XYLANASE PRODUCTION FROM BACILLUS SUBTILIS IN SUBMERGED FERMENTATION USING BOX-BEHENKEN DESIGN

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ABSTRACT
In this study, an attempt was made to optimize the nutritional conditions for xylanase production by Bacillus subtilis in submerged fermentation process using agricultural waste like corn cobs as substrate. Three variables with three levels such as corn cobs loading (1, 3, 5, % w/v), peptone (0.05, 0.275, 0.5%) and KH₂PO₄ (0.1, 0.3, 0.5%) were optimized through Box-Bhenken design of response surface methodology. It was revealed that the maximum yield of xylanase (295 U/ml) was achieved with 3% corn cobs as substrate, 0.05 (%) peptone, and 0.5 (%) KH₂PO₄. Analysis of variance reveals that the proposed model was significant having an F value of 188.77 and its corresponding p values 0.000 and, P>F<0.0001 shows the model’s accuracy. Higher R² values (98.93) of the model depicted that only 1.07% variations could not be explained by the model. Findings of this study could be utilized for industrial exploitation of the enzyme.

Keywords: Corn cobs, xylanase, Bacillus sp., Response Surface Methodology, submerged fermentation

INTRODUCTION
Xylan is a main constitue of plant hemicelluloses, and is a polymer of xylose molecules, which plays a significant role in holding plants cell wall together (Techapun et al., 2002). Several enzymes are needed for biodegradation of this complex component of plant cell wall. Along with main chain breaking enzymes few side chain breaking enzymes also take part in hydrolysis of xylan. Xylanase (E.C 3.2.1.8) is the enzyme which degrades β-1,4 xylan by cleaving β-1,4 glycosidic linkages thus forming useful products such as xylose, xylobiose like xylooligosaccharides (Bernier, et al., 1983; Chakrit, et al., 2006).

However joint action of many enzymes is needed for this complex process. Amongst them xylanase (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) is of great importance, because it cleaves internal linkage of β-1,4-xylose backbone (Whistler and Richard, 1970). After breaking these complex linkage, xylanase (E.C 3.2.1.8) converts xylan into useful products like xylobiose, xylooligosaccharides and xylose (Bernier et al., 1983; Chakrit, et al., 2006). Two methods are generally used for xylanase production, these are submerged fermentation (SmF) and solid-state fermentation (SSF). In sub-merged fermentation (SmF) microorganisms and substrate are homogeneously distributed in a liquid medium. It is noticed that approximately 80-90% of total xylanases are manufactured through submerged culture fermentation (Ho and Heng, 2015). Submerged fermentation is favored mostly, due to more accessibility to nutrients, sufficient supply of oxygen, and demand of small time duration for the fermentation (Gomes and Stiener, 1994; Hoq, et al., 1994; Veluz et al., 1999; Gouda, 2000).

SsF can be defined as growth of microorganisms on a wet sheet of solid substrate along with continuous supply of air for uninterrupted period (Gessesse and Mamo, 1999). In the process of SsF, flow of free water is not required, because it already contains enough moisture for microorganisms metabolism (Pandey et al., 1999; Halritch et al., 1996).

Xylanases have many applications and uses since their xylan degrading enzyme system is significantly present in Fungi (Belancic et al., 1995; Biely et al., 1985) as Actinomycetes (Elegir et al., 1995) and Bacteria (Dey et al., 1992). Xylanases produced by microbes have some advantages over xylanases from animal and plant source, since microbes have constant organization and easy genetic manipulation (Bilgrami and Pandy, 1992). An elevated level of extra-cellular xylanase has been reported to be exuded by bacterial species (Polizelli et al., 2005). Bacterial xylanases usually have more advantages and uses over xylanases obtained from yeasts (Frost and Moss, 1987). Moreover, due to simple genetic material their genetic handling and environmental manipulation became more convenient, that increases bacterial cell growth (Demain et al., 1971). To manufacture
significant level of extracellular xylanase, bacteria are the most suitable choice (Aarti et al., 2015; Nagar et al., 2013). As compared to all microorganisms, family of Bacillus is a more suitable choice to produce microbial enzymes. Bacillus family has been used for years due to its ability to manufacture huge quantity of enzymes like xylanase, protease, chitinase, amylase, lipase, pullulanase. And production of these enzymes represents about 60% worldwide, because their manufacturing is important commercially (Morikawa et al., 2006). Response surface methodology (RSM) is a most popular method which is frequently being practiced in optimization of medium components and other significant variables that are applied in manufacturing of different biomolecules (Xiong et al., 2004). Furthermore, in recent years RSM has become a popular method for different biochemical and biotechnological processes (Bas and Boyaci, 2007). RSM is a famous method used to improve the production of microbial xylanases (Coman and Bahrim, 2011). Basically, RSM is a fusion of mathematical and statistical tools that is frequently being practiced to study outcomes of elected sovereign variables (Myers and Montgomery, 1995).

