



Research Article

Humidified Column Solid-State Bioreactor for Enhanced Laccase Production under Forced Aeration

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Abstract: Solid-state fermentation (SSF) offers a promising route for enzyme production from lignocellulosic biomass; however, process scale-up remains constrained by gas–solid transport limitations within substrate beds. This study investigates the production of laccase by *Trichoderma asperellum* LBKURCC1 in a humidified column bioreactor using rice straw as the substrate. The effects of bed height, humidified airflow rate, and fermentation time were evaluated using Central Composite Design (CCD) combined with Response Surface Methodology (RSM). The quadratic model exhibited strong predictive capability ($R^2 = 0.964$, $p < 0.001$). Analysis of variance revealed that humidified aeration exerted the strongest nonlinear influence on reactor performance, highlighting its critical role in balancing oxygen availability and moisture retention. An operating window of 11 cm, a humidified airflow rate of $1 \text{ L} \cdot \text{min}^{-1}$, and a fermentation time of 4 days yielded a validated laccase activity of $253.8 \text{ U} \cdot \text{L}^{-1}$. Compared with the optimized flask-based SSF system previously reported for *Trichoderma asperellum* LBKURCC1 cultivated on rice straw, the humidified column bioreactor increased laccase activity from 57.7 to $253.8 \text{ U} \cdot \text{L}^{-1}$ (339.9%) while reducing the time required to reach peak activity from 8 to 4 days (50%). The findings demonstrate that the interaction among airflow, bed configuration, and fermentation time govern laccase production, providing an engineering framework for the design and scale-up of a humidified packed-bed column bioreactors for filamentous fungi cultivation under solid-state fermentation.

Keywords: Aeration; Bed Height; Laccase; Packed-bed bioreactor; