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Statistical Optimization for Enhanced Xylanase Production by In-house *Aspergillus fumigatus* PSF1 under Solid State Fermentation using Wheat Bran

V.H. Vinuthana[1], Santhoshkumar Subramaniam[1], A.S.M. Raja[2], S.K. Shukla[2], M. Gnanachitra[1], D. Ramesh[3], S. Haripriya[4], K. Chandrakumar[5] and Sivakumar Uthandi[1]*

[1]Biocatalysts Laboratory, Department of Agricultural Microbiology, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India.
[2]ICAR- Central Institute for Research on Cotton Technology, Matunga, Mumbai, Maharashtra, India.
[3]Department of Renewable Energy Engineering, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India.
[4]Horticulture College and Research Institute, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India.
[5]Department of Biochemistry, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India.

Abstract

Agro-residues constitute the largest reservoir of renewable carbon source on Earth. While their valorization has primarily focused on cellulose, hemicellulose is the second most abundant group of polysaccharides and remains underexploited. Glycosyl hydrolases (GHs) are key enzymes involved in the depolymerisation of plant biomass, and filamentous fungi are widely recognized as efficient producers of GHs enzymes. In the present study, an in-house isolate, *Aspergillus fumigatus* PSF1, was used to produce xylanase via solid-state fermentation (SSF) with wheat bran as the sole carbon source. Key process conditions, including moisture percent, inoculum load, temperature, and pH, were initially optimized using the One-Factor-at-a-Time (OFAT) method and subsequently statistically optimized using Response Surface Methodology (RSM). Under optimized conditions, xylanase activity reached 147.96 IU gds^{-1}, a 1.29-fold increase relative to unoptimized conditions. The study demonstrates that systematic optimization of critical fermentation parameters significantly enhances xylanase productivity in SSF by *A. fumigatus* PSF1. The findings suggest that wheat bran is an effective low-cost substrate and highlight the potential of this fungal strain for scalable, high-yield xylanase production.

Keywords: Response Surface Methodology, Solid-state Fermentation, Xylanase, Wheat Bran, Waste Valorization

***Correspondence:** usiva@tnau.ac.in

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INTRODUCTION

Management of enormous amounts of waste generated by food, industrial, and particularly agricultural sources poses a serious global problem. In developing countries, where agriculture is a primary economic activity, the waste generated is usually burned, dumped, or landfilled.[1] Approximately 1.3 billion tons of agricultural waste, valued at around $165 billion annually, are wasted worldwide, accounting for roughly one-third of total food production. 140 billion metric tons of waste originate from agriculture every year, of which half is disposed of in landfills or burned. Agricultural waste disposed of in landfills or burned pollutes ecosystems, emits harmful greenhouse gases, and endangers human health by releasing harmful substances.[2]

Agricultural residues are a primary renewable energy resource with negligible nutritional value for humans, produced mainly from wheat, rice, pearl millet, sorghum, barley, finger millet, sugarcane, potatoes, tubers, and pulses.[3,4] These lignocellulosic biomasses are abundant, low-cost, and rich in cellulose, hemicellulose, and lignin, making them promising feedstocks for diverse bioprocessing applications, including biofuel production, enzyme synthesis via solid-state fermentation (SSF), and animal feed formulation.[5] Among various lignocellulosic substrates, wheat bran is frequently reported as an excellent support and carbon source for fungal xylanase production, owing to its xylan content (18%-22%), favourable particle size, porosity, and balanced nutrient composition, which collectively support high enzyme yields under SSF.[6] Furthermore, xylan has extensive applications in the production of bio-based products, including packaging materials, hydrogels, ethanol, lactic acid, furfural, etc. Xylan is also used in bioplastic formulations, medical applications, and value-added processes to produce xylitol, xylanase, and xylooligosaccharides, among other products.[7,8] Valorization of wheat bran for xylanase and related bioproducts aligns with circular bioeconomy goals, transforming an abundant milling byproduct into a high-value biochemical input.

