane bagasse (124.24 mg/g substrate) as compared to pretreated (76.23 mg/g substrate) biomass. Furthermore, biophysical characterization of untreated and sodium carbonate pretreated sugarcane bagasse using Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD) and scanning electron microscopy (SEM), revealed the structural changes in the pretreated biomass.

Introduction

There is a major concern of environmental pollution due to the rising of agro-industrial waste generated from sugarcane bagasse, rice straw, wheat straw, wastes of vegetables, oil refinery, coconut husk, groundnut shell, etc. This leads to complexity in utilization and recycling of this waste. It consists of hemicelluloses (25%), lignin (25%) and cellulose (50%), which can be utilized by the microorganisms for the production of xylanases [1]. Solid-state fermentation is one of the eco-friendly alternatives to produce different value-added products (enzymes, biopesticides, bioflavor, biosurfactant, organic acids) via microbial fermentation using these agro-industrial residues which finally results in cleaner and greener environment [2]. Economical hemicellulosic

agricultural residues like sugarcane bagasse, rice straw, rice bran, wheat straw, wheat bran, corn cob and others have been reported as ideal solid substrate for the xylanase production using various fungal and bacterial genera including *Bacillus, Aspergillus*, *Clostridium*, *Rhizopus*, *Fusarium*, *Streptomyces*, *Micrococcus*, *Staphylococcus*, *Pseudomonas*, *Cellulomonas,* etc.[3].

Sugarcane bagasse is the major byproduct of the sugarcane industry, which consists of about 25% lignin, 25% hemicelluloses and 50% cellulose. The fibrous nature of sugarcane bagasse with high content of hemicellulose and cellulose induced the xylanase production by microorganisms. Due to huge availability, it is used as a perfect substrate for the production of various value-added products using microbial fermentations [4]. But the high content of lignin hinders the saccharification of sugarcane bagasse using enzymes. Therefore, pretreatment methods have been used to access cellulose and hemicellulose for enzymatic hydrolysis. Pretreatment disrupts the lignocellulosic matrix and removes lignin that is the main barrier of hydrolysis [5]. Xylan, the major constituent of hemicellulose, is a linear backbone of 1,4-β-linked D-xylose units [6]. Xylanases hydrolyse the β-1,4 D-xylopyranosyl

✉ Bijender Singh
ohlanbs@gmail.com

1 Laboratory of Bioprocess Technology, Department of Microbiology, Maharshi Dayanand University, Rohtak, Haryana 124001, India

2 Department of Biotechnology, Central University of Haryana, Jant-Pali, Mahendergarh, Haryana 123031, India

linkages of the β-1,4 D-xylans into monomeric and oligomeric sugars [7]. Xylanases are under enormous research due to their prospects in biofuel production, food industries, animal feed, paper and pulp, textiles industries and many others [3].

The economics of a microbial enzyme-based process can be determined by the cost of the enzyme which is one of the major factors. Therefore, optimizing the culture conditions is the main purpose of basic research for industrial applications [8]. RSM is a statistical technique used to design minimum number of experiments for large number of components to improve the enzyme activity. Using this, the connection between a group of controlled experimental factors and the observed results of one or more variables can be assessed [7]. Optimization of xylanase production was carried out in SSF using *B. subtilis* subsp. *subtilis* JJBS250. In the present research work, *B. subtilis* subsp. *subtilis* JJBS250 utilized low-cost sugarcane bagasse for maximum xylanase production followed by its applicability in saccharification of untreated and sodium carbonate pretreated sugarcane bagasse.

Materials and methods

Source and growth conditions of bacterial and fungal culture

Bacillus subtilis subsp. *subtilis* JJBS250 was isolated from soil sample and regularly grown on nutrient agar (pH 7.0) medium at 37 °C [9]. The bacterial culture was preserved in glycerol stocks at − 20 °C and on nutrient agar plates at 4 °C [10]. *Myceliophthora thermophila* BJTLRMDU3 was grown and maintained under conditions as described by Dahiya and Singh [11].

Cultivation in solid state fermentation

Bacterial xylanase cultivation medium containing 5 g of sugarcane bagasse substrate at a ratio of 4:1 with moistening medium [(%w/v) $(NH_4)_2SO_4$ 0.50, $MgSO_4 \cdot 7H_2O$ 0.10, K_2HPO_4 0.10 and NaCl 0.10] was autoclaved for 30 min at 121 °C. After cooling, the flasks were inoculated under sterile conditions and incubated at 35 °C. Similarly, fungal xylanase cultivation medium containing 5 g of substrate at a ratio of 1:7 with moistening medium [(% w/v) NH_4NO_3 2.0, K_2HPO_4 0.5, Sucrose 2.0 and Tween 20 3.0] was autoclaved for 30 min at 121 °C. After cooling, the flasks were inoculated under sterile conditions and incubated at 45 °C under static conditions for 4 days.

Table 1 Range of variables used for the response surface methodology

Independent variables	Levels				
	− α	− 1	0	+ 1	+ α
(A) Tryptone (%)	0.25	0.81	1.63	2.44	3.00
(B) Tween 20 (%)	0.25	0.81	1.63	2.44	3.00
(C) PEG 20000 (%)	0.10	0.49	1.05	1.61	2.00

Extraction of enzymes

After cultivation, 50 ml distilled water containing 0.1% Tween 80 was added to SSF medium and kept under shaking for 30 min at room temperature. Then, the obtained mixture was filtered, squeezed through the muslin cloth and the filtered crude supernatant was used as the xylanase source.

Xylanase assay

The xylanase assay was performed as described by Alokika et al. [9]. Using DNS method, the amount of reducing sugar liberation was estimated as reported by Miller [12]. One enzyme unit (IU) is defined as the amount of xylanase required to liberate 1µmole of xylose per min under the assay conditions.

