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A Statistical Design Approach for Xylanase Production by *Aspergillus niger* Using Soybean Hulls: Optimization and Determining the Synergistic Effects of Medium Components on the Enzyme Production

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Abstract

Xylanases are hydrolytic enzymes produced by different microorganisms which convert xylooligomers to its constituent xylose units. The growing interests in xylanase production could be linked to its diverse industrial applications. The use of agricultural residues contributed in overcoming one of the major challenges of enzyme production, which is the production cost. This study involves the use of Face centered central composite design (FCCCD) under Response surface methodology (RSM) to optimize the medium components for enhanced xylanase production using soybean hulls as renewable substrate by solid state fermentation. The optimum components that led to maximum xylanase activity of 139.73 U/g were 0.58% w/w of urea, 0.03% w/w of K_2HPO_4 and 0.04% w/w of Na_2CO_3. Coefficient of determination (R^2) used to check the fitness of the model showed the correlation between the experimental and predicted responses with a value of 0.8981. Thus, this study showed the potential use of soybean hulls for xylanase production

Keywords optimization, renewable substrate, soybean hulls, xylanase.

1. Introduction

Xylanase (endo-1, 4-β-D-xylanohydrolase; EC 3.2.1.8) is a xylan-degrading enzyme that aids in hydrolyzing xylan into its monomeric units (Mittal *et al.*, 2013). This enzyme is produced by various microbial systems including bacteria, yeast and fungi (Maheshwari *et al.*, 2014). The growing interest in xylanase production includes its utilization for various industrial applications such as biobleaching of pulp and paper, clarification of wine and juice, animal feed formulation, baking processes, and hydrolysis of lignocellulose for ethanol and xylitol production, among others (Bibi *et al.*, 2014; Coman and Bahrim, 2011).

Xylanase production is affected by various factors including the cost of raw materials, product inhibition, pH and thermal stability, as well as polymeric nature of xylans (Walia*et al.*, 2013). Although different agricultural substrates, such as wheat bran (Coman and Bahrim, 2011), tomato seed meal (Katapodis *et al*., 2006), apple pomace (Walia *et al.*, 2013), wheat straw (Garg *et al.*, 2011), sugarcane bagasse (Souza *et al.*, 1999), rice straw (Park *et al.*, 2002), rice husk (Singh *et al.*, 2013), sunflower meal and corn cobs (Irfan *et al.*, 2014), have been used for the production of xylanase through solid state fermentation as parts of the efforts on cost reduction, there is still a need for developing an effective fermentation system for enhanced levels of this enzyme for wider industrial applications.

Soybean, as a widely cultivated crop across the globe, has an annual production of more than 259 million tonnes; generating more than 20 million tonnes of hulls; thus, soybean hulls consist of 46–51% cellulose, 16–18% hemicellulose and 1.4–2% lignin (Corredor *et al.*, 2008; Cassales *et al.*, 2011); these make them attractive for ~~the~~ production of several value added products. Thus, xylan, being a major component of hemicellulose present in soybean hulls, could serve as an inducer for xylanase production.

Herein, Face Centered Central Composite Design (FCCCD) was employed for enhancing xylanase production using soybean hulls as a cheap and available substrate by *Aspergillus niger* AS-1. Preliminary screening of medium components using Placket-Burman design and one-factor-at-a-time method has been established by Salihu and Alam (2015). This design was selected based on its reliability with the ability to identify the separate and combined effects of various factors through a minimum number of experiments.

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2. Materials and Methods

2.1. Sample and Inoculum preparation

Soybean hulls of 1 mm particle sizes were prepared and kept under laboratory conditions at room temperature. A spore suspension of *Aspergillus niger* AS-1 cultured on potato dextrose agar plate was prepared as described by Alam *et al.* (2004) using 25 ml of sterile distilled water with the help of bent glass rod. Following the filtration of the suspension through Whatman No.1 filter paper under aseptic conditions; the spore count was found to be about 1×10^8 spores/ml and used as an inoculum in this study

2.2. Solid state fermentation and Xylanase production

The fermentation set up for xylanase production was prepared using 10 g of soybean hulls in 250 ml Erlenmeyer flask which was mixed with 10 ml of a mixture of urea, K_2HPO_4 and Na_2CO_3 at a different percentages (w/w) (as shown in Table 1), 0.5% w/w of peptone, 0.05% w/w each of $NaNO_3$, $(NH_4)_2SO_4$ and $CaCl_2$; so as to get the final solid to moisture ratio of 1:1 as described by Xu *et al.* (2008). The flasks were autoclaved at 121^0C for 20 min, and then cooled before inoculating with 1 ml of spore suspension of *A. niger* AS-1 (1×10^8 spores/ml). The set ups were incubated at 28 ± 1^0C for 5 days. Following the bioconversion, distilled water was added to each flask and then shaken on a rotatory shaker (180 rpm) for 30 min at room temperature. The content was centrifuged and the supernatant was used to assay for xylanase activity.

