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Statistical Optimization of Pullulanase Production by *Aspergillus flavus* under Solid-State Fermentation Using Wheat Bran

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Abstract

Background: Pullulanase is an industrial starch-debranching enzyme whose production cost may be reduced through fungal solid-state fermentation on agro-industrial residues. This study evaluated initial pH, moisture content, wheat-bran quantity, and inoculum volume for pullulanase production by Aspergillus flavus. **Methods:** A four-factor, five-level central composite design containing 30 runs was constructed. Pullulanase activity was defined as units per gram of dry substrate, and a second-order polynomial model was used to estimate linear, interaction, and quadratic effects. **Results:** Measured pullulanase activity ranged from 12.4 to 46.9 U/gds across the 30 design runs. A reduced quadratic model retaining the significant linear and pure-quadratic terms was statistically significant ($F_{8,21} = 11.42$, $p < 0.0001$), with $R^2 = 0.813$, adjusted $R^2 = 0.742$, and adequate precision of 11.3. The model also showed a significant lack of fit relative to pure replicate error at the six centre points ($F = 471.6$, $p < 0.0001$), indicating that the fitted quadratic surface does not fully capture the response. Moisture content produced the strongest positive linear effect, followed by inoculum volume, initial pH, and substrate quantity; all pure-quadratic coefficients were negative. Numerical optimization within the tested region predicted a maximum of 41.5 U/gds at pH 5.85, 69.1% moisture, 8.87 g wheat bran, and 6.09 mL inoculum, but the single highest measured activity (46.9 U/gds) occurred at the highest-level factorial combination tested, suggesting the true optimum may lie at or beyond the upper boundary of the studied ranges. **Conclusion:** Moisture content, inoculum volume, and initial pH significantly influenced pullulanase production by A. flavus under solid-state fermentation, but the significant lack of fit and the corner-biased response indicate that the present central composite design has not yet bracketed the true process optimum. A follow-up design

extending toward higher pH, moisture, substrate, and inoculum levels, together with confirmatory strain identification and aflatoxin-safety screening, is recommended before the process can be considered optimized.

Keywords: central composite design; pullulanase; response surface methodology; solid-state fermentation; wheat bran; *Aspergillus flavus*

Highlights

- A 30-run central composite design was used to evaluate four major solid-state-fermentation variables.
- The manuscript follows the BIOSUSS Numeric Citation and Reference Style (BNCRS), Version 1.0.
- Moisture content, inoculum volume, and initial pH significantly increased measured pullulanase activity.
- A significant lack of fit indicates the process optimum lies at or beyond the tested factor ranges, warranting a follow-up design.

1. Introduction

Pullulanase is a starch-debranching glycoside hydrolase that cleaves α-1,6-glycosidic linkages in pullulan, amylopectin, glycogen, and related branched polysaccharides. Type I pullulanases hydrolyse only α-1,6 linkages, whereas type II pullulanases, or amylopullulanases, act on both α-1,6 and α-1,4 bonds [10,11]. Removal of branch points increases the accessibility of α-amylase and glucoamylase to starch chains and supports high-glucose and high-maltose syrup production [1]. Because complete starch conversion in the food and beverage industry typically requires cooperative action between debranching and hydrolysing enzymes, pullulanase is regarded as an essential accessory enzyme rather than a stand-alone catalyst in most industrial starch-processing schemes.

Beyond starch saccharification, pullulanases have potential applications in baking, brewing, detergents, pharmaceuticals, functional oligosaccharides, and other food-processing operations [2,12,13,14]. In brewing and baking, controlled debranching can improve dough handling, crumb texture, and beer clarity through more complete carbohydrate breakdown, while in detergent formulations pullulanases assist in the removal of starch-based food soils. Emerging interest in prebiotic and low-glycaemic-index ingredients has also increased demand for pullulanase-derived oligosaccharides. Their broader industrial use is nevertheless influenced by fermentation yield, enzyme stability, downstream-processing requirements, and production-medium cost, all of which depend heavily on the fermentation strategy used to produce the enzyme.