Optimization of culture conditions for xylanase production

Xylanase production was first optimized by 'one variable at a time approach' and then by RSM a statistical approach under solid-state fermentation. Various culture conditions effect such as different substrates (wheat bran, wheat straw, rice bran, rice straw, sugarcane bagasse, cotton oil cake), pH (4.0–8.0), temperatures (30–45 ˚C), moistening media (tap water, distilled water, solution 1 [(% w/v) K_2HPO_4 0.1, $(NH_4)_2SO_4$ 1.0, $MgSO_4 \cdot 7H_2O$ 0.30, $CaCl_2$ 0.10, $FeSO_4 \cdot 7H_2O$ 0.10 and $MnSO_4$ 0.10], solution 2 [(% w/v) $(NH4)_2SO_4$ 0.50, $MgSO_4 \cdot 7H_2O$ 0.10, K_2HPO_4 0.10 and Nacl 0.10], solution3 [(% w/v) K_2HPO_4 1.0, $Cacl_2$ 0.03, $MgSO_4 \cdot 7H_2O$ 0.30 and $FeSO_4 \cdot 7H_2O$ 0.03]), different moisture ratio (1:2, 1:2.5, 1:3, 1:4), nitrogen sources (ammonium sulphate, ammonium nitrate, potassium nitrate, beef extract, yeast extract, tryptone, peptone), incubation time (24–96 h), surfactants (Tween 80, Tween 20, Triton X-100, SDS), polyethylene glycols (PEG 400, 4000, 8000, 20000) and water activity (100%, 0.95%, 0.92%, 0.87%, 0.82%).

On the basis of 'one variable at a time' approach, three variables viz. tryptone (A), tween 20 (B) and PEG 20000 (C) were selected as the most significant factors which affect

xylanase production. The basal medium containing sugarcane bagasse (5 g) optimized in the design was studied with five different levels of each variable at pH 7.0 (Table 1). With a set of 20 experiments, statistical software was used as described earlier [9].

Using quadratic equation, behaviour of the system was explained as follows:

$$
\begin{aligned}
Y = \ & \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 \\
& + \beta_{33} C^2 + \beta_1 \beta_2 AB + \beta_1 \beta_3 AC + \beta_2 \beta_3 BC
\end{aligned}
\tag{1}
$$

where Y denotes predicted response (Enzyme production), β_0 as intercept, β_1, β_2, β_3 as linear coefficient, β_{11}, β_{22}, β_{33} as squared coefficient, $\beta_{1,2}$, $\beta_{1,3}$, $\beta_{2,3}$ as interaction coefficients and A, B, C, A^2, B^2, C^2, AB, AC, BC as factors.

Pretreatment of sugarcane bagasse with sodium carbonate

Pretreatment of sugarcane bagasse was carried out using 10% (w/v) substrate and sodium carbonate (1% w/v) followed by autoclaving (121 °C) for 60 min. After autoclaving, the pretreated substrate was washed properly with distilled water and used for enzymatic hydrolysis after drying properly at 60 °C overnight.

Optimization of enzymatic hydrolysis

For enzymatic hydrolysis, 0.5 g of untreated and pretreated substrate supplemented with 20 ml potassium phosphate buffer (0.1 M) containing combination of bacterial and fungal xylanase was taken and kept under shaking conditions at 60˚C. Optimization was carried out using different parameters like enzyme doses (5–100 U/g), different pH (5.0, 7.0), different ratios of enzyme doses and different time intervals (0–48 h). Insoluble solids in the samples were removed after centrifugation at 10,000 rpm for 10 min and the amount of reducing sugars was evaluated as described earlier by Alokika et al. [9].

Biophysical characterization of sugarcane bagasse

Biophysical characterization of untreated and sodium carbonate pretreated samples of sugarcane bagasse was carried out using SEM, FTIR and XRD analysis. Surface images of untreated and pretreated sugarcane bagasse were obtained by JSM-6510 (JEOL) scanning electron microscope. For sample preparation, untreated and sodium carbonate pretreated sugarcane bagasse were mounted on aluminium stubs using double-coated tape with gold layer. Images were captured at 500 X magnification and 15.00 kV. FTIR analysis was carried out for the analysis of changes in functional group, morphological and structural modifications after the pretreatment of lignocellulosic biomass. The present work assessed chemical and structural changes of sodium carbonate pretreated sugarcane bagasse in comparison with untreated sugarcane bagasse. FTIR spectrum of samples was recorded between the resolutions of 500–4000 cm^{-1}, at 24 scans per sample [5]. XRD method was used for comparing cellulose crystallinity of untreated and pretreated sugarcane bagasse samples. First of all, the sugarcane bagasse samples were dried in oven at 60 °C overnight and then powdered samples were used for XRD (Rigaku Miniflex 600). Peaks were analysed between 10° to 70° 2-theta (deg) intensities and at a scanning speed of 0.002°/scan. Using following equation, cellulose crystallinity index was calculated [13]:

$$
X_c = \frac{I_{002} - I_{am}}{I_{002}} \times 100
\tag{2}
$$

where I_{am} and I_{002} are amorphous phase from the (002) lattice plane and peak intensity of crystalline.

Reducing sugar analysis using high-pressure liquid chromatography (HPLC)

The hydrolysates of untreated and sodium carbonate pretreated sugarcane bagasse of 24 h were evaluated using HPLC for analysing the presence of sugars. Column used for HPLC was Aminex 87hpx (BioRad, USA) using milli Q water as an eluent at flow rate of 0.3 ml/min at room temperature with refractive index (RI) detector.

