Valorization of de-oiled cakes for xylanase production and optimization using central composite design by *Trichoderma koeningi* isolate

Trichoderma koningii izolatının tarafından merkezi kompozit tasarım kullanılarak ksilanaz üretimi ve优化isyonu için de yağlanmış kek Valorizasyon

DOI 10.1515/tjb-2016-0290
Received April 9, 2016; accepted July 13, 2016; previously published online March 3, 2017

Abstract

**Aim:** Evaluation of the medium components and conditions for the optimization of xylanase production in submerged fermentation by *Trichoderma koeningi* isolate (KT006533).

**Methods:** *Trichoderma koeningi* was identified by the 18s rRNA. NaOH pre-treated corn cobs were used in order to reduce the crystallinity of corn cobs. De-oiled cakes proximate composition was analyzed according to AOAC. Plackett-Burman design (PBD) was used to screen the 19 media variables that affect xylanase production and optimized the media by central composite design (CCD).

**Results:** The isolate was identified as *Trichoderma koeningi*. Among the 19 variables more effective variables on xylanase production was mustard cake, xylose, FeSO$_4$·7H$_2$O, ZnSO$_4$·7H$_2$O and pH. The optimization of xylanase and protein were quantified as 74.84 U/mL and 2.94 mg/mL, respectively, owing to the influence of the above components at g/L 3.5, 6.0, 0.06, 0.04 at levels respectively and pH 6.5, by using a CCD. The coefficient of determination (R$^2$) is 0.92%. The correlations between the actual and coded results are found to be good.

**Conclusion:** NaOH pre-treated corn cobs were used with mustard cake powder as fermentation medium constituent to induce the xylanase production. From the results we conclude that mustard cake induced the xylanase production.

**Keywords:** De-oiled cakes; Xylanase; Submerged fermentation; Placket-Burman design; Central composite design.

**ÖZET**

**Amaç:** *Trichoderma koeningi* izolatı (KT006533) tarafından batık fermantasyon ksilanaz üretiminin optimizasyonu için orta bileşenleri ve koşulların değerlendirilmesi.

**Metod:** *Trichoderma koeningi* 18S rRNA ile teşhis edildi. NaOH önceden muamele edilmiş mısır koçanı, mısır koçanı kristal azaltmak için kullanılmıştır. yakan kompozisyon AOAC göre analiz edildi kek de yağlanmış. Plackett-Burman tasarım ksilanaz üretimini etkileyen ve merkezi kompozit tasarım ile medya optimize 19 medya değişkenleri ekrana kullanıldı.

**Bulgular:** izolat Trichoderma koeningi olarak tespit edilmştir. 19 değişkenler arasındaki ksilanaz üretim üzerinde daha etkili değişkenlerin hardal kek, ksiloz, FeSO$_4$·7H$_2$O, ZnSO$_4$·7H$_2$O ve pH olduğu. ksilanaz ve protein optimizasyonu kullanılarak mi 74.84 U/ve 2.94 mg/mL, sırasıyla seviyelerde g/L 3.5, 6.0, 0.06, 0.04, yukarıdaki bileşenlerin etkisi nedeniyle, sırasıyla pH 6.5 nicelleştirilmiş merkezi kompozit tasarımını belirlerle (R2) katsayısı
Introduction

In general, the production of xylanase based on microbial fermentation and biosynthesis renders its industrial application more feasible and economical. The use of submerged fermentation (SmF) for induction of cellulolytic and hemicellulolytic enzymes provides a means to use various agro-industrial byproducts, such as wheat bran, wheat straw, corn cobs, sugar beet pulp, apple pomace, and cassava waste [1]. Filamentous fungi have been widely used to produce hydrolytic enzymes for industrial application, including xylanase, whose levels in fungi are generally much higher than in yeast and bacteria. The most common industrial xylanase producing strains are the Aspergillus sp. and Trichoderma sp., as well as bacterial strains of the species Bacillus sp. [2]. Xylanase from filamentous fungi are extra-cellular, inducible enzymes. The nutrient medium compositions and culture factors strongly influence the xylanase production.

The optimization of fermentation conditions and screening of important nutritional factors are of essential importance to determine the optimal variables for efficient production and to avoid the wastage of nutrients in the production. As a result, the production cost for xylanase should be significantly reduced. However, optimization of all factors and establishment the best possible conditions by considering all interactions of parameters, numerous experiments have to be carried out, which is not economical and practical. For this type of cases, design of experiments (DOE) and statistical tools help to gain more information about the optimization conditions within a few trials [3]. There are two ways to solve this problem: conventional and statistical methods of DOE [4]. Plackett-Burman design (PBD) is a powerful statistical technique for screening medium components and has been widely used in optimization [5]. Response surface methodology (RSM) is one such technique based on the fundamental principles of statistics, such as randomization, replication and duplication, which simplifies the optimization by studying the mutual interactions among the variables over a range of values in a statistically valid manner [6]. The application of statistical experimental design in fermentation improves the product yield, and reduces the process variability, development time and overall costs [7].

The present objective on ability of Trichoderma koenigi isolate to utilize inexpensive substrate and an attempt was made to evaluate the medium components with PBD and statistically optimize the medium under submerged fermentation by using the central composite design (CCD).

Materials and methods

Microorganism and identification

The T. koeningi was isolate from the sorghum dumping soil using potato dextrose agar medium (PDA). The extraction of total DNA and its purification was followed by Purohit et al. [8]. Amplification of 581 base pair fragment within the gene coding for the small ribosomal subunit 18S rRNA of fungi was performed in a thermal cycler gene AMP cycler system using the fungal specific primers 18R5185′-GCATTTGCCAAGGATGTTTT-3′ and 18F5305′-TTCGTGCCAGCAGCCGCGG-3′. The purified and amplified 18S rDNA PCR products were sequenced by Xleris services (Ahmadabad, Gujarat, India). The resulted sequences were aligned using the Clustal W and then the sequence was compared with those from Gene Bank using BLAST [9]. The phylo-genetic tree was constructed by using joining bootstrap method [10]. The 18S rRNA partial sequences were submitted to gene bank database.

