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

Sobia Naz[1], Muhammad Irfan[1], Muhammad Umar Farooq[2]

[1]Department of Biotechnology, University of Sargodha, Sargodha Pakistan. [2]Food Science Department, Muhammad Nawaz Sharif University of Agriculture, Multan Pakistan Email; [1]m.irfan@uos.edu.pk, irfan.biotechnologist@gmail.com

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ABSTRACT

In this study, an attempt was made to optimize the nutritional conditions for xylanase production by *Bacillus subtilis* in submerge 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 KH_2PO_4 (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 (%) KH_2PO_4. 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 R^2 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 constitute 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 acessibility 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 signifycantly 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 (Polizeli *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 micro-organisms, 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 manufac-turing 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 35ºC 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 121ºC 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 35ºC 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 ºC. 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 spectrophoto-to-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.

$$\text{Xylanase activity (IU)} = \frac{\text{OD(Optimal density)} \times \text{DF(Dilution factor)} \times \text{Standard factor} \times 1000}{15\text{(Incubation period)}}$$

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 KH_2PO_4 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.

Independent Variable	Code	Code and actual factor level		
		−1	0	+1
Corncob concentration (%)	A	1	3	5
Peptone concentration (%)	B	0.05	0.275	0.5
KH_2PO_4 concentration (%)	C	0.1	0.3	0.5

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 corncob concentration as carbon source, peptone as nitrogen source and minerals like KH_2PO_4 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 295U/ml in all runs. Run no.15 gave maximum xylanase activity with residual difference of 0.11468 between observed and predicted with corncob concentration of 3.0%, peptone concentration of 0.05% and 0.5% of KH_2PO_4 concentration.

$$\text{Xylanase activity (U/ml)} = 277.7 - 18.85 (A) - 139.3 (B) + 348.5 C + 0.09 (A^2) - 587.1(B^2) - 568 (C^2) + 123.64 (A) \times (B) - 60.0 (A) \times (C) + 48.7 (B\times C) \quad \text{Eq. 1}$$

Table 2. Box Behnken design for xylanase production by *Bacillus subtilis.*

Run No.	A	B	C	Xylanase activity (IU/ml)		Residual.
				Observed	Predicted	
1	3	0.50	0.1	204.4512	204.5659	-0.11468
2	1	0.05	0.3	295.0986	292.8447	2.25389
3	5	0.275	0.1	275.8977	273.5291	2.36857
4	3	0.275	0.3	236.9548	244.6917	-7.73687
5	1	0.275	0.1	231.3298	234.7072	-3.37740
6	3	0.275	0.3	257.6990	244.6917	13.00733
7	1	0.275	0.5	216.8396	219.2082	-2.36857
8	3	0.275	0.3	239.4212	244.6917	-5.27047
9	5	0.275	0.5	165.3658	161.9884	3.37740
10	3	0.50	0.5	144.3020	145.4255	-1.12351
11	5	0.05	0.3	168.8797	172.3718	-3.49208
12	1	0.50	0.3	150.5290	147.0369	3.49208
13	3	0.05	0.1	244.6027	243.4792	1.12351
14	5	0.50	0.3	246.8581	249.1120	-2.25389
15	3	0.05	0.5	175.6945	175.5798	0.11468

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 values 0.000 and, P>F<0.0001 shows model's accuracy (Table 3). Higher R^2 values of 98.93, and adjusted R^2 values of 96.99% and predicted R^2 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 A×B, B×C, A×C, A^2 , B^2 , and C^2 were also found significant.

Table 3: Analysis of variance for xylanase production by *B. subtilis* in submerged fermentation.

Source	DF	Adj.SS	Adj.MS	F-value	P-value
Model	9	30205.1	3356.1	51.17	0.00
Linear	3	10624.0	3541.3	53.99	0.00
Substrate Conc. (A)	1	169.2	169.2	2.58	0.169
Peptone (B)	1	2385.2	2385.2	36.36	0.002
KH_2PO_4 (C)	1	8069.5	8069.5	123.03	0.000
Square	3	4874.0	1624.7	24.77	0.002
A^2	1	0.5	0.5	0.01	0.933
B^2	1	3262.0	3262.0	49.73	0.001
C^2	1	1903.6	1903.6	29.02	0.003
2-Way Interaction	3	14707.1	4902.4	74.74	0.000
A×B	1	12381.9	12381.9	188.77	0.000
(A×C)	1	2306.0	2306.0	2306.0	0.002
B×C	1	19.2	19.2	0.29	0.612
Error	5	328.0	65.6		
Lack-of-Fit	3	71.1	23.7	0.18	0.899
Pure Error	2	256.8	128.4		
Total	14	30533.0			