Results

Culture condition optimization for xylanase production

Bacillus subtilis subsp. *subtilis* JJBS250 has been reported for xylanase production in submerged fermentation [9]. In the present study, xylanase production was observed using sugarcane bagasse under SSF. Sugarcane bagasse favoured high xylanase production (20.35 U/g substrate) by *B. subtilis* subsp. *subtilis* JJBS 250 as compared to other substrates (Fig. 1a) moistened with moistening medium [(% w/v) $(NH_4)_2SO_4$ 0.50, $MgSO_4 \cdot 7H_2O$ 0.10, K_2HPO_4 0.10, NaCl 0.10] with moisture to substrate ratio of 4:1 at 40 °C and pH 7.0 after 48 h. The transport of several enzymes across the cell membrane and the solubility of some medium components is highly influenced by pH [14]. High xylanase production (19.00 U/g substrate) was observed by bacterial culture at pH 7.0 (Fig. 1b). Results revealed maximum xylanase production (26.53 U/g substrate) by
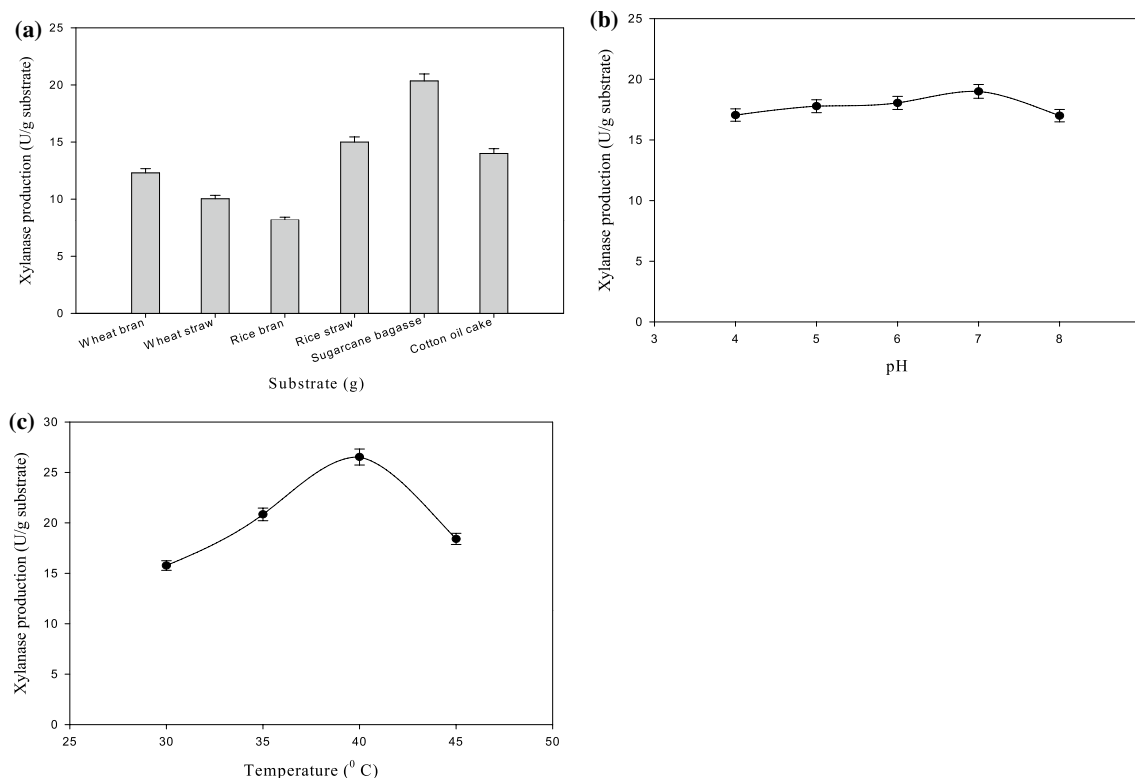
Fig. 1 **a** Effect of different substrates on xylanase production by *Bacillus subtilis* subsp. *subtilis* JJBS250. **b** Effect of different pH on xylanase production. **c** Effect of different temperatures on xylanase production by *Bacillus subtilis* subsp. *subtilis* JJBS250

bacterial culture at 40 °C followed by decline afterwards (Fig. 1c).

Maximum xylanase production (15.40 U/g substrate) was supported by solution 2 as compared to others (Table 2). Different moisture ratios were tested to check the optimum xylanase production. Results revealed that the moisture ratio of 1:4 was found optimum for high xylanase production (19.98 U/g substrate) (Table 2). The rate of water absorption by different substrates varies from one substrate to another [15]. Among all nitrogen sources, tryptone supported high xylanase titre (33.03 U/g substrate) by *B. subtilis* subsp. *subtilis* JJBS250 (Table 2). Using sugarcane bagasse as a substrate, xylanase production was observed maximally after 48 h (32.56 U/g substrate) (Table 2). Among all surfactants, high xylanase titre (32.59 U/g substrate) was observed by the addition of Tween 20 (Table 2). Xyalanse production was enhanced (32.54 U/g substrate) by the addition of PEG 20000 as compared to other (Table 2). Water activity effected xylanase production and high xylanase production (29.89 U/g substrate) was observed at 100% water activity (Table 2).