2.3. Determination of Xylanase activity

Xylanase activity was determined using xylan from beech wood as a substrate and the amount of reducing sugar released was measured by dinitrosalicylic acid (DNS) method using D-xylose as the standard (Biely *et al.*, 1985). The reaction mixture contains 900µl of 1% (w/v) xylan in 0.05 M citrate buffer, pH 5 and 100 µl of the enzyme solution incubated at 50^0C for 30 min. The reaction was terminated by the addition of DNS followed by incubating the tubes in boiling water bath for 5 min. The reducing sugar released was measured at 540 nm. One enzyme unit (U) was defined as the amount of enzyme that liberated 1 µmol of xylose per minute under the assay conditions. The results were expressed as U/g soybean hulls.

2.4. Statistical analysis and optimization

Face Centered Central Composite Design (FCCCD) under Response Surface Methodology (RSM) was used (Montgomery, 2001) to determine the effects of the medium components influencing xylanase production. Three medium components (urea, K_2HPO_4 and Na_2CO_3) were selected for the present study based on the results of Placket-Burman design and One-factor-at-a-time (OFAT) analysis (Salihu and Alam, 2015). Design Expert Statistical software (Version 6.0.8) was used to generate a set of seventeen (17) experiments with three center points so as to optimize the concentrations of these parameters on xylanase activity. The parameters were studied at three levels, low (–1), medium (0) and high (+1) as indicated in Table 1. The relationship between the dependent and independent variables is based on second order polynomial equation:

$$Y = \beta_o + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 \quad (1)$$

where Y represents the dependent variable (xylanase production); X_1, X_2 and X_3 are independent variables (urea, K_2HPO_4 and Na_2CO_3) ; β_0 is an intercept term; β_1, β_2 and β_3 are linear coefficients; β_{12},β_{13} and β_{23} are the interaction coefficients; β_{11},β_{22} and β_{33} are quadratic coefficients.

The results obtained were used to develop a regression model by analyzing the values of regression coefficient and analysis of variance (ANOVA). The quality of fit of the quadratic polynomial model equation was expressed by coefficient of determination, R^2. The validation experiment was carried out to determine the adequacy of the developed experimental model.

3. Results and Discussion

The present study involves the use of statistical design to determine the relationship between the selected medium components (urea, K_2HPO_4 and Na_2CO_3) for enhanced xylanase production. Through this design, linear and interactive effects of these components on the response could be ascertained.

Table 1 shows the FCCCD design with both the experimental and predicted response for the 17 experimental runs which constitute 8 star points, 6 axial points and 3 center points. The results showed that highest activity of xylanase (137.58 -140.17 U/g) was realized in runs associated with center points (6, 8 and 15), while the lowest was found in run 17 (113.61 U/g), where all the three components were at their low levels.

The relationship between the medium components and the response was predicted by the second-order polynomial equation in terms of coded factors:

$$\text{Xylanase activity (U/g)} = +139.03 - 0.16A + 0.26B + 3.49C - 5.24A^2 - 2.66B^2 - 5.89C^2 - 2.71AB - 1.49AC - 0.27BC \quad (2)$$

where Xylanase production is dependent on concentrations of urea (A), K_2HPO_4 (B) and Na_2CO_3 (C). The negative and positive signs before the terms show antagonistic and synergistic effects, respectively. The analysis of variance (ANOVA), as indicated in Table 2, helps in assessing the fitness as well as the significance of the developed model. As indicated in the Table, the model was found to be significant with *p*-value of 0.0095; while the linear term of Na_2CO_3 (C), quadratic terms of urea (A^2) and Na_2CO_3 (C^2) were significant at *p*-value less than or equal to 0.05.

The lack of fit was not found to be significant based on its *p*-value of 0.1008, as shown in Table 2; this indicates that there is only a 10.08% chance that this value could occur due to noise.

Similarly, coefficient of determination (R^2) is used to check the fitness of the model, the closer a value is to 1, the better the correlation between the experimental and predicted responses. Thus, R^2 value of 0.8981 and adjusted R^2 value of 0.7670 indicate that about 90% and 77% of variations could be accounted for by the model equations. Adequate precision value of 8.22 which measures the signal to noise ratio showed an adequate

signal since any value greater than 4 is considered desirable. In case of coefficient of variation, lower values are preferred as they indicate the precision and reliability of the experimental responses as presented in foot notes of Table 2.

Figure 1 shows the two and three dimensional surface plots of predicted xylanase production based on urea and K_2HPO_4. Although none of the interaction terms was significant, as indicated in Table 2, the AB (urea and K_2HPO_4) was the one with the lowest *p*-value (0.0627). It can be seen from ~~the~~ Figure 1, that xylanase production is affected by ~~the~~ increasing concentration of urea and K_2HPO_4, but at higher concentrations, xylanase production was found to be low.