Xylanases, key hemicellulolytic enzymes that break β-1,4-glycosidic linkages in the xylan polymer, thereby enabling the restyling of biomass into fermentable sugars and value-added products.[9,10] Xylanases are classified into GHs families, notably GH10 and GH11, with contributions from GH5, GH8, and GH43. GH11 xylanases are highly substrate-specific and efficient at cleaving β-1,4-glycosidic linkages in xylan, whereas GH10 xylanases often display broader substrate specificity and greater thermostability. The catalytic mechanism typically involves key glutamic and aspartic acid residues, and some xylanases operate via a double-displacement mechanism, thereby retaining the anomeric configuration.[11] Galactose, arabinose, and 4-O-methylglucuronic acid are present in the main chain of xylan, which is made up of xylose. These glycosidic linkages can also be of the type β-(1→3), β-(1→6), α-(1→2), α-(1→3), and α-(1→6)[12] Filamentous fungi, specifically *Aspergillus* spp., are widely preferred for xylanase production because of their robust secretory machinery, proliferation on low-cost residues, and the ability to yield extracellular enzyme titers suitable for industrial processes. SSF has gained prominence over submerged cultivation for such fungi as it mimics their natural habitat, enables high volumetric productivity, and can be implemented using inexpensive agricultural byproducts.[13,14] However, xylanase productivity under SSF is strongly influenced by multiple interacting factors, including substrate moisture content, initial pH, incubation temperature, inoculum load, and supplementation with nitrogen and mineral sources, which cannot be efficiently optimized by traditional one-factor-at-a-time (OFAT) approaches.

In recent years, statistical designs have been widely applied to optimize xylanase production, enabling quantitative evaluation of factor interactions, reducing the number of experimental runs, and significantly enhancing enzyme titers. Dual step statistical strategies, combining screening and response surface methodology (RSM), have delivered several-fold improvements in xylanase yields in bacterial and fungal systems, highlighting their utility for developing economically viable bioprocesses.[9] Nonetheless, there is limited information on the statistically optimized production of xylanase in *A. fumigatus* under SSF using wheat bran as a substrate despite the species' recognized

lignocellulolytic potential. Employing this strategy, this study was designed to enhance xylanase activity through SSF by optimizing key parameters, including moisture percentage, inoculum load, pH, and temperature, using the in-house *A. fumigatus* strain.

MATERIALS AND METHODS

Substrate processing and culture preparation

Wheat bran was procured from the local wheat mill in Coimbatore. The collected wheat bran was shade-dried for two days to retain its physical and chemical properties. The shade-dried sample was sieved using a 200 µm sieve and stored at room temperature for further studies. *A. fumigatus* PSF1 was collected from the Biocatalysts Laboratory, Department of Agricultural Microbiology, Tamil Nadu Agricultural University. The culture exhibited a white, floccose mycelial mat that later turned greenish due to profuse conidial sporulation on Potato Dextrose Agar (PDA). The culture was stored at 4 °C on PDA plates and sub-cultured frequently onto fresh PDA plates.[15]

Substrate preparation and xylanase production

SSF was performed in 90 mm borosilicate glass petri-dishes filled with 10 g of untreated wheat bran supplemented with synthetic medium consisted of 2 gL^{-1} K_2HPO_4, 0.5 gL^{-1} KCl, 0.01 gL^{-1} $FeSO_4 \cdot 7H_2O$, 0.15 gL^{-1} $MgSO_4 \cdot 7H_2O$, 7 gL^{-1}, KH_2PO_4, 1 gL^{-1} (NH_4) SO_4, and 1.2 gL^{-1} yeast extract at 50% moisture content and the pH was adjusted to 4.8 Prepared Petri dishes were autoclaved. Four discs of inoculum per plate were placed on sterile substrate and incubated at 40 °C for 10 days. The produced xylanase was extracted using a citrate-phosphate buffer (pH 3.4) by incubating at 40 °C for 30 min under shaking conditions, followed by filtration.[16]