Substrate preparation

Corn cobs were collected from agro-fields and local market of Tirupati (India) and pretreated with 2% of NaOH for 90°C at 90 min [11]. Mustard, soybean and ground nut cakes procured from a local small scale oil extracting units, was used as the supplementation for xylanase production. They were dried at 60°C for 72 h to reduce the moisture content and ground to a desired particle size (1 mm).

Cultivation of fungal spores and inoculum preparation

Fungal spores were obtained by growing the T. koeningi on PDA (Himedia, India) slants at room temperature. They
were harvested after 5 days of cultivation with saline containing 0.1% (w/v) Tween 80. The spore suspension at 1 × 10^8 spores/mL was used for inoculum preparation for SmF process.

**Chemical composition of de-oiled cakes**

It was analyzed according to method of AOAC as follows: Lipids content of the samples were determined by extracting the residue with petroleum ether (40–60°C) for 7 h in a Soxhlet apparatus. Crude fiber was determined as loss on dried lipid-free residue after digestion with 1.25% H₂SO₄ and ash was determined by ignition at 550°C in a muffle furnace to a constant weight. Moisture was determined by drying in an oven at 105°C for 8 h. Total carbohydrate content was determined according to Dubois et al. [12].

**Estimation of minerals in de-oiled cakes by ICP-OES**

Elemental analysis was carried out on an optima 2100 DV inductively coupled plasma-optical emission spectrometer (Dual view, Perkin Elmer life and analytical sciences). 1 g of sample (mustard, soybean and ground nut cakes) were taken and digested with 1 mL of 0.2% (v/v) HNO₃ and centrifuged for 20 min at 2000 rpm (Hettich Universal 30F, Tuttlingen, Germany). All sample vials, sample cups and glassware were cleaned by soaking in 10% (v/v) HNO₃ and rinsed with de-ionized water prior to use. The appropriate standards for each element were made within the concentration range of the elements in the samples. The results were obtained from triplicate measurements.

**Submerged fermentation (SmF) for xylanase production**

It was carried out in Erlenmeyer conical flasks (250 mL) that contained corn cobs (5 g) and the medium components of varying concentrations were added into the flask based on the PB design matrix basal medium. The contents were thoroughly mixed and autoclaved at 121°C for 20 min. The medium was inoculated with 5% (v/w) inoculum and incubated at 30°C for 7 days. The culture was incubated at 30°C on a rotary shaker at 200 rpm. The samples were withdrawn at regular intervals. The mycelium was removed by cooling centrifugation at 7000 rpm at 4°C for 15 min to obtain a clear supernatant. This supernatant was used for measurement of enzyme activities.

**Screening of media components by PBD**

A prior knowledge with understanding of the related bioprocesses is necessary for a realistic modeling approach. The PBD was used to determine variables which would significantly affect xylanase production by T. koening among the 19 variables. Each component (19 variables) was tested at low (−1) and high (+1) concentrations which are soybean (2–5 g/mL), mustard and groundnut cake (2–6 g/mL); glucose (5–8 mg/mL), xylose (5–10 mg/mL); yeast extract (0.5–1 mg/mL) tryptone (0.5–1.5 mg/mL), peptone (2–5 mg/mL), urea (0.5–1 mg/mL), FeSO₄·7H₂O and ZnSO₄·7H₂O (0.01–0.05 mg/mL), MnSO₄·7H₂O (0.1–0.5 mg/mL), MgSO₄·7H₂O (0.2–1 mg/mL), CaCl₂ (0.05–1.5 mg/mL), CuSO₄·7H₂O (0.01–0.05 mg/mL), NaCl (0.1–0.5 mg/mL), pH (6–9); temperature (30–40°C) and incubation time (48–120 min) and screened in 20 experimental runs and insignificant ones were eliminated to obtain a smaller, manageable set of factors. The experiments were carried out according to the design matrix in 250 mL conical flasks. The inoculum concentration and particle size were maintained at 5%, and 1 mm, respectively.

**Optimization of selected variables for xylanase production by CCD**

CCD with quadratic model was employed to study the combined effect of RSM [13], which was employed to optimize the selected five significant independent variables and three levels each with four concentric point combinations was used for the enhancement of dependent variable such as xylanase (U/mL) production. Actual and coded levels of variables along with response variables for CCD are presented in (Table 1). The overall second order polynomial mathematical relationship of the response Y₁, Y₂ and the five variables, can be approximated by the quadratic Eq. (1).

\[
Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{55}X_5^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{15}X_1X_5 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{25}X_2X_5 + b_{34}X_3X_4 + b_{35}X_3X_5 + b_{45}X_4X_5
\]

where Y is the predicted responses, X₁, X₂, X₃, X₄ and X₅ are independent variables, b₀ is the offset term, b₁, b₂, b₃, b₄ and b₅ are linear effects, b₁₁, b₂₂, b₃₃, b₄₄ and b₅₅ are squared effects and b₁₂, b₁₃, b₁₄, b₁₅, b₂₃, b₂₄, b₂₅, b₃₄, b₃₅, b₄₅ are interaction terms. The significance of all terms in the polynomial functions were assessed statistically using F-value
at a probability (p) of 0.001, 0.01 or 0.05. The regression coefficients were then used to generate response surface graphs from the regression models. The response surface graphs were generated by keeping one variable constant at the center point and varying the other variables within the experimental range.

The experimental design for the variables, i.e. mustard cake (2–6 g/L), xylose (2–8 g/L), FeSO$_4$$\cdot$7H$_2$O (0.02–0.08 g/L), ZnSO$_4$$\cdot$7H$_2$O (0.02–0.06 g/L) and pH (5–15) were taken for optimization and for measuring the enzymatic activities. The design was applied for selection range of each variable was minimum and maximum. Total 50 experiments were designed by the model and performed in triplicate. The obtained results in the performed assays, executed in terms of xylanase production have been recorded. The model was validated by running the experiments within the experimental range. The data validation runs were also statistically analyzed to find out the correlation between observed actual and predicted values.