Nowadays xylanases have wide range of applications in many industrial processes such as softening of fruits, extraction of plant oils, digestibility of animal feed, clarification of juices and beer bio-bleaching of kraft pulp and Biocon-version of agricultural wastes and degumming of plant fibers (Khosarvi-Darani and Karamad 2016; Dhiman et al., 2008; Eriksson, 1990).

Regardless of this reality, that use of xylanase in industries has got well acknowledgement, but in paper industry use of xylanase is still facing problems due to expenditure on its manufacturing (Lei et al., 2008). It is reported that enzymes impart a significant role in baking industry and xylanase has been widely used in making of bread (Beg et al., 2001). Xylanases are also helpful in manufacturing of beer, because it increases sugar production by fermentation of barley (Garg et al., 2010). Xylanases are also involved in germination of plant seeds, as they change stored food into assaulted end product. It is suggested that xylanase plays in important part in cell elongation and reduction of fruit hardness (Kulkarni et al., 1999). The main aim of this study was optimization of medium components for enhanced xylanase production through response surface methodology using corn cobs as substrate in submerged fermentation.

**MATERIALS AND METHODS**

**Chemicals/Biochemicals:** All the chemicals/biochemicals used in present study were of analytical grade and purchased from Sigma (USA), Merck (Germany), Fluka (Switzerland) and Acros (Belgium). Agricultural residue such as corn cobs, was purchased from the local market of Sargodha city.

**Microorganism:** Bacillus subtilis was obtained from Microbiology laboratory, Department of Food Science and Technology, University of Sargodha, Sargodha, Pakistan.

**Inoculum preparation:** Inoculum was prepared inoculating a loop full of 24 h old strain of Bacillus subtilis in nutrient broth and incubated at 35°C with shaking speed of 120 rpm for 24 h. The cell culture obtained after 24h was used as an inoculum source.

**Fermentation Technique:** Twenty-five milliliter of medium components as designed through response surface methodology was taken into 250-ml Erlenmeyer flask which was cotton plugged and autoclaved at 121°C for 15 min and 15 psi. After sterilization, the medium was allowed to cool at room temperature and inoculated by 1 ml cell suspension of 24 h old Bacillus subtilis strain and incubated at 35°C for 24 h of fermentation time. After the termination of the fermentation period, the culture broth was centrifuged at 10,000 x g for 10 min at 4 °C. The cell free supernatant obtained was used as the crude source of xylanase enzyme.

**Xylanase Assay:** Xylanase activity was assayed by incubating 0.5 ml of appropriately diluted culture filtrate with 0.5 ml of 1% birchwood xylan (Sigma) solution prepared in citrate buffer (0.05 M, pH 5.0) for 15 min at 50°C. After incubation, the reaction was stopped by the addition of 1.75 ml of 3, 5-dinitrosalicylic acid (Miller 1959) and heated for 10 min in boiling water bath. After cooling the reducing sugars liberated were measured by spectropho-metrically at 550 nm and expressed as xylose equivalent. Xylose was taken as standard.

One unit of activity was defined as the amount of enzyme, which liberates reducing sugar
(equivalent to xylose) from 1.0% Birch wood xylan under standard assay conditions.

Xylanase activity (IU) = OD(Optimal density) × Dilution factor × Standard factor × 1000 / (Incubation period)

**Experimental design**

Box-Behnken design with three variables and three levels like substrate concentration (1, 3, 5%), peptone concentration (0.05, 0.275, 0.5%) and KH₂PO₄ concentration (0.1, 0.3, 0.5%) were optimized by *Bacillus subtilis* in submerged fermentation. The coded and actual values were mentioned in Table 1.

**Table 1:** Code and actual level of the three independent variables for the design of enzyme production experiment used in the BBD.

<table>
<thead>
<tr>
<th>Independent Variable</th>
<th>Code</th>
<th>Code and actual factor level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn cob concentration (%)</td>
<td>A</td>
<td>1 -1 3 5</td>
</tr>
<tr>
<td>Peptone concentration (%)</td>
<td>B</td>
<td>0.05 0.275 0.5</td>
</tr>
<tr>
<td>KH₂PO₄ concentration (%)</td>
<td>C</td>
<td>0.1 0.3 0.5</td>
</tr>
</tbody>
</table>

**Statistical analysis:** Minitab v. 17.0 Version of Statistical software package was used to plot the response surfaces and regression analysis of experimental data. Statistical parameters were examined through Analysis of variance (ANOVA). And values differences were showed in terms of probability p < 0.05 values.