Xylanase extraction

The 3,5-dinitrosalicylic acid (DNS) assay was used to measure xylanase activity.[17] 0.5 mL of enzyme extract was added to 1 mL of substrate (1% xylan in 50 mM sodium phosphate buffer, pH 6.5), mixed thoroughly, and incubated at 50 °C for 30 min. The activity was terminated by adding 1.5 ml of the DNS reagent, followed by boiling in a water bath for 5 min. Absorbance was measured at 540 nm using a UV-Vis spectrophotometer (Spectramax i3x, USA) to quantify the enzyme activity of the resulting solution. One international unit (IU) of xylanase activity was defined as the amount of enzyme that liberates 1 µmol of xylose per minute under the specified assay conditions.

Pre-optimization of process parameters for xylanase production

In the present study, culture and substrate conditions, including moisture percentage, inoculum load, temperature, and pH, were optimized to enhance xylanase production under SSF using an OFAT approach. The effect of moisture percent (60%, 65%, 70%, 75% and 80%), inoculum load (4, 5, 6, 7 and 8 disc per plate), temperature (32 °C, 34 °C, 36 °C, 38 °C, and 40 °C), and pH levels (4.0, 4.5, 5.0, 5.5, and 6.0) on xylanase produced from *A. fumigatus* PSF1 was studied.

Production optimization of xylanase by response surface methodology (RSM)

The Box-Behnken design (BBD) is well documented for evaluating the effects of individual factors and their interactions on experimental responses with considerably fewer experiments. This approach yields more accurate and reliable results, providing efficient, rapid process conditions by using second-order polynomial equations as response classifiers and highlighting the significance of key variables. The BBD approach drastically reduces the number of trials, making it highly advantageous for optimization.[18] Xylanase production under SSF depends critically on variables such as inoculum load, substrate moisture content, temperature, and pH. The optimal response conditions for xylanase production using wheat bran were systematically identified using RSM. Four independent variables, such as moisture percent (%), inoculum load (disc per plate), temperature (°C), and pH, were utilised for optimization. The second-order model used to describe the influence of independent factors on the response variable was presented as

$$Y_i = \beta_0 + \sum_{i=1}^{k} \beta_{ij} x_i + \sum_{i=1}^{k} \beta_{ij} x_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} x_i x_j + \varepsilon$$

In this model, Y_i denotes the output variable; x denotes the independent variable; β_0 is the constant term; and β_i, β_{ii}, and β_{ij} denote the linear, quadratic, and interaction regression coefficients, respectively. The β coefficients are calculated by the least-squares method (Table 1). The BBD generated 29 experimental runs, comprising four variables, using Design-Expert software 10.0 (Stat-Ease, Inc., USA).[19]

Statistical analysis

Experiments were performed in triplicate; results were expressed as mean ± standard error. The RSM models and optimization procedures are constructed using Design-Expert software 10.0 (Stat-Ease, Inc., USA). Analysis of Variance (ANOVA) was also evaluated using Design-Expert software 10.0.

RESULTS AND DISCUSSION

Pre-optimization of process parameters for xylanase production

Role of moisture percent on xylanase activity

Moisture percent is a crucial determinant in SSF, as excessive moisture alters substrate porosity and particle structure, whereas insufficient moisture increases water tension and

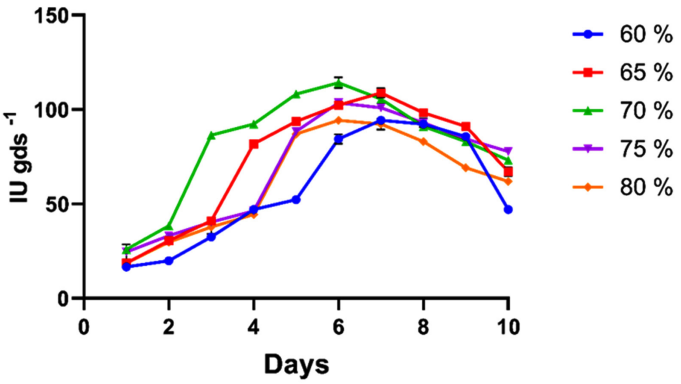

Figure 1. Pre-optimization studies for xylanase activity in solid-state fermentation at different moisture percentages (60, 65, 70, 75 and 80). All the data plotted indicates the mean of three replications accomplished by standard error (SE)

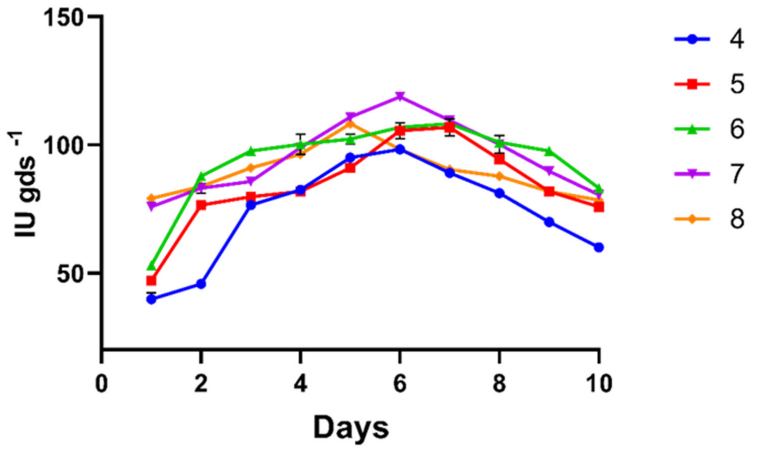

Figure 2. Pre-optimization studies for xylanase activity in solid-state fermentation at different inoculum loads in discs (4, 5, 6, 7, and 8). All the data plotted indicates the mean of three replications accomplished by standard error (SE)

reduces nutrient solubility. Therefore, optimizing the moisture content is crucial for maximizing enzyme yield.[20] In the current study, the effect of five moisture levels on xylanase activity was evaluated. Xylanase activity elevated with rising moisture and achieved a maximum of 114.14 IU gds^{-1} at 70 % moisture level, beyond which activity declined significantly (Figure 1). Higher moisture levels limit oxygen diffusion and increase particle clumping, thereby restricting hyphal penetration. Similarly, Khanahmadi et al. reported a peak xylanase activity of 2070 U/g of fermented dry matter at 70% moisture, using wheat bran as the substrate under SSF by *A. niger* CCUG 3399.[14] Yet another study reported maximum xylanase activity of 310 IU g^{-1} with wheat bran at 70% moisture, supporting the present findings.[21]

Role of inoculum load on xylanase activity

The effect of inoculum load on xylanase production showed that increasing the inoculum load from 4 to 7 discs (9 mm) significantly elevated xylanase production, with a maximum activity of 118.74 IU gds^{-1} (Figure 2). Further increase in inoculum load reduced activity, attributed to nutrient limitation. Conversely, a low inoculum load produced insufficient biomass to utilize available nutrients, resulting in reduced enzyme

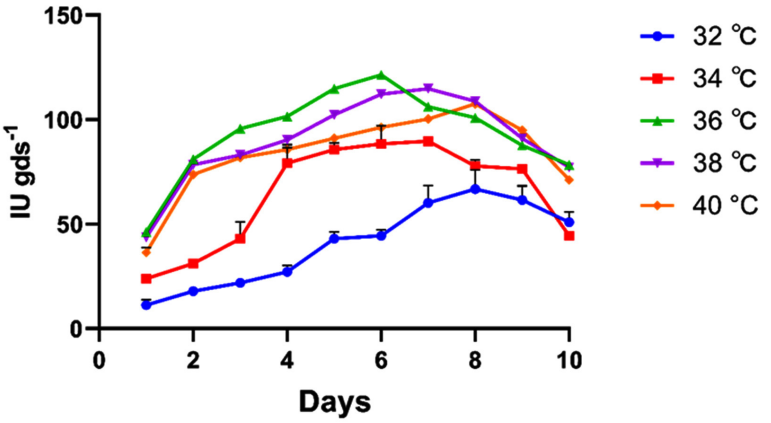

Figure 3. Pre-optimization studies for xylanase activity under SSF at different temperatures (32 °C, 34 °C, 36 °C, 38 °C, and 40 °C). All the data plotted indicates the mean of three replications accomplished by standard error (SE)