### Protein estimation

The protein content in the culture filtrate was carried out by Folin-Ciocalteu reagent using bovine serum albumin (BSA) as standard [14].

### Enzyme assays

Xylanase activity was quantitatively assayed in cell free supernatant [15]. The reaction mixture (2.0 mL) containing 1.0 mL of 1.0% (w/v) oat spelt xylan in 0.1 M citrate buffer, pH 5.0; 0.9 mL citrate buffer and 0.1 mL of a suitably diluted enzyme solution was incubated at 50°C for 5 min. The reaction was stopped by adding 3 mL of 1.0% dinitro-salicylic acid reagent. The reaction mixture was then kept on boiling water bath for 10 min. Amount of reducing sugars liberated was determined by measuring absorbance of the resulting color at 540 nm in a UV-vis spectrophotometer using xylose as standard [16]. One unit (IU) of xylanase activity was defined as the amount of enzyme that produced 1 μmol of xylose equivalent per min of reaction and per mL of enzyme solution under the assay condition. The controls without either enzyme or substrate were run simultaneously.

### Results and discussion

#### Fungal genome identification

It was carried out by 18s rRNA sequencing, and the sequence was obtained, followed to BLAST against the total fungal genome database in National Center for Biotechnology Information (NCBI). The BLAST result showed 98% identity with existence fungal species. According to Dhiman et al. [17] the maximum identity 1 or 2% difference between isolate and existed strains showed that isolate was considered as a novel organism and thus was found to be a new strain (named BROV1). Hence, this sequence was deposited in the GenBank and was provided with a new accession number (KT006533).

#### Evolutionary relationship

It was inferred using phylo-genetic neighbor-joining program, using bootstrap consensus test with 500 in MEGA 5 and the branch lengths are in the same as those of the evolutionary distances used to infer the phylo-genetic tree. The newly isolated strain was closely related to T. koeningi with 98% similarity. Based on this similarity the isolated new fungal strain was identified as a T. koeningi BROV1 (Figure 1).

### Selection of media components by PBD

Conventional single dimensional search involves changing one independent variable at a time while fixing the
others at a constant level, which gives unreliable results, inaccurate conclusion, and even frequent interactions of two or more factors. PBD is a powerful statistical technique for screening medium components in a shake flask and has been widely used in optimization of fermentation [2, 5]. From the Pareto chart (Figure 2), the nutrients, namely mustard cake, xylose, FeSO\textsubscript{4}·7H\textsubscript{2}O, ZnSO\textsubscript{4}·7H\textsubscript{2}O and pH were found to be significant for the production of xylanase by \textit{T. koeningi} using corn cobs. Hence, these nutrients were selected for further optimization using RSM to maximize the production of xylanase. The results obtained in this study, the highest and lowest xylanase production were found to be 46.69 U/mL and 12.85 U/mL as observed in runs 4 and 20, respectively (Data not shown).

The \textit{Penicillium} sp. WX-Z1 achieved the maximum xylanase production of 29.72 U/mL [18], when compared to present results to earlier result highest production was observed in this study using PBD with different variables.

### Optimization of xylanase production by CCD

The optimization (selected variables) was attempted to improve the enzymatic yield under SmF on corn cobs. SmF was carried out according to the experimental design model for 5 days. The influences of independent variables on xylanase and protein results were summarized in (Table 2). The model was analyzed by multiple regression analysis and a second order polynomial equation (Eq. 1) was derived to represent the xylanase production as a function of the independent variables tested. The high value 5.27 for linear coefficient of xylose concentration illustrates the significant, positive effect of variable on the enzyme activity. Linear coefficient indicates the increase in enzyme activity with more increase in the xylose concentration supplementation. The positive effects of other variables also have significance on enzyme production. The negative quadratic coefficient of other variables.

**Figure 1:** Phylogenetic tree constructed on the basis of 18s rRNA sequence of the study and those of other strains obtained from GeneBank database (\textit{T. koeningi} BROV1 is used only).

**Figure 2:** Pareto chart analysis of variables.

| A: Soybean cake | B: Mustard cake | C: Groundnut cake | D: Glucose | E: Xylose | F: Yeast extract | G: Tryptone | H: Peptone | J: Urea | K: FeSO\textsubscript{4}·7H\textsubscript{2}O | L: ZnSO\textsubscript{4}·7H\textsubscript{2}O | M: MnSO\textsubscript{4}·7H\textsubscript{2}O | N: MgSO\textsubscript{4}·7H\textsubscript{2}O | O: CaCl\textsubscript{2} | P: CuSO\textsubscript{4}·5H\textsubscript{2}O | Q: NaCl | R: pH | S: Temperature | T: Incubation time |
|----------------|----------------|------------------|-----------|---------|-----------------|-----------|----------|------|----------------|----------------|----------------|----------------|----------------|-----------------|----------------|-----------|----------------|---------------|
Table 2: CCD used to optimize the medium consequents for the production of xylanase by *T. koeningi*.