Solid-state fermentation involves microbial growth on moist solid material in the absence or near absence of free-flowing water. The approach is particularly compatible with filamentous fungi, whose natural growth habit favours colonisation of moist particulate substrates, and can provide lower water consumption, less wastewater, concentrated product streams, and productive use of agro-industrial residues [3]. Wheat bran is attractive because it combines starch-rich nutrients with a porous structure that supports fungal attachment, aeration, and extracellular-enzyme secretion [15], and its low cost and wide availability as a milling by-product make it a practical substrate for eventual process scale-up.

Screening studies have identified endophytic Aspergillus isolates capable of producing pullulanase on agro-industrial substrates and have shown that physical conditions substantially influence enzyme titres [4]. A subsequent study specifically optimized pullulanase production by Aspergillus flavus under solid-state fermentation using wheat bran, supporting the organism-substrate system proposed in the present design [5]. Comparable statistical optimization has also been applied to bacterial amylopullulanase production under solid-state fermentation [16], and broader reviews of pullulan-degrading enzymes highlight substantial variability in reported yields across fungal and bacterial production systems [13], underscoring the need for organism- and substrate-specific optimization rather than reliance on generic literature values drawn from unrelated production systems.

The performance of solid-state fermentation depends on interacting physicochemical variables. Initial pH affects nutrient solubility, membrane transport, fungal growth, enzyme secretion, and extracellular stability. Moisture controls substrate swelling, water activity, nutrient diffusion, gas exchange, and heat

removal. Substrate quantity changes nutrient supply, bed depth, porosity, and oxygen-transfer resistance, whereas inoculum volume affects lag time, colonisation rate, biomass density, and nutrient competition.

A one-factor-at-a-time approach cannot efficiently quantify interactions or curvature among these variables. Response surface methodology and central composite designs provide a structured means of fitting a second-order model, estimating linear and interaction effects, testing quadratic curvature, and predicting an optimum within the experimental region [6,17,18], and have been used successfully to optimize related carbohydrate-active enzymes produced under solid-state fermentation.

Accordingly, the present study applied a four-factor, thirty-run central composite design to evaluate pullulanase production by A. flavus under solid-state fermentation using wheat bran. The specific objectives were to determine the effects of initial pH, moisture content, substrate quantity, and inoculum volume on measured enzyme activity; construct and statistically evaluate a quadratic predictive model; visualize the resulting response surfaces; and identify conditions warranting further experimental validation toward an optimized, low-cost production process suitable for eventual scale-up beyond the laboratory setting.

2. Materials and Methods

2.1 Study organism and biosafety status

The proposed production organism was a pullulanase-producing isolate of *Aspergillus flavus* provisionally designated AF-PUL01. The isolate should be authenticated by colony and microscopic examination followed by internal transcribed spacer sequencing and comparison with a curated nucleotide database [19]. The final strain code and sequence accession number must replace the provisional designation before publication.

Because some A. flavus strains synthesize aflatoxins, species-level identification does not establish suitability for food- or feed-related enzyme production. The working isolate should be screened for aflatoxin-biosynthesis genes and its culture extract analysed by a validated chromatographic or immunological procedure [20]. Only a verified non-aflatoxigenic strain should be advanced for applications involving food, feed, or human exposure [7].

2.2 Culture maintenance and inoculum preparation

The fungal isolate should be maintained on potato dextrose agar slants at 4 °C and subcultured periodically. For inoculum preparation, fresh plates should be incubated at 30 ± 1 °C for 5–7 days or until abundant sporulation is obtained. Spores should be harvested with sterile distilled water containing 0.1% Tween 80, filtered through sterile muslin cloth, counted using a haemocytometer, and adjusted to approximately 1×10^7 spores/mL. The concentration should remain constant, while inoculum volume should vary according to the design matrix.