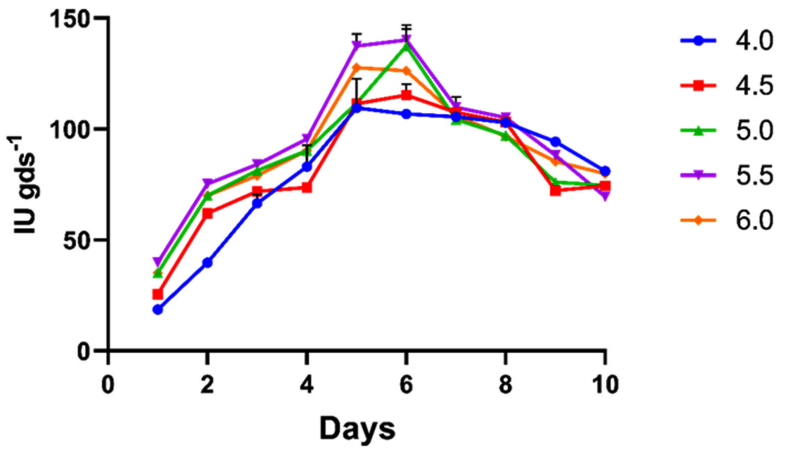

Figure 4. Pre-optimization studies for xylanase activity under SSF at different pH levels (4.0, 4.5, 5.0, 5.5, and 6.0) All the data plotted indicates the mean of three replications accomplished by standard error (SE)

production effectively. Ramanjaneyulu and co-workers conducted a similar study to improve xylanase production by *Fusarium* sp. BVKT R2 and found the inoculum load of 6 agar discs (0.5 mm) as the optimum inoculum load, and higher loads led to a significant decline in activity.[22]

Role of incubation temperature on xylanase activity

The influence of temperature on xylanase showed an increasing trend from 32-34 °C, reaching a maximum of 121.40 IU gds^{-1} at 36 °C. Further increase in temperature from 36-40 °C significantly reduced enzymatic activity (Figure 3). This trend in enzymatic activity at suboptimal temperatures is consistent with an Arrhenius-type relationship, in which elevated temperature promotes molecular motion and facilitates the reaction to overcome its activation energy barrier. At temperatures above the optimum, protein denaturation predominates, leading to enzyme deactivation and reduced activity.[23] Another study on the optimization of xylanase production using wheat bran as a substrate with the *A. niger* strain

Table 1. Independent variables and levels of variation in Box-Behnken design (BBD)

Study type	Response Surface	Subtype	Randomized			
Design type	Box-Behnken		29 Runs			
Design mode	Quadratic					
Factor	**Name**	**Units**	**Type**	**Minimum**	**Maximum**	**Mean**
A	Moisture percent	%	Numeric	60	80	70
B	Inoculum load	disc	Numeric	4	8	6
C	temperature	°C	Numeric	32	40	36
D	Initial pH		Numeric	4	6	5
Response	**Name**		**obs**	**Analysis**		
R1	Xylanase	IU gds^{-1}	29	Polynomial		

Table 2. Analysis of variance (ANOVA) for optimization of xylanase activity under solid-state fermentation