| Std | A | B | C | D | E | Xylanase (U/mL) Actual | Predicted | Protein (mg/mL) Actual | Predicted |
|-----|---|---|---|---|---|------------------------|-----------|------------------------|-----------|
| 1   | -1| -1| 0 | 0 | 0 | 69.52                  | 65.12     | 2.36                   | 2.10      |
| 2   | 1 | -1| 0 | 0 | 0 | 9.74                   | 15.86     | 0.08                   | 0.13      |
| 3   | -1| 1 | 0 | 0 | 0 | 24.94                  | 24.31     | 0.52                   | 0.50      |
| 4   | 1 | 1 | 0 | 0 | 0 | 67.84                  | 67.73     | 2.18                   | 1.98      |
| 5   | 0 | 0 | -1| -1| 0 | 17.51                  | 24.71     | 0.41                   | 0.43      |
| 6   | 0 | 0 | 1 | -1| 0 | 28.53                  | 40.23     | 0.79                   | 1.31      |
| 7   | 0 | 0 | -1| 1 | 0 | 64.43                  | 58.17     | 1.91                   | 1.73      |
| 8   | 0 | 0 | 1 | 1 | 0 | 27.63                  | 25.47     | 0.59                   | 0.42      |
| 9   | 0 | -1| 0 | 0 | -1| 26.53                  | 42.09     | 0.25                   | 0.43      |
| 10  | 0 | 1 | 0 | 0 | -1| 63.86                  | 61.83     | 2.13                   | 2.01      |
| 11  | 0 | -1| 0 | 0 | 1 | 26.59                  | 41.12     | 0.98                   | 1.32      |
| 12  | 0 | 1 | 0 | 0 | 1 | 47.49                  | 42.44     | 1.89                   | 2.13      |
| 13  | -1| 0 | -1| 0 | 0 | 34.87                  | 37.87     | 0.75                   | 0.84      |
| 14  | 1 | 0 | -1| 0 | 0 | 32.84                  | 39.24     | 0.74                   | 0.90      |
| 15  | -1| 0 | 1 | 0 | 0 | 38.45                  | 38.57     | 0.81                   | 0.83      |
| 16  | 1 | 0 | 1 | 0 | 0 | 17.83                  | 21.36     | 0.09                   | 0.12      |
| 17  | 0 | 0 | 0 | -1| -1| 19.04                  | 17.82     | 0.19                   | 0.24      |
| 18  | 0 | 0 | 0 | 1 | -1| 69.75                  | 72.19     | 2.34                   | 2.65      |
| 19  | 0 | 0 | 0 | -1| 1 | 56.74                  | 52.65     | 1.96                   | 1.71      |
| 20  | 0 | 0 | 0 | 1 | 1 | 17.42                  | 16.99     | 0.09                   | 0.06      |
| 21  | 0 | -1| -1| 0 | 0 | 27.42                  | 16.25     | 0.41                   | 0.38      |
| 22  | 1 | 1 | 0 | 0 | 0 | 74.84                  | 79.91     | 2.94                   | 3.05      |
| 23  | 0 | -1| 1 | 0 | 0 | 69.53                  | 60.79     | 2.08                   | 1.82      |
| 24  | 0 | 1 | 1 | 0 | 0 | 11.94                  | 18.82     | 0.05                   | 0.12      |
| 25  | -1| 0 | 0 | -1| 0 | 19.32                  | 22.67     | 0.18                   | 0.29      |
| 26  | 1 | 0 | 0 | -1| 0 | 36.84                  | 35.58     | 0.83                   | 0.76      |
| 27  | 1 | 0 | 0 | 1 | 0 | 50.64                  | 52.84     | 1.43                   | 1.22      |
| 28  | -1| 0 | 0 | 1 | 0 | 26.52                  | 24.11     | 0.16                   | 0.19      |
| 29  | 0 | 0 | -1| 0 | -1| 46.32                  | 38.27     | 1.31                   | 1.72      |
| 30  | 0 | 0 | 1 | 0 | -1| 64.15                  | 52.66     | 2.10                   | 1.62      |
| 31  | 0 | 0 | -1| 0 | 1 | 47.48                  | 51.07     | 1.74                   | 1.82      |
| 32  | 0 | 0 | 1 | 0 | 1 | 19.34                  | 19.50     | 0.21                   | 0.25      |
| 33  | -1| 0 | 0 | 0 | -1| 57.82                  | 58.92     | 1.83                   | 1.76      |
| 34  | 1 | 0 | 0 | 0 | -1| 23.59                  | 25.32     | 0.14                   | 0.22      |
| 35  | 2.27| 0 | 0 | 0 | 0 | 27.84                  | 23.06     | 0.29                   | 0.34      |
| 36  | 1 | 0 | 0 | 0 | 1 | 44.83                  | 40.82     | 1.36                   | 1.21      |
| 37  | 0 | 2.27| 0 | -1| 0 | 64.85                  | 56.91     | 2.14                   | 1.83      |
| 38  | 0 | 1 | 0 | -1| 0 | 28.75                  | 21.01     | 0.27                   | 0.72      |
| 39  | 0 | -1| 2.27| 1 | 0 | 16.42                  | 19.83     | 0.38                   | 0.48      |
| 40  | 0 | 1 | 0 | -0.03| 0 | 61.32                  | 34.50     | 3.37                   | 1.74      |
| 41* | 0 | 0 | 0 | 2.27| 0 | 57.79                  | 46.16     | 2.13                   | 1.83      |
| 42* | 0 | 0 | 0 | 0 | -0.03| 70.21                  | 24.18     | 0.40                   | 3.15      |
| 43* | 0 | 0 | 0 | 0 | 2.27| 49.85                  | 35.17     | 2.19                   | 1.98      |
| 44* | 0 | 0 | 0 | 0 | 0 | 73.19                  | 73.19     | 2.90                   | 2.74      |
| 45* | 0 | 0 | 0 | 0 | 0 | 73.19                  | 73.19     | 2.90                   | 2.74      |
| 46* | 0 | 0 | 0 | 0 | 0 | 73.19                  | 73.19     | 2.90                   | 2.74      |
| 47* | 0 | 0 | 0 | 0 | 0 | 73.19                  | 73.19     | 2.90                   | 2.74      |
| 48* | 0 | 0 | 0 | 0 | 0 | 73.19                  | 73.19     | 2.90                   | 2.74      |
| 49* | 0 | 0 | 0 | 0 | 0 | 73.19                  | 73.19     | 2.90                   | 2.74      |
| 50* | 0 | 0 | 0 | 0 | 0 | 73.19                  | 73.19     | 2.90                   | 2.74      |