**RESULTS**

**Box- Behnken design for xylanase production by *Bacillus subtilis* in submerged fermentation**

In our study, we used Box-Behnken design (BBD) of response surface methodology to investigate the effect of 3 parameters with three levels on xylanase production. These three parameters used were corn cob concentration as carbon source, peptone as nitrogen source and minerals like KH₂PO₄ concentration which were abbreviated as A, B and C respectively. Experiments were conducted as per design created through Minitab software version 17 and the response obtained was calculated through second order polynomial regression equation (Eq. 1). Table 2 shows that xylanase activity range from 144 to 295 U/ml in all runs. Run no. 15 gave maximum xylanase activity with residual difference of 0.11468 between observed and predicted with corn cob concentration of 3.0%, peptone concentration of 0.05% and 0.5% of KH₂PO₄ concentration.

Xylanase activity (U/ml) = 277.7 - 18.85 (A) - 139.3 (B) + 348.5 C + 0.09 (A²) - 587.1(B²) - 568 (C²) + 123.64 (A) × (B) - 60.0 (A) × (C) + 48.7 (B×C)  

**Analysis of Variance for xylanase production by *Bacillus subtilis* in submerged fermentation**

Data was statistically analyzed by analysis of variance (ANOVA). The model’s F value of 188.77 and its corresponding p value 0.000 and, P>F<0.0001 shows model’s accuracy (Table 3). Higher R² values of 98.93, and adjusted R² values of 96.99% and predicted R² of 94.38, indicates that actual results were in accordance with the values predicted by the model (Fig. 1). A p-value of less than 0.05 shows significance of the model. Model terms A, B, C and quadratic term interactions AxB, BxC, AxC, A², B², and C² were also found significant.
Table 3: Analysis of variance for xylanase production by *B. subtilis* in submerged fermentation.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Adj.SS</th>
<th>Adj.MS</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>9</td>
<td>30205.1</td>
<td>3356.1</td>
<td>51.17</td>
<td>0.00</td>
</tr>
<tr>
<td>Linear</td>
<td>3</td>
<td>10624.0</td>
<td>3541.3</td>
<td>53.99</td>
<td>0.00</td>
</tr>
<tr>
<td>Substrate Conc. (A)</td>
<td>1</td>
<td>169.2</td>
<td>169.2</td>
<td>2.58</td>
<td>0.169</td>
</tr>
<tr>
<td>Peptone (B)</td>
<td>1</td>
<td>2385.2</td>
<td>2385.2</td>
<td>36.36</td>
<td>0.002</td>
</tr>
<tr>
<td>KH₂PO₄ (C)</td>
<td>1</td>
<td>8069.5</td>
<td>8069.5</td>
<td>123.03</td>
<td>0.000</td>
</tr>
<tr>
<td>Square</td>
<td>3</td>
<td>4874.0</td>
<td>1624.7</td>
<td>24.77</td>
<td>0.002</td>
</tr>
<tr>
<td>A²</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>0.01</td>
<td>0.933</td>
</tr>
<tr>
<td>B²</td>
<td>1</td>
<td>3262.0</td>
<td>3262.0</td>
<td>49.73</td>
<td>0.001</td>
</tr>
<tr>
<td>C²</td>
<td>1</td>
<td>1903.6</td>
<td>1903.6</td>
<td>29.02</td>
<td>0.003</td>
</tr>
<tr>
<td>2-Way Interaction</td>
<td>3</td>
<td>14707.1</td>
<td>4902.4</td>
<td>74.74</td>
<td>0.000</td>
</tr>
<tr>
<td>AxB</td>
<td>1</td>
<td>12381.9</td>
<td>12381.9</td>
<td>188.77</td>
<td>0.000</td>
</tr>
<tr>
<td>(AxC)</td>
<td>1</td>
<td>2306.0</td>
<td>2306.0</td>
<td>2306.0</td>
<td>0.002</td>
</tr>
<tr>
<td>BxC</td>
<td>1</td>
<td>19.2</td>
<td>19.2</td>
<td>0.29</td>
<td>0.612</td>
</tr>
<tr>
<td>Error</td>
<td>5</td>
<td>328.0</td>
<td>65.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lack-of-Fit</td>
<td>3</td>
<td>71.1</td>
<td>23.7</td>
<td>0.18</td>
<td>0.899</td>
</tr>
<tr>
<td>Pure Error</td>
<td>2</td>
<td>256.8</td>
<td>128.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>30533.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1: Observed values versus mathematical model prediction of xylanase activity.

**2D contour plots**

Figure 2 depicted contours plots for xylanase activity which were made from two selected independent variables keeping the value of third variable persistent at its central value to get optimum conditions for maximum xylanase production. These plots were represented by different colors which indicated different levels of xylanase production between two independent parameters and keeping third parameter at constant value. These graphs indicated that each parameter had significant impact on xylanase production by *Bacillus subtilis* in submerged fermentation.
Figure 2: Effect of KH\textsubscript{2}PO\textsubscript{4}, peptone and corncobs concentration on xylanase production by \textit{Bacillus subtilis} in submerged fermentation.