Source	Sum of Squares	df	Mean Square	F-value	P-value	
Model		14	332.18	7.78	0.0002	Significant
A-Moisture percent	4650.56	1	21.48	0.5028	0.4899	
B-Inoculum load	21.48	1	15.99	0.3743	0.5505	
C-Temperature	15.99	1	19.92	0.4663	0.5059	
D-pH	19.92	1	589.74	13.81	0.0023	
AB	589.74	1	48.60	1.14	0.3042	
AC	48.60	1	78.55	1.84	0.1965	
AD	78.55	1	12.33	0.2886	0.5995	
BC	12.33	1	83.93	1.96	0.1828	
BD	83.93	1	6.99	0.1636	0.6919	
CD	6.99	1	2.21	0.0518	0.8233	
A^2	2.21	1	166.08	3.89	0.0687	
B^2	166.08	1	48.74	1.14	0.3035	
C^2	48.74	1	1525.93	35.72	<0.0001	
D^2	1525.93	1	848.31	19.86	0.0005	
Residual	848.31	14	42.71			
Lack of Fit	597.99	13	45.98	193.16	0.0563	Not significant
Pure Error	597.75	1	0.2380			
Cor Total	0.2380	28				
R^2	0.88		C.V **%**	5.92		
Adjusted R^2	0.77		Adeq Precision	9.69		
Predicted R^2	0.66					

BG also reported 36 °C as the optimal temperature within the range of 22-40 °C (4084.75 IU gds^{-1}), which is in agreement with the current findings.[24]

Role of pH on xylanase activity

The initial pH of the medium is a key determinant of enzyme synthesis by influencing enzyme stability, membrane transport, nutrient solubility, and the activity of regulatory proteins. The role of initial pH on xylanase was optimized. As depicted in Figure 4, the highest xylanase activity was achieved at pH 5.5 (140.23 IU gds^{-1}). Enzyme activity declined markedly at acidic pH values (≤ 5.0) due to reduced growth and decreased enzyme stability. In contrast, the decline at pH 6.0 indicates that slightly alkaline conditions are also unsuitable

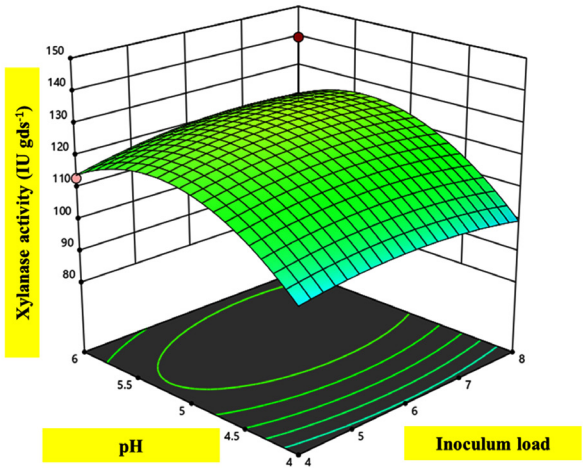

Figure 5. 3D surface plots for the response of xylanase activity (IU gds^{-1}) due to physical and nutritional parameters: pH vs. inoculum load. The dependent effect is visualized by colors ranging from blue to red (blue, green, and red). Blue denotes least significant, green is moderately significant, and red is highly significant

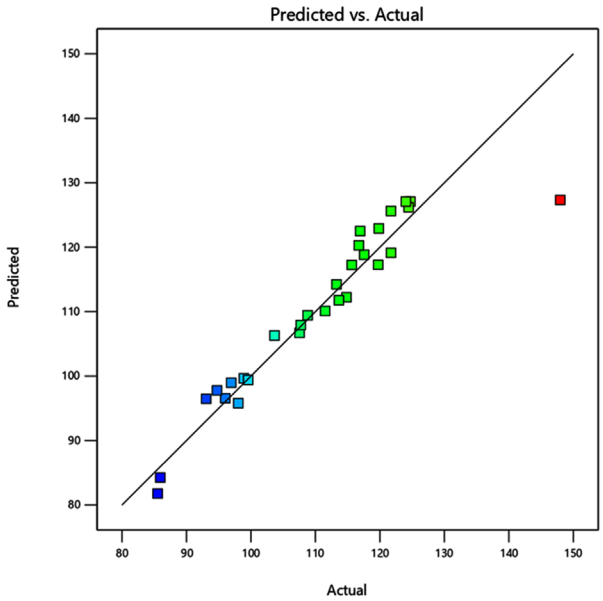

Figure 6. Correlation matrix between predicted value and actual values of xylanase activity. The interactive effect is represented with the color ranging from blue to red (blue, green, red); blue is least significant, green is moderately significant, and red is highly significant

for enzyme production. Similarly, a study on the production of xylanase from a white-rot fungus under SSF using wheat bran also concluded that pH 5.0 was optimal for maximum xylanase production.[25]