The values in parenthesis are the predicted values; Std, standard run order; *Central value; A, mustard cake (g/L); B, xylose (g/L); C, FeSO₄·7H₂O (g/L); D, ZnSO₄·7H₂O (g/L); E, pH.
indicates the existence of maximum activity to a point beyond that the entire variable had an inhibitory effect on enzyme activity.

\[
Y_1 (\text{U/mL}) = 73.19 + 3.96X_1 + 5.27X_2 - 4.29X_3 + 4.68X_4
+ 5.09X_5 - 21.14X_1^2 - 11.30X_2^2 - 17.99X_3^2 - 18.25X_4^2
- 15.02X_1X_2 + 25.67X_1X_3 - 4.65X_1X_5 - 10.41X_2X_3
+ 12.84X_2X_4 - 26.25X_2X_5 + 23.22X_3X_4 - 4.61X_3X_5
+ 12.05X_4X_5 - 11.49X_5 - 22.59X_5
\]

(2)

\[
Y_2 (\text{mg/mL}) = 0.93 + 0.57X_1 + 1.21X_2 - 2.02X_3 + 0.76X_4
+ 0.62X_5 - 5.64X_1^2 - 2.45X_2^2 - 3.51X_3^2 - 4.95X_4^2
- 3.90X_1X_2 + 2.67X_1X_3 - 1.95X_1X_4 + 1.53X_1X_5 + 3.72X_1X_5
- 8.92X_2X_3 + 6.41X_2X_4 - 3.91X_2X_5 + 2.31X_3X_4 - 4.25X_3X_5
- 6.87X_4X_5
\]

(3)

where, \(Y_1\) and \(Y_2\) are the predicted response of xylanase and protein. \(X_1, X_2, X_3, X_4\) and \(X_5\) are the coded values of mustard cake, xylose, \(\text{FeSO}_4\cdot7\text{H}_2\text{O}\), \(\text{ZnSO}_4\cdot7\text{H}_2\text{O}\) and pH, respectively.

Statistical testing of the CCD model was performed with the Fisher's statistical test for analysis of variance (ANOVA) using design expert software and the results of ANOVA for xylanase activity and protein is shown in (Table 3). The F-value is the ratio of the mean square due to regression to the mean square and indicates the influence of each controlled factor on the tested model. ANOVA of the quadratic regression model suggest that the model is significant with a computed F-value of 15.84 and \(P<0.05\). The model determination coefficient \(R^2 (0.9269)\) suggested that the fitted model could explain 92.69% of the total variation and unable to explain only 7.31% of the total variation. This implies a satisfactory representation of the process by the model. The “Pred R-Squared” of 0.7076 is in reasonable agreement with the “Adj R-Squared” of 0.8684. The determination coefficient value is closer to 1.0, the better is the correlation between the observed and predicted values and the \(R^2\) value obtained indicated a better correlation. A lower value

| Source | Sum of squares | DF | Mean square | F-Value A | F-Value B | p-Value prob > A | p-Value prob > B |
|--------|----------------|----|-------------|-----------|-----------|-----------------|-----------------|
| Model  | 20187.3        | 20 | 1009.3      | 15.85     | 86.25     | <0.0001         | <0.0001         |
| A      | 250.67         | 1  | 250.67      | 3.94      | 1.72      | 0.0583          | <0.0001         |
| B      | 443.63         | 1  | 443.63      | 6.97      | 0.59      | 0.0141          | 0.0018          |
| C      | 295.07         | 1  | 295.07      | 6.53      | 4.82      | 0.0412          | <0.0001         |
| D      | 349.88         | 1  | 349.88      | 5.49      | 2.42      | 0.0273          | 0.0275          |
| E      | 414.84         | 1  | 414.84      | 6.52      | 9.82      | 0.0172          | 0.0287          |
| AB     | 2635.80        | 1  | 2635.8      | 41.40     | 0.082     | <0.0001         | <0.0001         |
| AC     | 86.40          | 1  | 86.40       | 1.36      | 4.82      | 0.2551          | 0.1840          |
| AD     | 433.47         | 1  | 433.47      | 6.81      | 82.62     | 0.0151          | 0.0372          |
| AE     | 695.46         | 1  | 695.46      | 10.36     | 173.6     | 0.0036          | 0.0427          |
| BC     | 2756.78        | 1  | 2756.7      | 43.30     | 1.63      | <0.0001         | 0.0285          |
| BD     | 2156.21        | 1  | 2156.2      | 33.86     | 295.6     | <0.0001         | <0.0001         |
| BE     | 84.92          | 1  | 84.92       | 1.33      | 8.32      | 0.2591          | 0.378           |
| CD     | 581.29         | 1  | 581.29      | 9.13      | 2.51      | 0.0057          | 0.0638          |
| CE     | 528.31         | 1  | 528.31      | 8.30      | 4.90      | 0.0080          | 0.0042          |
| DE     | 2026.35        | 1  | 2026.3      | 31.82     | 7.26      | <0.0001         | 0.0095          |
| A²     | 3898.83        | 1  | 3898.8      | 61.23     | 0.83      | <0.0001         | 0.0840          |
| B²     | 1113.97        | 1  | 1113.9      | 17.50     | 69.28     | 0.0003          | <0.0001         |
| C²     | 2762.95        | 1  | 2762.9      | 43.39     | 27.31     | <0.0001         | 0.0532          |
| D²     | 2907.92        | 1  | 2907.9      | 45.67     | 8.32      | <0.0001         | <0.0001         |
| E²     | 1969.64        | 1  | 1969.6      | 30.93     | 5.94      | <0.0001         | 0.0064          |
| Residual | 1591.81      | 25 | 63.67       |           |           |                 |                 |
| Lack of fit | 27.53     | 20 | 79.59       |           |           |                 |                 |
| Pure error | 0.000     | 5  | 0.000       |           |           |                 |                 |
| Cor total | 21779.1     | 45 |             |           |           |                 |                 |