In order to investigate the applicability of the developed second-order model based on the three parameters (urea, K_2HPO_4 and Na_2CO_3), numerical optimization was used to determine the most desirable concentrations of the components that lead to maximum outcome for validation (Table 3a). The desirable model solution was validated as indicated in Table 3b; the experimental and the predicted results appeared to be in good agreement.

Accordingly, the concentrations of the three medium components optimized in the present study were lower than those reported by Xu *et al.* (2008) for maximum xylanase production using wheat bran and *A. niger* XY-1 at optimum concentrations of urea, Na_2CO_3 and $MgSO_4$ of 4.2%, 0.3% and 1.1%, respectively.

Park *et al.* (2002) reported a combined optimization based on medium and process parameters by fractional factorial design that led to enhanced xylanase activity of 5,071 U/g using rice straw by *A. niger*. The yield was several times higher than what was observed in the present study, but there is a possibility of getting an enhanced production in soybean hulls containing medium when considering some parameters such as temperature, moisture content, inoculum concentration, pH, pretreatment methods and particle size. Similarly, xylanase yield of 10.9 U/ml was obtained following CCD analysis of alkali-pretreated rice husk by *A. niger* ITCC 7678. The optimum operating conditions were found to be pH of 5.5, temperature of 32.5 °C, and $NaNO_3$ of 0.35 % (w/v) (Singh *et al.*, 2013).

In case of sugarcane bagasse, effects of process parameters were analyzed using RSM for enhanced xylanase production by *Thermoascus aurantiacus*. Initial moisture content of 81% and bagasse mass of 17g influenced xylanase activity with a maximum yield of 2700 U/g (de O Souza *et al.*, 1999). Based on these findings, de O Souza *et al.* (1999) suggested that xylanase production by several fungal species using agricultural residues depends on the composition of the substrates, choice of fermentation type and conditions as well as downstream processing of the produced enzyme.

Ang *et al.* (2013) reported the potential of untreated oil palm trunk for xylanase production by *A. fumigates* SK1. High extracellular xylanase activity of 418.70 U/g was obtained when the moisture content, initial pH and inoculum concentration were at 80%, 5.0 and 1×10^8 spore/g, respectively. When a 10 liter-capacity stainless steel horizontal bioreactor was used for the production of xylanase by *Sporotrichum thermophile* in an optimized medium containing wheat bran and wheat straw; maximum xylanase yield was found to be 320 U/g (Topakas *et al.*, 2003). Additionally, application of CCD resulted in an enhanced xylanase production by *Cellulosimicrobium* sp. CKMX1 using apple pomace based medium. Some of the parameters that supported xylanase production include particle size, fermentation time, temperature, initial pH and inoculum concentration; with a maximum yield of 535.6 U/g (Walia *et al*., 2013).

Thus, the findings of the present study indicate that FCCCD was suitable for optimizing the medium components for enhanced xylanase production by *A. niger* AS-1 using soybean hulls; further studies involving the process parameters will give the overall picture in terms of yield and production cost economics of the process.

Table 1: Face centered central composite design matrix indicating the experimental and predicted responses

Run	Urea (% w/w)	K_2HPO_4 (% w/w)	Na_2CO_3 (% w/w)	Xylanase activity (U/g)	
				Experimental	Predicted
1	0.8 (+1)	0.04 (+1)	0.03 (–1)	117.74	120.92
2	0.8 (+1)	0.03 (0)	0.04 (0)	132.74	133.63
3	0.6 (0)	0.03 (0)	0.05 (+1)	133.51	136.63
4	0.8 (+1)	0.02 (–1)	0.05 (+1)	128.23	129.81
5	0.8 (+1)	0.02 (–1)	0.03 (–1)	127.33	125.26
6	0.6 (0)	0.03 (0)	0.04 (0)	139.33	139.03
7	0.8 (+1)	0.04 (+1)	0.05 (+1)	127.95	124.37
8	0.6 (0)	0.03 (0)	0.04 (0)	140.17	139.03
9	0.6 (0)	0.02 (–1)	0.04 (0)	136.03	136.11
10	0.4 (–1)	0.03 (0)	0.04 (0)	134.84	133.95
11	0.6 (0)	0.03 (0)	0.03 (–1)	132.77	129.65
12	0.4 (–1)	0.04 (+1)	0.05 (+1)	131.01	133.08
13	0.6 (0)	0.04 (+1)	0.04 (0)	136.71	136.63
14	0.4 (–1)	0.02 (–1)	0.05 (+1)	130.87	127.69
15	0.6 (0)	0.03 (0)	0.04 (0)	137.58	139.03
16	0.4 (–1)	0.04 (+1)	0.03 (–1)	125.26	123.68
17	0.4 (–1)	0.02 (–1)	0.03 (–1)	113.61	117.19