Statistical optimization of xylanase production using Response Surface methodology

A BBD comprising 29 runs was considered optimal for four significant parameters: moisture percentage, inoculum load, temperature, and pH source to achieve elevated production of the xylanase enzyme. The quadratic model yielded predicted and adjusted R^2 values of 0.66 and 0.77, respectively, for xylanase activity. The predicted R^2 closely matched the adjusted R^2 suggesting that the model is reliable. Additionally, a precision value of 9.69 indicates a highly favorable signal-to-noise ratio. The second-order equation for xylanase optimization, expressed in terms of coded factors and derived from the BBD, is as follows:

$$\text{Xylanase (IU gds}^{-1}) = 126.36 - 1.90A + 1.35B + 1.56 + 8.57D - 3.87AB - 5.17AC - 2.51AC + 5.21BC + 1.69BD + 0.8731 - 6.32A^2 - 3.39B^2 - 18.56C^2 - 14.29D^2$$

The effects and significance of variable interactions on xylanase response in the quadratic model were evaluated using Analysis of variance (ANOVA). A model F-value of 7.78 indicates that the model is significant. A small F-value for the model is undesirable as it suggests that the variance is attributable to random, unexplained disturbances (noise). Among the interactions, D, C^2, and D^2 showed significant positive effects on the production process, with P-values <0.05. The Coefficient of variance (CV) value of 5.92% further demonstrated that the model possessed high precision and reliability (Table 2). The maximum xylanase activity of 147.96 IU gds^{-1} was obtained with 70% moisture, seven discs of inoculum load, under 36 °C, and pH 5.5 (Figure 5). Plotting the actual value curve as a function of the predicted values, which displays the points dispersed around the regression line confirmed the correlation (Figure 6). In a similar study, to elevate the xylanase activity, various physicochemical parameters were initially optimized using OFAT, then the xylanase production showed three folds increase after optimization through RSM with BBD by *A. niger* utilizing wheat bran as a sole carbon source.[26] A concurrent study showed *A. terreus* GU227345.1 isolated from local bio waste sources enhanced xylanase activity to 80 IU gds^{-1} at optimized conditions of 6 g substrate, pH 6.0, 60% moisture, inoculum of 2.75 mL at 35 °C for 4 days.[27] Another study reported elevated xylanase activity of 146 IU gds^{-1} using a fungi *Fomes fomentarius* under RSM optimized conditions.[28] A recent study used RSM optimization to enhance the laccase production to 260 IU gds^{-1} using *Fomes fomentarius.*[29] RSM is also used to optimize industrially important enzymes such as complex pectinolytic enzymes[15] and lipases.[30]

CONCLUSION

This study demonstrates the production and optimization of xylanase from the in-house potential strain *A. fumigatus* PSF1 using wheat bran, nutrient-rich substrate, under SSF. Preliminary optimization was performed using OFAT. Subsequent optimization using RSM (BBD) significantly enhanced xylanase yield up to 1.29-fold compared to unoptimized conditions. The model exhibited good predictive capability, as indicated by acceptable R^2 values, significant model terms, and satisfactory experimental reliability. Optimal conditions included 70% moisture, an inoculum load of seven discs, incubation at 36 °C, and a pH of 5.5; these were experimentally validated and confirmed their suitability for maximizing enzyme output. The study's findings emphasize the potential of *A. fumigatus* PSF1 as a promising fungal candidate for cost-efficient xylanase production from agro-residues. The optimized process provides a scalable strategy for sustainable enzyme production and the valorization of agricultural biomass, thereby contributing to broader goals of waste minimization and a circular bioeconomy.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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