[Xylanase]: Coefficient of variation (CV) = 18.08%; \(R^2 = 0.9269\); adjusted \(R^2 = 0.8684\); Pred \(R^2 = 0.7076\); mean = 44.15. A, Mustard cake (g/L); B, Xylose (g/L); C, \(\text{FeSO}_4\cdot7\text{H}_2\text{O}\) (g/L); D, \(\text{ZnSO}_4\cdot7\text{H}_2\text{O}\) (g/L); E, pH.
for the coefficient variation suggests higher reliability of the experiment and in this case the obtained CV value of 18.08% demonstrated a greater reliability of the trails. The more significant terms having a lower p value and values of “Prob > F” less than 0.05 for A, B, C, D, E, AE, BD, A², B², C², D² and E² indicated the model terms are more significant. Values greater than 0.1000 indicate the model terms are not significant (Design-expert, 2012). The natural logarithm (ln) of the residual SS (sum of square) against λ is one, dip suddenly with a minimum in the region of the best optimum value 0.95, 0.62 and 0.48. The data do not require a transformation, as current value of confidence interval it contains (λ) very close and near to the optimum value.

The 3D response surface plots (Figure 3) were obtained by plotting the response (enzyme activity) on Z-axis against any two variables while keeping other variable at its ‘0’ level. The iso-response contour and surface plots for the optimization of conditions for enzyme activity was follows.

Figure 3: Response surface plots for the interactive effect variables showing on xylanase production of (A) Mustard cake and xylose, (B) Mustard cake and FeSO₄·7H₂O, (C) Mustard cake and ZnSO₄·7H₂O, (D) Mustard cake and pH, (E) xylose and FeSO₄·7H₂, (F) Xylose and ZnSO₄·7H₂O.
Effect of mustard cake and xylose on xylanase production

The data on xylanase production as influenced by mustard cake and xylose levels along with the variables such as FeSO$_4$-H$_2$O, ZnSO$_4$-H$_2$O, and pH are presented in (Figure 3A). Increased xylanase production was observed at FeSO$_4$-H$_2$O 0.06, ZnSO$_4$-H$_2$O 0.04 and pH 6.5, respectively. The production of xylanase significantly influenced by the mustard cake ($p < 0.005$) and the mustard cake ($p < 0.005$) in interaction terms. The mustard cake ($p < 0.005$) and xylose were ($p < 0.005$) significant in terms of squared terms. The negative interactive coefficient (Eq. 2) suggests that decreasing the level increases the enzyme activity while mustard cake at 3.96 g/L.

Effect of mustard cake and FeSO$_4$-7H$_2$O on xylanase production

The interaction effects plotted for mustard cake and FeSO$_4$-H$_2$O show that there are no significant interactions between these variables that affect xylanase yield (Figure 3B). The enzyme production was influenced by the KH$_2$PO$_4$ in linear term ($p > 0.05$) and MgSO$_4$ ($p > 0.05$) though their individual p-value is not significant. However this confirmed that optimal mustard cake range lied between 2 and 3.5 g/L and the optimal FeSO$_4$-H$_2$O was between 0.04 and 0.06 g/L. The (Figure 3B and Table 2) show that amount of xylanase production under these conditions were 17.83 U/mL. The remaining variables at their optimal level support the enzyme production.

Effect of mustard cake and ZnSO$_4$-7H$_2$O on xylanase production

The enzyme production was influenced by the mustard cake in linear term ($p > 0.05$) and ZnSO$_4$-7H$_2$O ($p > 0.05$) though their individual p value is significant and also both KH$_2$PO$_4$ and MgSO$_4$ ($p < 0.005$) are having significant squared terms and they showed significant interaction between these two variables that should influence the xylanase production (Table 2). The positive interactive coefficient (Eq. 2) of these two variables suggested that the enzyme activity was significantly influenced by these variables by fixing other variables at their fixed levels. The Figure 3C shows that optimal xylanase activity could be obtained when the mustard cake was in the range of 2–3.5 g/L, and the ZnSO$_4$-7H$_2$O levels between 0.02 and 0.04 g/L, at the optimal level. The maximum yield of enzyme activity was 50.64 U/mL.

Effect of mustard cake and pH on xylanase production

The Figure 3D shows interactive effect of mustard cake and pH level on xylanase yield. Increase in mustard cake level up to 3.5 g/L and pH up to 6.5, improved the xylanase yield at the optimum variables were xylose 6 g/L, FeSO$_4$-7H$_2$O 0.06 g/L and ZnSO$_4$-7H$_2$O 0.04 g/L. The linear terms and squared terms of mustard cake and pH are positive and also the positive interactive coefficient (Eq. 2) of these variables suggested the significant increase in the xylanase yield. The Table 2 shows the actual yield was 44.83 U/mL, with the predicted xylanase yield of 40.82 U/mL, at optimum level.

Effect of xylose and FeSO$_4$-7H$_2$O on xylanase production

Regardless of FeSO$_4$-7H$_2$O, the maximum xylanase yield was obtained in between 0.04 and 0.06 g/L of xylose and concentration variations in FeSO$_4$-7H$_2$O did not affect the xylose optima between 4 and 6 g/L. It confirms the lack of interaction between these parameters. The negative interactive coefficient (Eq. 2) suggests that decreasing the level of enzyme yield while the FeSO$_4$-7H$_2$O in negative form and also squared terms of these variables. The Figure 3E shows the maximum actual yield of xylanase was 11.94 U/mL and predicted yield of xylanase was 18.82 U/mL, at other variables at their optimum conditions.