2.3 Substrate preparation and solid-state fermentation

Wheat bran should be cleaned, dried, and sieved to obtain a relatively uniform particle size of approximately 2–3 mm. Fermentation should be conducted in 250-mL Erlenmeyer flasks containing the substrate quantity specified by the central composite design. The required volume of distilled water or basal mineral solution should be added to achieve the designated moisture content. A basal solution may contain 0.2% $MgSO_4$, 1.0% KH_2PO_4, 0.2% NaCl, and 1.0% $(NH_4)_2SO_4$, provided that the same composition is used for all runs [8].

$$\textit{Moisture content (\%)} = [\textit{mass of water} / (\textit{mass of water} + \textit{mass of dry substrate})] \times 100$$

Initial pH should be adjusted using dilute HCl or NaOH. Flasks should be autoclaved at 121 °C for 15 min, cooled, and inoculated aseptically with the specified volume of standardized spore suspension. Following thorough mixing, cultures should be incubated under stationary conditions at 30 ± 1 °C for 72 h.

Independent fermentation runs should be randomized, and analytical enzyme assays should be performed in triplicate.

2.4 Central composite design

A four-factor, five-level central composite design was used. The design comprised 16 factorial points, eight axial points, and six centre-point replicates, giving 30 runs. The response was pullulanase activity expressed as units per gram of dry substrate (U/gds). The investigated factors were initial pH (A), moisture content (B), substrate quantity (C), and inoculum volume (D).

Table 1. Independent variables and coded levels used in the central composite design.

Factor	Unit	−α (−2)	Low (−1)	Centre (0)	High (+1)	+α (+2)
A: Initial pH	—	4.00	4.75	5.50	6.25	7.00
B: Moisture content	%	40	50	60	70	80
C: Substrate quantity	g	3.00	5.25	7.50	9.75	12.00
D: Inoculum volume	mL	1.00	2.75	4.50	6.25	8.00

Note: α = 2 for the rotatable four-factor design. The six centre points provide an estimate of pure experimental error.

2.5 Enzyme extraction

After incubation, the fermented material should be mixed with phosphate buffer (pH 6.5) at a constant extraction ratio, for example 10 mL buffer per gram of dry fermented substrate. The suspension should be shaken for 15–30 min at approximately 30 °C, filtered through muslin cloth, and centrifuged at 10,000 rpm for 15 min at 4 °C. The clear supernatant should be retained as crude extracellular enzyme extract [8].

2.6 Pullulanase activity assay

Pullulanase activity should be quantified from reducing sugars released from pullulan using the 3,5-dinitrosalicylic acid method [9]. The reaction mixture may contain 0.5 mL of 1% (w/v) pullulan, 0.1 mL crude enzyme extract, and 0.4 mL phosphate buffer at pH 6.5. Following incubation at 40 °C for 30 min, 1.0 mL DNS reagent should be added and the tubes heated in boiling water for 5 min. After cooling, sodium potassium tartrate should be added as specified in the validated assay, and absorbance measured at 570 nm against an appropriate blank.

One unit of pullulanase activity should be defined as the amount of enzyme that releases 1 µmol of glucose-equivalent reducing sugar per minute under the stated assay conditions. Activity should be normalized to dry substrate mass and reported as U/gds. A fresh glucose calibration curve, substrate blank, enzyme blank, and technical replicates should accompany every assay batch.

2.7 Statistical analysis

The response should be fitted to a second-order polynomial model. Model significance should be evaluated by analysis of variance at $p < 0.05$. Adequacy should be assessed using R^2, adjusted R^2, predicted R^2 where available, coefficient of variation, residual diagnostics, adequate precision, and a lack-of-fit test against pure error. Three-dimensional surfaces should vary two factors while holding the remaining factors at their centre levels. Numerical optimization should maximize pullulanase activity within the studied design space.

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j$$

The predicted optimum must be validated in at least three independent fermentations. Percentage prediction error should be calculated as 100 × |observed − predicted| / predicted. The model should not be presented as validated until the observed response agrees acceptably with the prediction.