Figure 3 explains the desirability chart for xylanase production by \textit{Bacillus subtilis} using corncob as substrate in submerged fermentation. This chart showed that if corncob concentration of 3%, peptone concentration of 0.275% and KH\textsubscript{2}PO\textsubscript{4} concentration of 0.3% was used, the xylanase production was ranged from minimum 123.53 to maximum 310.33 U/ml. In our experiment, when we used these concentration, maximum xylanase production of 257.66 U/ml was obtained which were within the range as per prediction.

Figure 3: Desirability chart of xylanase activity. Describing predicted and desired values for 3 parameters A, B and C i.e substrate concentration, peptone and KH\textsubscript{2}PO\textsubscript{4} respectively.

**DISCUSSION**

The results of the present study revealed that \textit{B. subtilis} can produce xylanase enzyme using corn cobs as a cheapest substrate in submerged fermentation. In this study, bacterial strains were used for xylanase enzyme production which has advantage of short period of growth as compared to the fungi. Our study indicated that nutrients and cultural properties played a pivotal role in enzyme production. Earlier studies reported that organic nitrogen sources have been found to stimulate xylanase production in \textit{Bacillus} species (Battan et al., 2007). In our study, we used peptone as nitrogen source for media supplementation. Different concentration was used but maximum yield was obtained with media comprising of 0.05% of peptone as nitrogen source. Among all the tested inorganic and organic nitrogen sources, tryptone and (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} are best for \textit{B. subtilis} BS04 while KNO\textsubscript{3} and malt extract for \textit{B. megaterium} BM07 for xylanase synthesis in submerged fermentation (Irfan et al., 2016). Walia et al., (2013) also found in their study, that basal salt yeast extract medium (BSYEM) has significant role enhancing xylanase (Sharma 1998) production on apple pomace. And reduction in enzyme yield in Basal salt medium (BSM) without yeast extract lead to the reduction in growth of the organism.

The results of our study also conclude that corn cobs which are inexpensive as well as abundantly produce higher amount of activity of xylanase.
among the natural substrates used. Since pure commercial xylan is too expensive to be used as substrate. So it is suggested that corn cobs may be a good alternative for xylanase production from industrial point of view run number 15 with 3% of substrate, i.e., corn cobs as carbon source yielded maximum xylanase. The effect of easily metabolizable sugars (glucose, xylose, fructose, maltose, cellulbiose and lactose) on xylanase production by Aspergillus tamarii in solid-state fermentation (SSF) was studied by de Souza et al., (2001) using wheat bran, corn cob and sugar cane bagasse as substrate. Kumar et al., (2009) also reported that xylanase production may be enhanced to 3299 ± 95 U ml⁻¹ by using corn cobs as carbon source under optimized growth conditions.

In this study we also used different concentrations of KH₂PO₄ as mineral source among different concentrations used 0.5% KH₂PO₄ give xylanase activity up to 295 (U/ml). Along with other parameters, minerals also play pivotal role in xylanase production. Chaturvedi et al., (2015) used different sources of minerals such as KH₂PO₄, K₂HPO₄, FeSO₄, MgSO₄, MnSO₄ to attain max-imum xylanase activity. The optimum conditions for maximum xylanase activity were wheat bran (5%), yeast extract and peptone (1%), MgSO₄ (1%) and xylan (1%) which on validation produced xylanase activity of 205.3 IU/ml. And these results were in good confirmation with the predicted values thus proving the accuracy of the model. Irfan et al (2012) reported maximum xylanase activity from Bacillus subtilis using (g/L) 0.5 KH₂PO₄, 0.5 yeast extract, 0.2 NaCl, 0.16 MgSO₄, 7H₂O, sucrose 20 and sugarcane bagasse 20 in submerged fermentation at 37°C for 48h.

CONCLUSIONS
The results of the present study revealed that Bacillus subtilis can produce xylanase enzyme using agricultural residue like corn cobs as a substrate in submerged fermentation. In this study, bacterial strains were used for xylanase enzyme production which has advantage of short period of growth as compared to the fungi. Results of this study indicated that nutrients and cultural properties played a pivotal role in enzyme production. After analysis of variance data showed that highest level xylanase was produced from media comprising of 3% substrate, 0.05% peptone, and 0.5% KH₂PO₄. The optimization of all the process parameters are being considered as pre-requisites to make the process of enzyme production cost effective at large scale. The projected model is valid to be used for sugar and manufacturing of other industrially important products, such as bio-ethanol, bio-fuel, paper pulp bleaches food and feed production.

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