Optimization of fermentation parameters using statistical design for improved xylanase production

Maximum xylanase production (20.35 U/g substrate) was achieved by *B. subtilis* subsp. *subtilis* JJBS250 using 'one variable at a time approach' at 40 °C, pH 7.0 after 48 h. To enhance xylanase production, medium components were further optimized using RSM and xylanase production ranged from 61.58 U/g to 98.16 U/g (Table 3). The results were analysed using analysis of variance (ANOVA) and xylanase production (Y) was calculated from regression analysis as a function of these variables using the following equation:

$$
\begin{aligned}
\text{Xyalnase production (U/g :)} \; & 61.60 + 4.64A - 5.98B - 3.92C \\
& + 8.94A^2 + 7.17B^2 + 2.49C^2 \\
& + 1.19AB - 1.04AC - 2.07BC \\
& - 1.22B^3
\end{aligned}
$$
$$(3)$$

Different criteria like correlation coefficient (predicted R^2), coefficient of determination (adjusted R^2), adequate precision, model F and values of $P > F$ can be used for

Table 2 Optimization of different parameters using 'one variable at a time' approach

Culture conditions	Xylanase production (U/g substrate)
Moistening medium	
Tap water	7.65 ± 0.23
Distilled water	7.69 ± 0.23
Solution 1	11.34 ± 0.34
Solution 2	15.40 ± 0.46
Solution 3	15.10 ± 0.45
Substrate to moisture ratio	
1:2.0	17.57 ± 0.52
1:2.5	18.76 ± 0.56
1:3.0	18.97 ± 0.56
1:4.0	19.98 ± 0.59
Nitrogen source (0.5%)	
Peptone	28.76 ± 0.86
Tryptone	33.03 ± 0.99
Beef extract	28.76 ± 0.86
Yeast extract	20.64 ± 0.61
Ammonium sulphate	26.12 ± 0.78
Ammonium nitrate	21.45 ± 0.64
Potassium nitrate	15.97 ± 0.47
Incubation time (h)	
24	24.50 ± 0.73
36	27.95 ± 0.83
48	32.56 ± 0.97
56	29.37 ± 0.88
72	27.31 ± 0.81
96	25.67 ± 0.77
Surfactant (0.2%)	
Control	28.56 ± 0.85
Tween 20	32.59 ± 0.97
Tween 80	18.00 ± 0.54
SDS	14.95 ± 0.44
Triton-X 100	25.11 ± 0.75
PEG (0.2%)	
Control	25.30 ± 0.75
PEG 400	28.08 ± 0.84
PEG 4000	25.45 ± 0.76
PEG 8000	27.65 ± 0.82
PEG 20000	32.54 ± 0.97
Water activity (%)	
Control	29.89 ± 0.89
0.95	23.89 ± 0.71
0.92	21.86 ± 0.65
0.87	22.26 ± 0.66
0.82	21.25 ± 0.63

interpreting the quality of the model as given in Table 4. All these values justify the significance and variability in the model. The 3D plots of interactions between the variables showed maximum xylanase production upto the optimum level followed by decline afterwards (Fig. 2). The experimental value for xylanase production was observed as 98.16 U/g substrate, whereas predicted value was 97.71 U/g substrate. The experimental and predicted values are very close, hence, revealing that the model is significant for xylanase production. As a result of statistical analysis, there was 4.82-fold improvement in the xylanase production by *B. subtilis* subsp. *subtilis* JJBS250 using sugarcane bagasse as a substrate under SSF.

Optimization of enzymatic hydrolysis of untreated and sodium carbonate pretreated sugarcane bagasse

Hydrolysis of untreated and sodium carbonate pretreated sugarcane bagasse was carried out using bacterial and fungal xylanases and reducing sugars were analysed. Results revealed that from the hydrolysis of untreated sugarcane bagasse, high amount of reducing sugars was attained by both bacterial (36.39 mg/g substrate) and fungal xylanases (34.19 mg/g substrate) at 20 U/g (Fig. 3a). Among different pH values, high amount of reducing sugars was released by the synergistic action of bacterial and fungal xylanases at pH 7.0 (37.80 mg/g substrate) (Fig. 3b). A combination comprising of 12 U/g bacterial xylanase and 8 U/g fungal xylanase resulted in enhanced saccharification of sugarcane bagasse (36.17 mg/g substrate). High amount of reducing sugars was released from untreated sugarcane bagasse (124.24 mg/g substrate) as compared to sodium carbonate pretreated biomass (76.23 mg/g substrate) after 48 h (Fig. 3c).

Biophysical characterization using SEM, FTIR and XRD analysis

SEM produces images by scanning the surface of samples with a focused beam of electrons. In our study, surface images of untreated and sodium carbonate pretreated sugarcane bagasse were compared. Figure 4a showed rough, compact, rigid, ordered structure of untreated sugarcane bagasse while pretreated sugarcane bagasse showed loosening of fibrous network, with appearance of lignin droplets and pores (Fig. 4b).

FTIR spectroscopy is used to examine morphological, structural changes of untreated and pretreated lignocellulosic biomass [5, 16]. The FTIR spectrum of untreated,

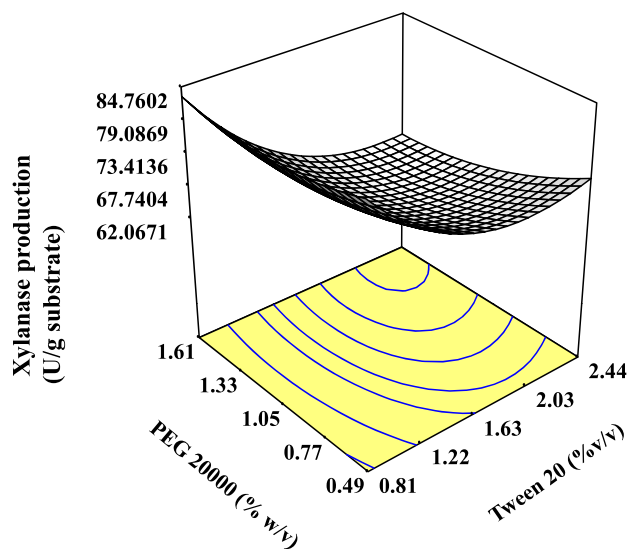**Table 3** Experimental design and results of central composite design of response surface methodology