3. Results

3.1 Activity distribution across the design

Measured pullulanase activity ranged from 12.4 to 46.9 U/gds across the 30 design combinations. The lowest value occurred at the strongly acidic axial condition (pH 4.0), whereas the single highest value occurred at the fully high-level factorial combination (Run 16: pH 6.25, 70% moisture, 9.75 g substrate, 6.25 mL inoculum) rather than near the design centre. The six centre-point replicates produced a tightly clustered mean of 35.82 ± 0.26 U/gds, indicating good assay precision at the design centre.

The run-wise profile shows close agreement between measured and model-fitted activity within the factorial block, but larger deviations in the axial block (Figure 1), consistent with the significant lack of fit reported in Section 3.3.

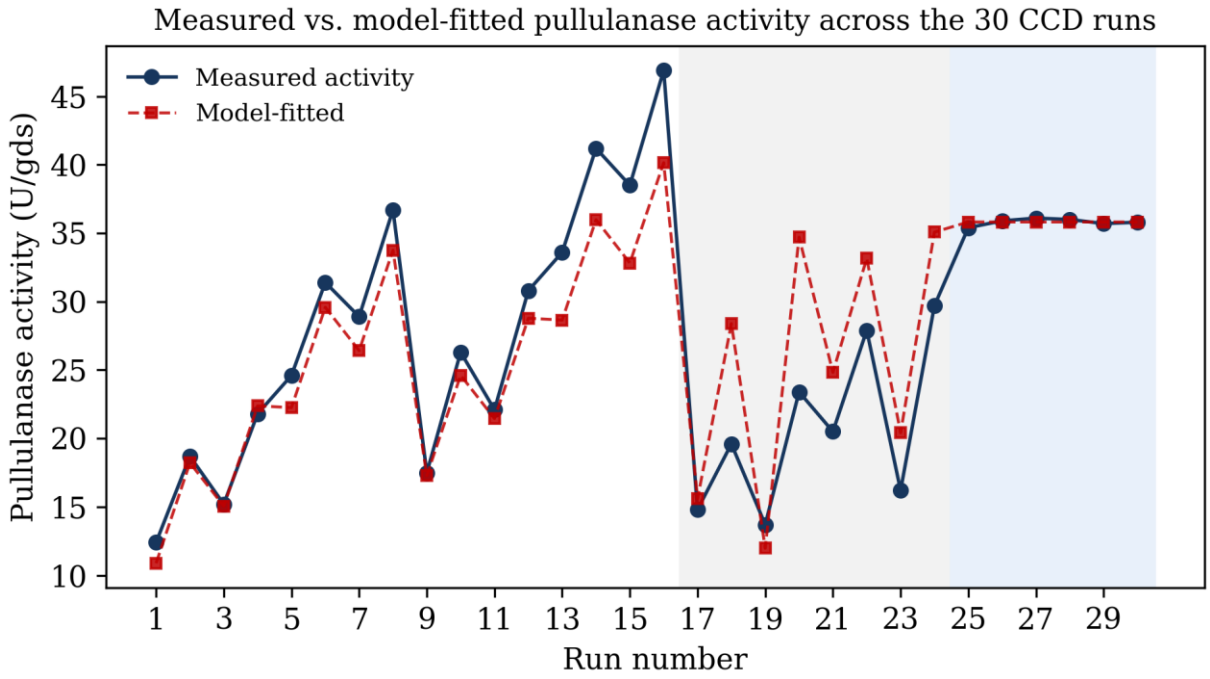

Figure 1. Measured pullulanase activity across the 30 central composite design runs and the corresponding quadratic-model fit. Runs 1–16 are the factorial block, 17–24 the axial block (shaded), and 25–30 the centre-point replicates (shaded).