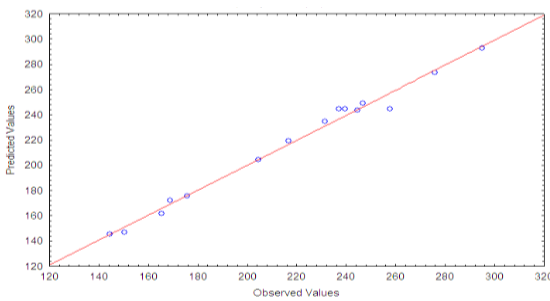
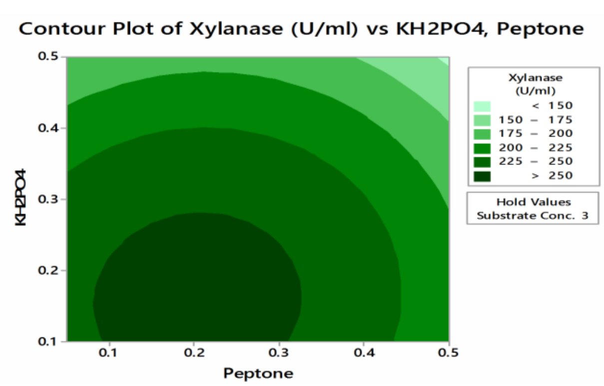
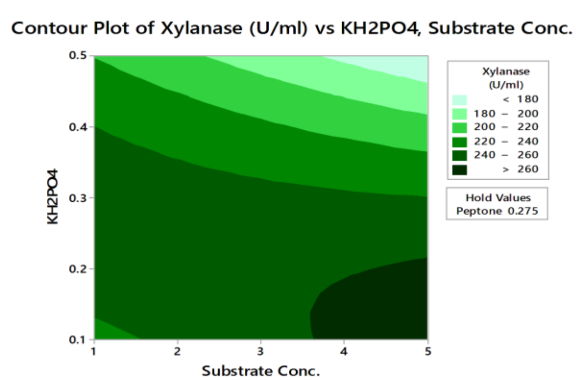


Figure 1: Observed values versus mathematical model prediction of xylanase activity.

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.





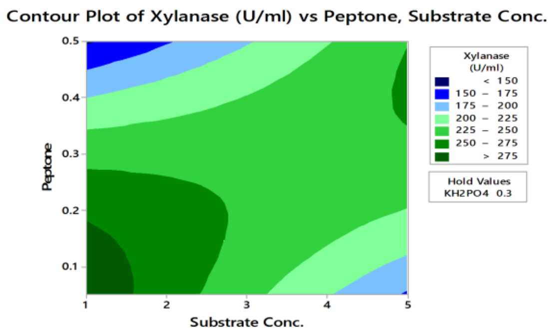


Figure 2: Effect of KH_2PO_4 , peptone and corncobs concentration on xylanase production by *Bacillus subtilis* in submerged fermentation.

Figure 3 explains the desirability chart for xylanase production by *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_2PO_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.

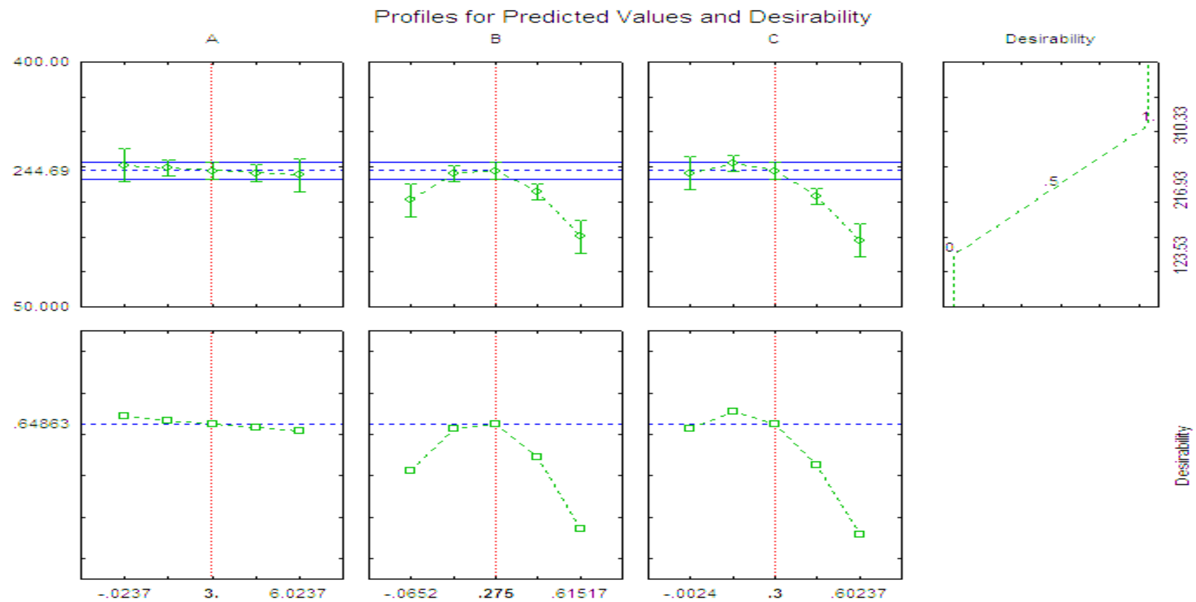


Figure 3: Desirability chart of xylanase activity. Describing predicted and desired values for 3 parameters A, B and C i.e substrate concentration, peptone and KH_2PO_4 respectively.

DISCUSSION

The results of the present study revealed that *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 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 $(NH_4)_2SO_4$ are best for *B. subtilis* BS04 while KNO_3 and malt extract for *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 corncobs as carbon source yielded maximum xylanase. The effect of easily metabolize able sugars (glucose, xylose, fructose, maltose, cellobiose 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 $\pm$ 95 U ml^{-1} by using corn cobs as carbon source under optimized growth conditions.

In this study we also used different concentrations of KH_2PO_4 as mineral source among different concentrations used 0.5 (%) KH_2PO_4 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 KH_2PO_4, K_2HPO_4, $FeSO_4$, $MgSO_4$, $MnSO_4$ to attain maxi-mum xylanase activity. The optimum conditions for maximum xylanase activity were wheat bran (5 %), yeast extract and peptone (1 %), $MgSO_4$ (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 KH_2PO_4, 0.5 yeast extract, 0.2 NaCl, 0.16 $MgSO_4$. $7H_2O$, sucrose 20 and sugarcane bagasse 20 in submerged fermentation at 37ºC 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% KH_2PO_4. 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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