Run no	Tryptone (%)	Tween 20 (%)	PEG 20000 (%)	Xylanase production (U/g substrate)
1	+1	−1	+1	67.70±2.03
2	0	0	0	61.58±1.84
3	0	0	0	61.58±1.84
4	0	0	0	66.90±2.00
5	+1	−1	−1	70.94±2.12
6	0	0	+α	72.58±2.17
7	0	0	0	61.58±1.84
8	−1	+1	+1	64.44±1.93
9	0	0	0	61.58±1.84
10	−1	−1	+1	82.72±2.48
11	−1	+1	−1	71.76±2.15
12	+α	0	0	95.72±2.87
13	0	0	−α	91.66±2.74
14	−α	0	0	87.98±2.63
15	0	−α	0	98.16±2.94
16	0	+α	0	66.48±1.99
17	0	0	0	61.58±1.84
18	0	0	0	61.58±1.84
19	−1	−1	−1	62.00±1.86
20	+1	+1	−1	76.64±2.29

Table 4 Analysis of variance and regression analysis for xylanase production by *Bacillus subtilis* subsp. *subtilis* JJBS250 in SSF

Source	Sum of squares	DF	Mean square	F value	Prob > F
Model	3215.24	10	321.52	755.74	<0.0001
A	294.45	1	294.45	692.10	<0.0001
B	101.42	1	101.42	238.40	<0.0001
C	211.13	1	211.13	496.25	<0.0001
A^2	1152.52	1	1152.52	2709.00	<0.0001
B^2	739.78	1	739.78	1738.86	<0.0001
C2	90.52	1	90.52	212.76	<0.0001
AB	11.28	1	11.28	26.52	0.0006
AC	8.61	1	8.61	20.24	0.0015
BC	34.20	1	34.20	80.38	<0.0001
B^3	16.42	1	16.42	38.60	0.0002

R^2: 0.9988, Adj. R^2: 0.9975, Pred R^2: 0.9767, Adeq precision: 74.647

Fig. 2 Three-dimensional graphs showing the effect and interaction of PEG 20000 and Tween 80 on xylanase production

sodium carbonate pretreated and enzymatic hydrolyzed sugarcane bagasse showed relative changes in the absorbance at specific band positions (Fig. 4c). According to the previous studies, the region between 3100 and 3600 cm^{-1} has been assigned to the OH stretching and 2890–2940 cm^{-1} for C–H stretching. Various other ranges like between, 1660–1630, 1600–1610, 1505–1510 are associated with conjugated C=O stretch aromatic skeletal vibrations. Bands between 1380–1370, 1060–1040 showed

C–H deformation in cellulose and hemicelluloses and C-O stretch in cellulose and hemicelluloses, respectively. In our result, untreated and enzyme treated sugarcane bagasse shows highest absorbance but sodium carbonate pretreated ones show smallest absorbance. The absorption bands and corresponding assignments are given in Table 5. Results
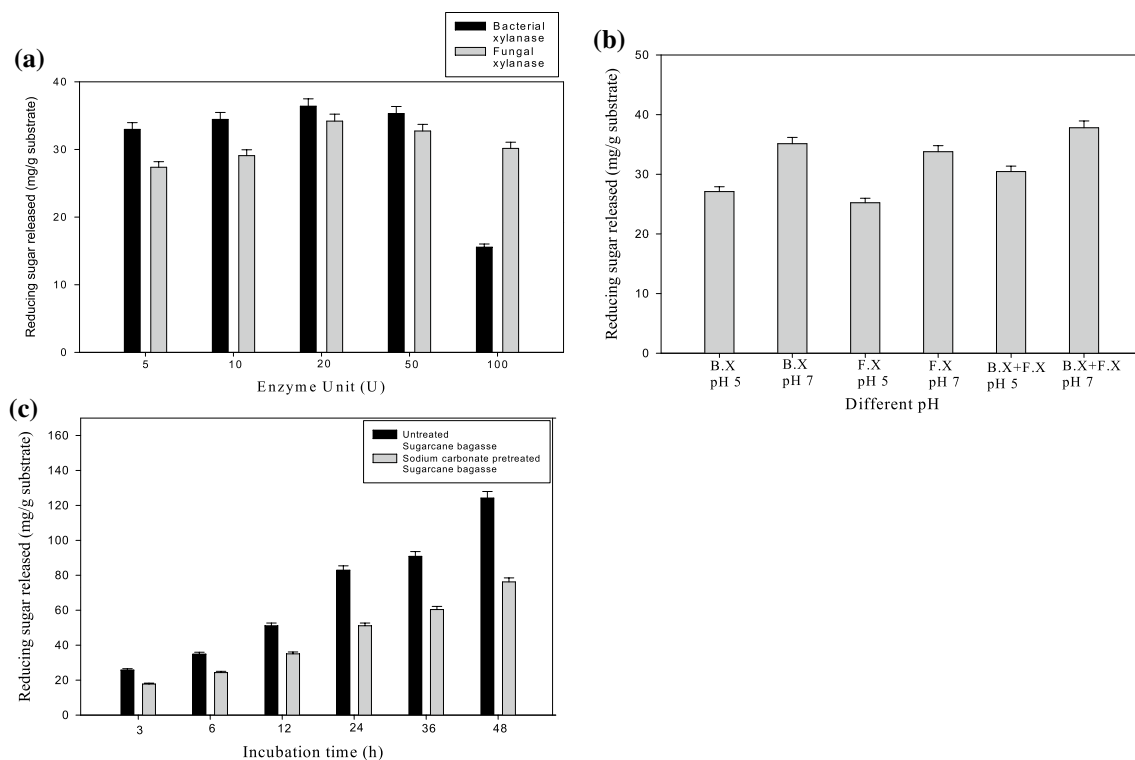
Fig. 3 Optimization of enzymatic hydrolysis of untreated sugarcane bagasse at **a** different enzyme doses, **b** enzyme doses with different pH, BX and FX represents bacterial xylanase and fungal xylanase, respectively and **c** effect of reaction time on the saccharification of untreated and sodium carbonate pretreated sugarcane bagasse

revealed that the peak around 3330 cm^{-1} assigned to the OH stretching and 2897 to the C–H stretching. Bands at 1602 cm^{-1}, 1631 cm^{-1} and 1633 cm^{-1} are associated with conjugated C=O stretch aromatic skeletal vibrations. Peaks around 1514 cm^{-1} and 1031 cm^{-1} attributed to aromatic ring vibrations and C–O stretch (primary alcohol).