3.2 Quadratic response model

Backward elimination removed the six two-factor interaction terms (AB, AC, AD, BC, BD, CD), none of which was statistically significant ($p > 0.65$). The reduced fitted equation in coded variables was:

$$Y = 35.8167 + 3.2000A + 5.6833B + 2.0833C + 3.6667D - 3.4562A^2 - 3.1187B^2 - 1.7062C^2 - 2.0187D^2$$

All linear coefficients were positive: moisture content (B) had the largest effect, followed by inoculum volume (D), initial pH (A), and substrate quantity (C), which was the weakest but still significant linear effect ($p = 0.042$). All pure-quadratic coefficients were negative, indicating curvature; however, as shown in Section 3.4, this curvature did not fully capture the measured response.

3.3 Analysis of variance and model adequacy

The reduced quadratic model was statistically significant ($F_{8,21} = 11.42$, $p < 0.0001$). The coefficient of determination was $R^2 = 0.813$, adjusted $R^2 = 0.742$, and adequate precision was 11.3 (values above 4 indicate a usable signal). However, the lack-of-fit test was highly significant ($F = 471.56$, $p < 0.0001$; Table 2): the model's deviations from the data are far larger than the pure replicate error measured at the

six centre points. This indicates that a second-order polynomial does not adequately describe the true response surface across the full tested range, and the numerical optimum reported in Section 3.5 should be interpreted with caution.

Table 2. Analysis of variance for the reduced quadratic response-surface model fitted to the measured pullulanase activity data.

Source	Sum of squares	df	Mean square	F-value	p-value
Model	**2025.3970**	**8**	**253.1746**	**11.42**	**<0.0001**
A - pH	245.7600	1	245.7600	11.09	0.0032
B - Moisture content	775.2067	1	775.2067	34.97	<0.0001
C - Substrate quantity	104.1667	1	104.1667	4.70	0.0418
D - Inoculum volume	322.6667	1	322.6667	14.55	0.0010
A^2	327.6525	1	327.6525	14.78	0.0009
B^2	266.7868	1	266.7868	12.03	0.0023
C^2	79.8525	1	79.8525	3.60	0.0716
D^2	111.7811	1	111.7811	5.04	0.0356
Residual	**465.5767**	**21**	**22.1703**		
Lack of fit	**465.2683**	**16**	**29.0793**	**471.56**	**<0.0001**
Pure error	**0.3083**	**5**	**0.0617**		
Corrected total	**2490.9737**	**29**			

Note: The AB, AC, AD, BC, BD, and CD interaction terms were removed by backward elimination ($p > 0.65$ for all) and are not shown. The significant lack of fit indicates the model should be refined — for example by expanding the design toward higher factor levels — before the fitted optimum is treated as final.

3.4 Response-surface interpretation

The pH–moisture response surface (Figure 2) rises from the low corner toward the high corner rather than forming a clear interior dome, consistent with the significant lack of fit. Within the sampled ranges, higher pH, moisture, substrate quantity, and inoculum volume were generally associated with higher activity, and the single highest measured value occurred at the highest tested level of all four factors simultaneously (Run 16). This corner-biased pattern, rather than an interior peak, is the main driver of the lack-of-fit result: a true optimum located at or beyond the boundary of the tested region is difficult for a second-order model centred on the design midpoint to represent accurately.