Effect of xylose and ZnSO$_4$-7H$_2$O on xylanase production

The enzyme production was affected by xylose in linear term ($p > 0.05$) and ZnSO$_4$-7H$_2$O ($p > 0.05$) though their individual p value was significant. However, both xylose and ZnSO$_4$-7H$_2$O are having not significant in squared terms and they showed significant interaction between these two variables should influence on the xylanase production. The positive interactive coefficient (Eq. 2) of these two variables suggested that the enzyme activity
was significantly influenced by these variables by fixing other variables at their fixed levels. The Figure 3F shows that optimal xylanase activity could be obtained when the xylose was in the range of 4–6 g/L, and the ZnSO₄·7H₂O levels between 0.04 and 0.06 g/L, at the optimal level. The maximum actual yield of enzyme was 74.84 U/mL and predicted yield of xylanase was 79.91 U/mL. The other variables tested were at their optimal levels. Xylose could induce xylanase production by *Trichosporon cutaneum* SL409 and *Aspergillus nidulans*, respectively [19, 20]. The results are observed in the present work was high when compared to *T. longibrachitum*, and also with *Penicillium* sp. [21].

**Experimental validation**

The adequacy of the model was validated by performing a total five verification experiments within the experimental range is given in (Table 4). The data of the validation runs were also statistically analyzed to find out the correlation between actual observed and predicted values. The determination of coefficient (R²) of experimental and predicted values was found to be 0.9651 indicating that the group of experimental values is in good agreement with that of predicted, showing thereby the accuracy of the model.

**Composition of de-oiled cakes**

The proximate composition of different de-oiled cakes used in the present study was depicted in the (Table 5).

From the data it is observed that soybean, mustard and ground nut cake contained crude protein 35.56±0.05, 41.03±0.06 and 21.64±0.02%, respectively, whereas crude fiber 7.18±0.02, 10.63±0.05 and 21.64±0.02%, respectively. Similarly the crude fat present in the soybean, mustard and ground nut cake is 21.5±0.04, 18.39±0.06 and 17.63±0.02%, respectively, whereas total carbohydrates are 29.72±0.06, 32.73±0.02 and 27.31±0.04%, respectively. Crude protein was high in mustard cake than the others, fat and fiber values are lower in ground nut cake, followed by soybean cake and mustard cake. The total carbohydrate content is higher in mustard cake compared to soybean and ground nut due to the higher cellulose content of mustard cake. Similarly the nitrogenous substance i.e. crude protein also high in mustard cake, this resembles that mustard cake increases the xylanase production. There are several reports describing production of various enzymes using oilseed cakes as supplements in the production medium. Oil cakes are ideally suited nutrient support in SmF rendering both carbon and nitrogen sources, and reported to be good substrate for enzyme production using fungal species. This is due to the fact that oilseed cakes are among the agricultural residues commonly used for xylanase production, since they contain some residual nutrients that can serve as carbon, nitrogen inducer sources, and have been reported to be good substrates for microbial enzyme production [22]. Canola oil seed cake (CaOC) has been used as a substrate for xylanase production by *Trichoderma reesei*. The results suggested that xylanase yields were better in CaOC than from Solka-Xoc, xylan or glucose. The enzyme system produced using CaOC also contained a higher proportion

### Table 4: Model validation experiments.

| S. No | A  | B  | C  | D  | E  | Experimental values xylanase (U/mL) | Predicted values xylanase (U/mL) | Experimental values protein (mg/mL) | Predicted values protein (mg/mL) |
|-------|----|----|----|----|----|-------------------------------------|---------------------------------|----------------------------------|-------------------------------|
| 1     | 5  | 3  | 1  | 4  | 3  | 60.6                                | 59.4                            | 2.18                             | 1.98                          |
| 2     | 5  | 5  | 2  | 5  | 2  | 61.4                                | 65.6                            | 2.97                             | 2.36                          |
| 3     | 8  | 5  | 3  | 8  | 6  | 69.8                                | 67.4                            | 3.62                             | 3.52                          |
| 4     | 10 | 5  | 3  | 8  | 5  | 60.2                                | 61.8                            | 2.81                             | 2.02                          |
| 5     | 7.5| 4  | 3  | 6  | 4  | 81.2                                | 80.9                            | 4.62                             | 4.31                          |

A, Mustard cake (g/L); B, Xylose (g/L); C, FeSO₄·7H₂O (g/L); D, ZnSO₄·7H₂O (g/L); E, pH.

### Table 5: Composition of the oil seed cakes (on dry matter basis %).

| Type of oil seed cake | Moisture | Ash       | Crude protein | Crude fiber | Crude fat | Total Carbohydrates |
|-----------------------|----------|-----------|---------------|-------------|-----------|---------------------|
| Soybean               | 6.02±0.05| 7.16±0.05| 35.56±0.05    | 7.18±0.02   | 21.5±0.04 | 29.72±0.06          |
| Mustard               | 8.07±0.04| 10.26±0.02| 41.03±0.06    | 10.63±0.05  | 18.39±0.06| 32.73±0.02          |
| Groundnut             | 5.04±0.02| 5.64±0.03| 21.64±0.02    | 21.3±0.03   | 17.63±0.02| 27.31±0.04          |

The experiments were performed in triplicate.
of acetyl-xylan esterase, cellulase, and xylosidase activities [23]. These results indicate that xylanase production was enhanced by some oil cakes. Generally the carbohydrates stimulated the growth of fungi [24]. In the present investigation the de-oiled mustard cake contained higher amount of total carbohydrates (32.73 ± 0.02% w/v) than the other cakes that served as growth promoters and supported the production of xylanase in the medium along with the corn cobs.

The mineral content of the de-oiled cakes is shown in Figure 4. The data showed the presence of nitrogen, calcium, magnesium, manganese, zinc, and iron (1.84, 1.09, 0.69, 0.71, 0.96, and 1.52 mg/100 mg, respectively) in mustard cake. The phosphorus (1.01 mg/100 mg) content was high in ground nut cake and copper (0.64 mg/100 mg) was high in soybean cake. Although ions of Mg, Ca, Fe, Mn and Zn were chosen in the PBD, the result showed that only Fe²⁺ and Zn²⁺ had a significant impact on xylanase production. Some researchers have indicated the importance of Mg²⁺ as a trace element in xylanase production by Aspergillus fischeri Fxn 1 [25]. Zn²⁺, Mg²⁺ and Fe²⁺ could also stimulate the yield of xylanase by Streptomyces thermodiasticus [26]. In addition to these elements, the mustard cake having the higher amounts of Zn and Fe appear to support xylanase production indirectly.