X-ray diffraction (XRD) analysis is a suitable technique which revealed the crystallinity index of lignocellulosic biomass. Crystallinity index (Crl, %) is used to measure the relative amount of crystalline material present in the lignocellulosic biomass and it also affects the enzymatic saccharification of biomass [5, 17]. After various pretreatments, cellulose structure and crystallinity in the biomass could change due to disrupting inter and intra chain hydrogen bonding of cellulose fibrils [16]. Our both samples showed typical XRD peaks of cellulose at 2-theta of 20–30° (degree) (Fig. 4d). Sodium carbonate preteated sugarcane bagasse showed more elevated peak in comparison to untreated biomass.

Reducing sugar analysis

HPLC profiles of hydrolysates of untreated and sodium carbonate pretreated sugarcane bagasse showed release of different sugars like glucose, xylose, xylohexose, cellobiose and arabinose (Fig. 5a, b). These sugars can be utilized in many fermentation processes. A good amount of xylose, xylohexose and cellobiose released by untreated bagasse is also advantageous because many microorganisms converted these sugars into value-added products like biofuels.

Discussion

Solid-state fermentation by utilizing less cost agricultural residues provides various economic advantages for the production of enzymes that can be used in various industrial applications [14]. In our study, sugarcane bagasse favoured high xylanase production by *Bacillus subtilis* subsp. *subtilis* JJBS250 as compared to other substrates. The high xylanase yield on sugarcane bagasse was due to relatively high levels of hemicellulose in this substrate [15]. Similarly, high xylanase titre was reported by *Aspergillus niger* [18], *Penicillium citrinum* [2], *Trichoderma viride*-IR05 [19] using sugarcane bagasse as a substrate under SSF conditions. High xylanase production was observed by bacterial culture at pH 7.0. Similarly, Ho and Heng [20] observed maximum xylanase production at pH 6.5 by *Bacillus subtilis.* Some other investigators reported highest xylanase titre at pH 7.0 by *Streptomyces* sp [21]. and by *Bacillus* sp. at pH 8.0 under SSF [14]. Maximum xylanase production by *B. subtilis* subsp. *subtilis*
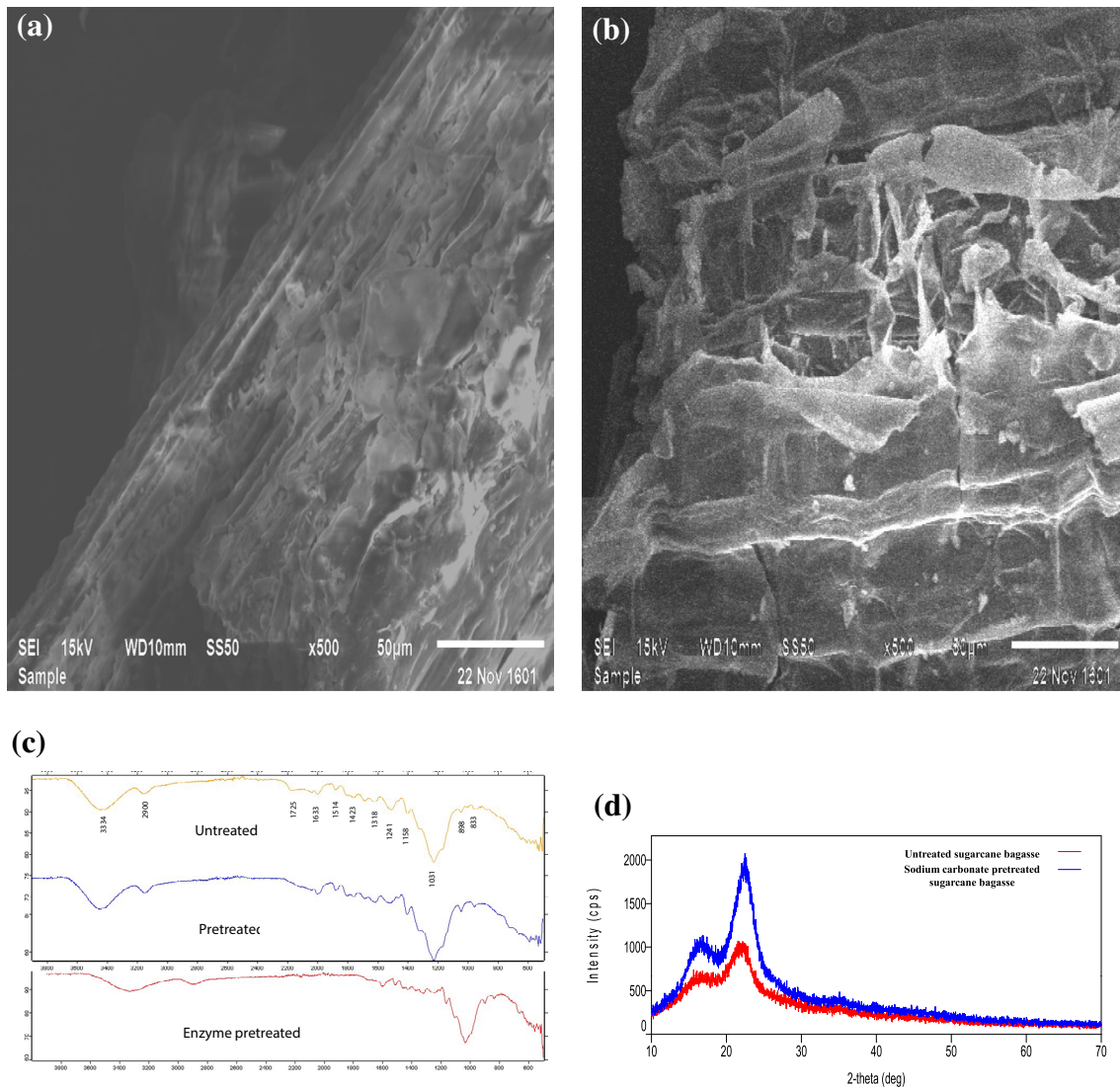
Fig. 4 Scanning electron microscopy of **a** untreated and **b** sodium carbonate pretreated sugarcane bagasse. **c** Fourier-transform infrared spectra of untreated, pretreated and enzymatic treated sugarcane bagasse. **d** X-ray diffraction spectra of untreated (blue) and pretreated (red) sugarcane bagasse (color figure online)