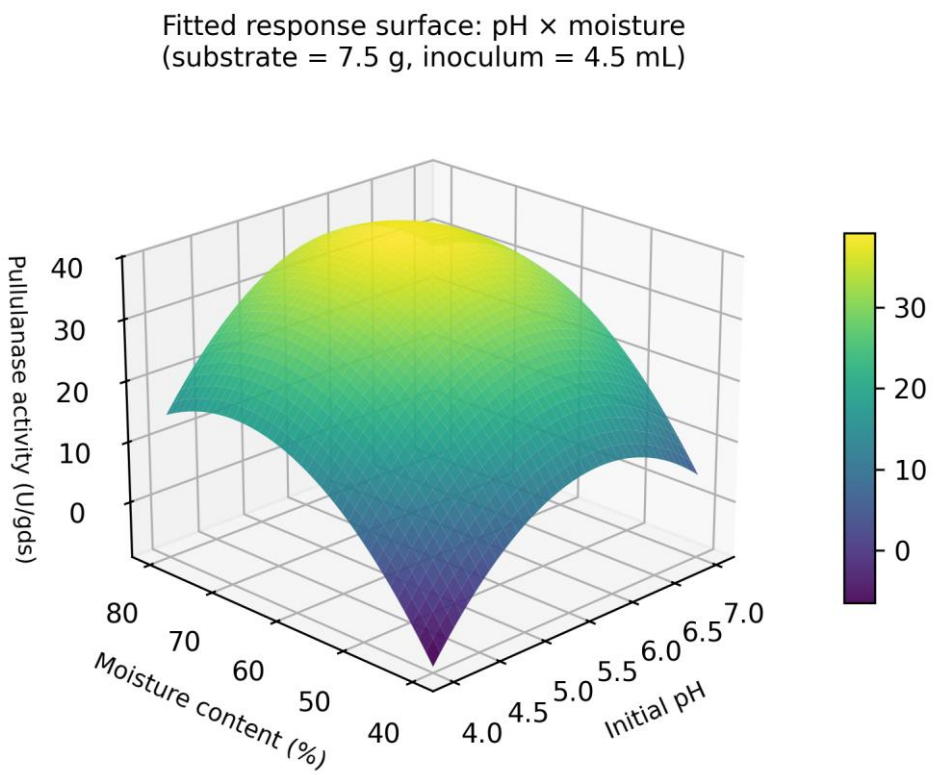

Figure 2. Fitted response surface for the combined effects of initial pH and moisture content on pullulanase activity at 7.5 g substrate and 4.5 mL inoculum, based on the reduced quadratic model.

Substrate quantity and inoculum volume showed the same pattern: measured activity increased toward the highest levels tested (12.0 g substrate; 8.0 mL inoculum) rather than turning over within the sampled range, reinforcing that the current design has not yet bracketed the process optimum.

3.5 Predicted optimum

Numerical optimization within the tested design space identified a stationary point rather than a boundary maximum, summarized in Table 3. Given the significant lack of fit described above, this predicted optimum should be treated as a provisional estimate rather than a validated maximum.

Table 3. Model-predicted optimum and model-summary statistics from the reduced quadratic model.

Parameter	Predicted value
Initial pH	5.85
Moisture content	69.1%
Wheat-bran quantity	8.87 g
Inoculum volume	6.09 mL
Predicted pullulanase activity	41.5 U/gds
Model R^2	0.813
Adjusted R^2	0.742

Note: Because the model shows significant lack of fit, this optimum should be confirmed — or superseded — by a follow-up experimental design (e.g., steepest-ascent runs or a second CCD centred beyond the current upper boundary) before it is adopted as a process target.

Table 4. Complete central composite design matrix with measured pullulanase activity, model-fitted values, and residuals.