Table 2: Analysis of variance (ANOVA) of the developed model

Source	Sum of squares	F-value	*p*-value	Remark
Model*	739.4869	6.8516	0.0095	Significant
A (Urea)	0.2562	0.0214	0.8879	Not significant
B (K_2HPO_4)	0.6762	0.0564	0.8191	Not significant
C (Na_2CO_3)	121.5252	10.1338	0.0154	Significant
A^2	73.4355	6.1237	0.0425	Significant
B^2	18.8904	1.5752	0.2497	Not significant
C^2	92.8006	7.7385	0.0272	Significant
AB	58.6469	4.8905	0.0627	Not significant
AC	17.7026	1.4762	0.2638	Not significant
BC	0.6047	0.0504	0.8287	Not significant
Lack of Fit	80.4522	9.2154	0.1008	Not significant

*R^2 = 0.8981, Adjusted R^2 = 0.7670, Coefficient of variation (CV) = 2.65, adequate precision = 8.22

Table 3a: Constrains for the parameters in numerical optimization

Parameters	Ultimate goal	Experimental range
Urea	range	0.4 – 0.8 %
K_2HPO_4	range	0.02 – 0.04 %
Na_2CO_3	range	0.03 – 0.05 %
Xylanase production (U/g)	Maximum production	113.61 – 140.17 U/g

Table 3b: Optimization and model validation

Urea (% w/w)	K_2HPO_4 (% w/w)	Na_2CO_3 (% w/w)	Xylanase production (U/g)	
			Experimental	Predicted
0.58	0.03	0.04	139.73 ± 0.19	139.572

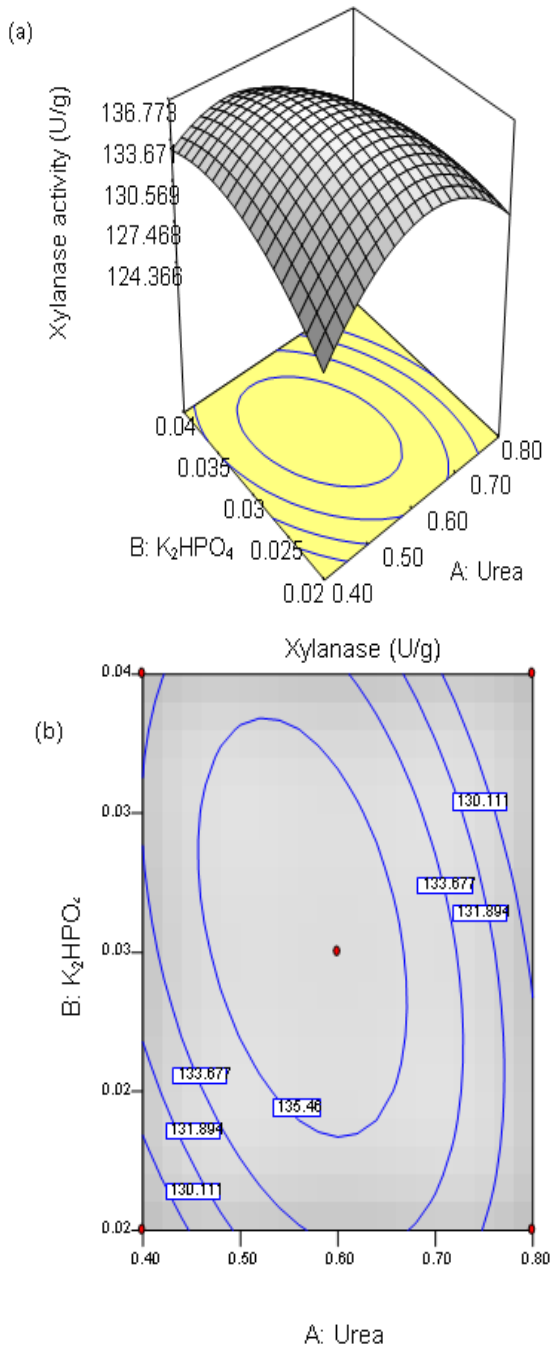

Figure 1: Plots of the effects of urea and K_2HPO_4 on xylanase activity by *A. niger* AS-1 using soybean hulls: (a) response surface 3D and (b) contour plot (2D).

4. Conclusion

Statistical optimization using FCCCD for xylanase production by *A. niger* AS-1 using soybean hulls as raw material indicated the dependence of the production on the selected medium components (urea, Na_2CO_3 and K_2HPO_4). Under the optimized condition, maximum xylanase activity of 139.73 U/g was obtained. Thus, the utilization of soybean hulls as an inexpensive and renewable substrate for xylanase production boosts its industrial relevance when compared with its current usage.

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