JJBS250 was recorded at 40 °C that declined afterwards. Similarly, high xylanase production by *B. subtilis* [20], *B. subtilis* ASH [22], *B. megatherium* [23], *Bacillus* sp. PKD [14] and *B. pumilus* VLK-1 [24] was attained at 37 ˚C in SSF. Abdel-Sater and El-Said [25] observed high xylanase titre at 35 ˚C using *Trichoderma harzianum.*

The maximum xylanase production was supported by solution 2 [(% w/v) $(NH_4)_2SO_4$ 0.50, $MgSO_4 \cdot 7H_2O$ 0.10, K_2HPO_4 0.10 and Nacl 0.10]. Ghoshal et al. [2] observed high xylanase titre by *P. citrinum* using Czapek yeast extract media (201.91 U/gds) as compared to other moistening media. In the study of Sanghi et al. [22] high xylanase production (7988 ± 10.41 U/g) was reported using moistening solution by *Bacillus subtilis* ASH. The moisture ratio of 1:4 was found optimum for high xylanase production by *Bacillus*

subtilis subsp. *subtilis* JJBS250 as also reported by Panwar et al. [14]. The available water can be absorbed by substrate particles, which provides best conditions for microbial metabolic activity and growth [4]. There is lower degree of swelling and higher water tension of the nutrients of the solid substrate due to lower moisture content [26]. In some other findings, highest xylanase production was reported at a substrate to moisture ratio of 1:2.5 and 1:2 by *B. pumilus* VLK-1 [24] and *B. subtilis* ASH [22], respectively.

Among all nitrogen sources, tryptone supported high xylanase titre by *B. subtilis* subsp. *subtilis* JJBS250. Similarly, Irfan et al. [19] found high xylanase production by *T. viride*-IR05 using tryptone as a nitrogen source. In contrast, Kumar et al. [24] and Shangi et al. [22] observed maximum xylanase production using yeast extract by *B. pumilus*

Table 5 FTIR spectra bands of untreated, sodium carbonate pretreated and enzyme treated sugarcane bagasse

Bands (cm^{-1})	Assignment
3325–3335	O–H stretching
2890–2900	C–H stretching
1725	Unconjugated C=O stretch in xylans
1630–135, 1600–1605, 1500–1520	Conjugated C=O stretch aromatic skeletal vibrations
1420–1425	Aromatic ring vibration and C–H deformation
1315–1320	Syringyl nuclei (C–O stretching)
1240–1245	Guaiacyl nuclei (C–O stretching)
1155–1165	C–O–C vibration in cellulose and hemicellulose
1025–1035	C–O stretch in cellulose and hemicellulose
895–900	C–H deformation in cellulose
830–835	C–H out of plane vibration in lignin
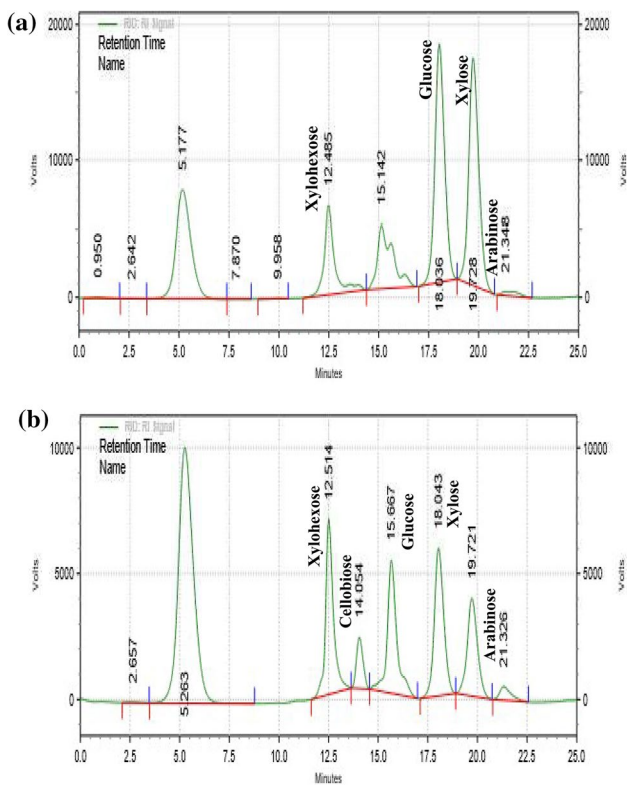
Fig. 5 High performance liquid chromatography profiles of enzymatic hydrolysate of **a** untreated sugarcane bagasse and **b** sodium carbonate pretreated sugarcane bagasse after 24 h of enzymatic hydrolysis