Run	Point type	pH	Moisture (%)	Substrate (g)	Inoculum (mL)	Measured activity (U/gds)	Model fitted (U/gds)	Residual
1	Factorial	4.75	50.0	5.25	2.75	12.4	10.883	+1.517
2	Factorial	4.75	50.0	5.25	6.25	18.7	18.217	+0.483
3	Factorial	4.75	50.0	9.75	2.75	15.2	15.050	+0.150
4	Factorial	4.75	50.0	9.75	6.25	21.8	22.383	-0.583
5	Factorial	4.75	70.0	5.25	2.75	24.6	22.250	+2.350
6	Factorial	4.75	70.0	5.25	6.25	31.4	29.583	+1.817
7	Factorial	4.75	70.0	9.75	2.75	28.9	26.417	+2.483
8	Factorial	4.75	70.0	9.75	6.25	36.7	33.750	+2.950
9	Factorial	6.25	50.0	5.25	2.75	17.5	17.283	+0.217
10	Factorial	6.25	50.0	5.25	6.25	26.3	24.617	+1.683
11	Factorial	6.25	50.0	9.75	2.75	22.1	21.450	+0.650
12	Factorial	6.25	50.0	9.75	6.25	30.8	28.783	+2.017
13	Factorial	6.25	70.0	5.25	2.75	33.6	28.650	+4.950
14	Factorial	6.25	70.0	5.25	6.25	41.2	35.983	+5.217
15	Factorial	6.25	70.0	9.75	2.75	38.5	32.817	+5.683
16	Factorial	6.25	70.0	9.75	6.25	46.9	40.150	+6.750
17	Axial	4.00	60.0	7.50	4.50	14.8	15.592	-0.792
18	Axial	7.00	60.0	7.50	4.50	19.6	28.392	-8.792
19	Axial	5.50	40.0	7.50	4.50	13.7	11.975	+1.725
20	Axial	5.50	80.0	7.50	4.50	23.4	34.708	-11.308
21	Axial	5.50	60.0	3.00	4.50	20.5	24.825	-4.325
22	Axial	5.50	60.0	12.00	4.50	27.9	33.158	-5.258
23	Axial	5.50	60.0	7.50	1.00	16.2	20.408	-4.208
24	Axial	5.50	60.0	7.50	8.00	29.7	35.075	-5.375
25	Center	5.50	60.0	7.50	4.50	35.4	35.817	-0.417
26	Center	5.50	60.0	7.50	4.50	35.9	35.817	+0.083
27	Center	5.50	60.0	7.50	4.50	36.1	35.817	+0.283
28	Center	5.50	60.0	7.50	4.50	36.0	35.817	+0.183
29	Center	5.50	60.0	7.50	4.50	35.7	35.817	-0.117
30	Center	5.50	60.0	7.50	4.50	35.8	35.817	-0.017

Note: Residuals are largest in the axial block (Runs 17–24), consistent with the significant lack of fit reported in Table 2.

4. Discussion

The design shows how pH, moisture content, substrate quantity, and inoculum volume jointly influence pullulanase production by A. flavus under solid-state fermentation. All four main effects were positive within the tested range, and three of the four pure-quadratic terms were significant, indicating some curvature. However, the significant lack of fit and the corner-biased response described in Section 3.4 show that activity was still rising toward the upper boundary of every factor at the end of the tested range, rather than turning over inside the design space. This is a common and informative outcome in a first-pass central composite design: it indicates that the experimental window should be shifted upward before a genuine optimum can be located, rather than that no optimum exists. Practically, this means the process has not yet been pushed to its ceiling, and further gains in enzyme titre are likely available simply by testing higher factor combinations.

Moisture content produced the largest positive linear coefficient ($\beta = 5.68$). In solid-state fermentation, water availability governs substrate swelling, nutrient dissolution, diffusion, water activity, heat transfer, and fungal metabolism. Because measured activity continued to increase up to the highest tested level (80%), insufficient rather than excessive moisture appears to have limited production across the present range. Comparable fungal carbohydrase studies under solid-state fermentation have similarly reported strong moisture dependence [5,8,17].

The pH effect was also positive and significant across the tested range (4.0–7.0), with the highest measured activity occurring near neutrality (pH 6.25, Run 16) rather than under the mildly acidic conditions often considered favourable for fungal carbohydrase secretion. This may reflect strain-specific behaviour of the AF-PUL01 isolate or an interaction with the other factors tested, and should be examined directly in a

narrower, higher-resolution pH study centred closer to neutrality, ideally paired with direct pH monitoring of the substrate bed during fermentation rather than relying on the initial adjusted value alone.

Wheat bran provided both nutrients and physical support for fungal growth across all runs; its starch fraction may support induction of amylolytic systems, while its porous particles facilitate mycelial attachment, aeration, and extracellular-enzyme secretion. Previous screening and optimization studies have supported wheat bran and other agro-industrial residues as low-cost substrates for pullulanase production [4,5,8], consistent with the present results, and its low cost and wide availability make it an attractive substrate for eventual scale-up.

The measured activity range (12.4–46.9 U/gds) is broadly consistent with the order of magnitude reported for other solid-state-fermentation pullulanase systems, though direct comparison is limited by differences in strain identity, medium composition, incubation period, extraction ratio, assay substrate, temperature, reducing-sugar standard, and enzyme-unit definition across studies [1,2,5].