Conclusions

Utilization of cheaply available lignocellulosic substrates such as corn cobs for fermentation process would eventually reduce the environmental problems, which may be caused by their accumulation. The use of by-products of oil-seed processing industry such as cakes in fermentation medium will also reduce the cost of microbial products.

The effect of cakes (mustard, soybean and groundnut) in SmF was evaluated, and optimization to maximize the xylanase production was carried out. Optimization of these parameters had a significant effect on the enzyme production. The actual optimum experimental value of xylanase activity of 74.84 U/mL is a good fit with the predicted value of 79.91 U/mL, which showed the model accuracy.

Acknowledgements: We are thankful to Dr. S.C. Basappa, former Deputy Director and Scientist, Central Food Technological Research Institute (CFTRI), Mysore, for his encouragement and critical comments on the manuscript.

Conflict of interest: The authors have no conflict of interest.

References

1. Pandey A, Selvakumar P, Soccol CR, Nigam P. Solid state fermentation for the production of industrial enzymes. Curr Sci 1999;77:149–62.
2. Salihu A, Alam MZ, Abdul Karim MI, Salleh HM. Optimization of lipase production by Candida cylindracea in palm oil mill effluent based medium using statistical experimental design. J Mol Catal Enzym 2011;69:66–73.
3. Krishna Prasad K, Venkata Mohan S, Sreenivas Rao R, Ranjan Pati B, Sarma PN. Laccase production by Pleurotus ostreatus 1804: optimization of submerged culture conditions by Taguchi DOE methodology. Biochem Eng J 2005;24:17–26.
4. Montgomery D. Design and analysis of experiments, 5th ed. Wiley, New York: 2001.
5. Chen XS, Tang L, Li S, Liao LJ, Zhang JH, Mao ZW. Optimization of medium for enhancement of epsilon Poly-L-Lysine production by Streptomyces sp.M-Z18 with glycerol as carbon source. Bioresour Technol 2011;102:1727–32.
6. Jayaswal RK, Fernandez MA, Schroeder RG. Isolation and characterization of a Pseudomonas strain that restricts growth of various phytopathogenic fungi. Appl Environ Microbiol 1990;56:1053–8.
7. Elibol M. Optimization of medium composition for actinorhodin production by Streptomyces coelicolor A3 with response surface methodology. Process Biochem 2003;39:1057–62.
8. Purohit HJ, Kapley A, Moharikar AA, Narde G. A novel approach for extraction of PCR-compatible DNA from activated sludge samples collected from different biological effluent treatment plants. J Microbiol Methods 2003;52:315–23.
9. Thompson JD, Higgins DG, Gibson TJ. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acid Res 1994;22:4673–80.
10. Naveena B, Sakhiselvan P, Elayaraju P, Partha N. Ultra sound induced production of thrombinase by marine actinomyces: kinetic and optimization studies. Biochem Eng J 2012;61:34–42.
11. Ramesh B, Vijaya Kumar P, Reddy OV. Enhanced production of xylanase by solid state fermentation using *Trichoderma koenungi* isolate: effect of pretreated agro-residues. 3 Biotech 2014;4:655–64.

12. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;38:265–73.

13. Box GE, Behnken DW. Some new three level designs for the study of quantitative variables. Technometrics 1960;2:455–75.

14. Lowry OH, Rosbrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:257–323.

15. Bailey MJ. Inter-laboratory testing of method for assay of xylanase activity. J Biotechnol 1992;23:257–70.

16. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 1959;31:426–8.

17. Dhiman SS, Sharma J, Battan B. Pretreatment processing of fabrics by alcalothermophilic xylanase from *Bacillus stearothermophilus* SDX. Enzyme Microb Technol 2008;43:262–9.

18. Li Y, Cui FJ, Liu ZQ, Xu YY, Zhao H. Improvement of xylanase production by *Penicillium oxalicum* ZH-30 using response surface methodology. Enzyme Microb Technol 2007;40:1381–8.

19. Fernándezespinar M, Pinaga F, Deegraaf L, Visser J, Ramon D, Valles S. Purification, characterization and regulation of the synthesis of an *Aspergillus nidulans* acidic xylanase. Appl Microbiol Biotechnol 1994;42:555–62.

20. Liu W, Lu YL, Ma GR. Induction and glucose repression of endo-β-xylanase in the yeast *Candida cylindracea* in palm oil mill effluent based medium using statistical experimental design. J Mol Catal Enzym 2003;69:66–73.

21. Haltrich P, Nidetzky B, Kulbe KD, Steiner W, Zupansias S. Production of fungal xylanase. Bioresource Technol 1996;58:137–61.

22. Ramachandran S, Singh SK, Larroche C, Soccol CR, Pandey A. Oil cakes and their biotechnological applications. Bioresour Technol 2009;58:137–61.

23. Gattinger LD, Duvnjak Z, Khan AW. The use of canola meal as a substrate for xylanase production by *Trichoderma reesei*. Appl Microbiol Biotechnol 1990;33:257–70.

24. Katapodis P, Christakopoulou V, Kekos D, Christakopoulos P. Optimization of xylanase production by *Chaetomium thermophilum* in wheat straw using response surface methodology. Biochem Eng J 2007;35:136–41.

25. Senthilkumar SR, Ashok Kumar B, Chandra Raj K, Gunasekaran P. Optimization of medium composition for alkali-stable xylanase production by *Aspergillus fischeri* Fxn 1 in solid-state fermentation using central composite rotary design. Bioresour Technol 2005;96:1380–6.

26. Techapun C, Sinsuwongwat S, Watanabe M, Sasaki K, Poosaran N. Production of cellulase-free xylanase by a thermotolerant *Streptomyces* sp. grown on agricultural waste and media optimization using mixture design and Plackett-Burman experimental design methods. Biotechnol Lett 2002;24:1437–42.