VLK-1 and *B. subtilis* ASH, respectively. There was gradual increase in enzyme production upto 48 h by the bacterial culture in SSF as reported by Ho and Heng [20]. After that there was gradual decrease in enzyme activity that could be

due to the depletion of essential growth nutrients and accumulation of toxic products in the medium [26]. Duration of incubation time affects the growth rate of microorganism and enzyme production. Therefore, in case of bacteria as compared to fungi, less time is required for enzyme production due to their faster doubling time [22]. In contrast, Poorna and Prema [27] and Sanghi et al. [22] reported high xylanase titre after 72 h by the microorganisms in SSF.

Among all surfactants, high xylanase titre was observed by the addition of Tween 20 as reported in *Streptomyces* sp. ESRAA-301097 [21]. In some other reports, enhanced xylanase production was observed by addition of Tween 80 under SSF [19, 24, 28]. Xyalanse production by bacterial culture was also enhanced by the addition of PEG 20000 as reported in *B. pumilus* MK001 by Kapoor et al. [29]. Sharma et al. [30] also observed that addition of PEG improved laccase production from *Ganoderma* sp. kk-02. Maximum xylanase production by the bacterial culture was observed at 100% water activity (aw 0.99) as reported for xylanase production by *Sporotrichum thermophile* BJAMDU5 [28]. In contrast, Singh and Satyanarayana [31] and Kumar and Satyanarayana [32] reported high phytase and glucoamylase production by *S. thermophile* and *Thermomucor indicae-seudaticae* at low water activity (aw 0.95).

In our study, as a result of RSM, there was 4.82-fold improvement in the xylanase production by *B. subtilis* subsp. *subtilis* JJBS250 using sugarcane bagasse as a substrate under SSF. Khusro et al. [7] observed 3.7-fold improvement in xylanase production as a result of RSM by *B. subtilis* ARMATI. Panwer et al. [14] observed 1.30-fold increase in the production of xylanase by *Bacillus* sp. PKD after statistical optimization.

Usually high amounts of reducing sugars have been reported after pretreatment of lignocellulosic biomass. But present work showed antagonistic results in terms of liberation of reducing sugars. In this study, high amounts of reducing sugars were liberated from untreated sugarcane bagasse (124.24 mg/g substrate) as compared to sodium carbonate pretreated bagasse (76.23 mg/g substrate). This may be due to pretreatment of bagasse by physical grinding used for reducing particle size. Saccharification of sugarcane bagasse was significantly increased after various pretreatment using xylanolytic and cellulolytic enzymes of *S. thermophile* [28]. Chong et al. [33] reported that dilute alkali pretreatment enhanced the saccharification of sugarcane bagasse. Similarly, Chandel et al. [17] observed high reducing sugars (48.50 g/l) after acid/base pretreatment of sugarcane bagasse.

Morphological analysis of untreated and pretreated sugarcane bagasse using SEM showed rough, rigid and highly ordered structure of untreated sugarcane bagasse as compared to pretreated one which showed partial

deconstruction of biomass surface, exposure of fibres and high disruption [5]. Similarly, untreated sugarcane bagasse showed rigid, highly ordered and compact structures while more fragmented structures were observed in pretreated samples [33]. Chandel et al. [17] reported rough, compact and thick-walled parallel fibres in untreated sugarcane bagasse, while disrupted cell wall with loose matrix and pores was shown in pretreated biomass.

In our result, untreated and enzyme-treated sugarcane bagasse showed higher absorbance values as compared to sodium carbonate pretreated biomass. This is due to the structural changes in the biomass as a result of pretreatments that modifies the structures of all the macromolecules like cellolose, hemicelluloses and lignin present in plat biomass [5, 17, 34]. Bala and Singh [5] observed structural and functional changes in the pretreated biomass. Nosratpour et al. [34] reported reduction in total crystallinity index of sodium carbonate pretreated biomass. Our results revealed that peaks of untreated and pretreated sugarcane bagasse for cellulose was recorded at 2-theta of 20–30°. Sodium carbonate preteated sugarcane bagasse showed more elevated peak as compared to untreated biomass revealing the removal of non-crystalline components after pretreatment. Similar findings have been observed earlier for chemically pretreated sugarcane bagasse [16, 17].

In our study, HPLC profile of enzymatic hydrolysates of untreated and sodium carbonate pretreated sugarcane bagasse showed the release of many sugars like glucose, xylose, xylohexose, cellobiose and arabinose as reported by Bala and Singh [5] In another study, saccharification of green liquor-ethanol pretreated sugarcane bagasse showed the presence of xylose, glucose, glycerol, ethanol and acetic acid using HPLC [35].

Conclusions

Xylanase production by *B. subtilis* subsp. *subtilis* JJBS250 in SSF using sugarcane bagasse was enhanced due to optimization. Optimization of three significant variables using RSM resulted in 4.82-fold higher xylanase production. Moreover, sodium carbonate pretreatment does not affect enzymatic hydrolysis of sugarcane bagasse because untreated sugarcane bagasse liberated high amounts of reducing sugars as compared to untreated biomass. Biophysical characterization using SEM, FTIR and XRD analysis showed structural changes in untreated and pretreated sugarcane bagasse. Furthermore, liberation of various reducing sugars in the enzymatic hydrolysate was confirmed by HPLC data.

Compliance with ethical standards

Conflict of interest The authors declared that they have no conflict of interest.

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