None of the six two-factor interaction terms reached significance ($p > 0.65$), indicating that, within the present range, the four factors acted largely independently on measured activity. This contrasts with some published solid-state-fermentation optimization studies that report significant interaction effects [17], and may itself be a consequence of the unmodelled curvature revealed by the lack-of-fit test: a model that has not captured the true surface shape can also misrepresent its interaction structure. The lack-of-fit finding should therefore be resolved, via an expanded design, before interaction effects are ruled out or reintroduced into a revised model.

The R^2 (0.813) and adjusted R^2 (0.742) obtained here are realistic for a biological fermentation dataset, and considerably lower than the near-unity values typical of simulated or noise-free data, reflecting genuine fermentation, extraction, and assay variability. The very small pure-error variance at the centre points (MS = 0.062) confirms that assay precision itself was high; the residual variability instead reflects model-form error, reinforcing that the design, not the assay, should be revised. A practical next step would be a steepest-ascent series moving from the current centre point in the direction of increasing predicted response, followed by a second central composite design centred on the new region, which typically resolves this type of boundary-biased result more efficiently than expanding the original design symmetrically in all directions.

The choice of A. flavus requires particular biosafety caution. Aflatoxin production is strain-dependent, and a production isolate cannot be considered food-safe merely because it produces a useful enzyme. Molecular and chemical confirmation of a non-aflatoxigenic phenotype is essential before industrial claims are made [7,20]. If such verification is unavailable or the isolate proves toxigenic, a non-toxigenic fungal or bacterial pullulanase producer, several of which have been characterized in the literature [11,16], should be selected instead before any food- or feed-related application is pursued.

5. Conclusions

A four-factor, 30-run central composite design was used to evaluate pullulanase production by Aspergillus flavus on wheat bran under solid-state fermentation. Measured activity ranged from 12.4 to 46.9 U/gds. Moisture content was the strongest positive linear factor, followed by inoculum volume, initial pH, and substrate quantity, and all four pure-quadratic terms were negative. However, the reduced quadratic model showed a statistically significant lack of fit ($F = 471.56$, $p < 0.0001$), and the highest measured activity occurred at the highest tested level of all four factors simultaneously (Run 16) rather than near the design centre. Together, these results indicate that the true process optimum lies at or beyond the upper boundary of the ranges tested here, and the model-predicted stationary point (pH 5.85, 69.1% moisture, 8.87 g substrate, 6.09 mL inoculum, 41.5 U/gds) should be treated as provisional. A follow-up design — for example, steepest-ascent runs or a second central composite design centred at higher factor levels — is recommended to locate and confirm the true optimum. Strain-level identification and aflatoxin-safety

testing of isolate AF-PUL01 remain outstanding requirements before any industrial application is considered.

Declarations

Author contributions: Sonia Aziz: Conceptualization, Investigation, Data curation, Writing—original draft. Rabbia Shafqat: Methodology, Formal analysis, Visualization, Writing—review and editing. Fahad Mehmood: [CRediT role(s) — to be confirmed by author]. Syed Muhammad Hamza Abidi: Supervision, Project administration, Writing—review and editing, Corresponding author. All authors have read and agreed to the published version of the manuscript.

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Institutional review board statement: Not applicable to a laboratory microbial-fermentation study. Institutional biosafety approval should be reported where required by local policy.

Informed consent statement: Not applicable.

Data availability: The complete central composite design matrix, measured pullulanase activity values, model-fitted values, and residuals are provided in Table 4. Raw assay data, calibration curves, and replicate measurements are available from the corresponding author upon reasonable request.

Conflicts of interest: The authors declare no conflict of interest.

Acknowledgements: To be completed by the authors.

Use of artificial intelligence tools: The authors must disclose any use of generative AI for language assistance or manuscript preparation according to the journal policy. AI tools cannot be listed as authors and cannot replace author verification of data, citations, or conclusions.

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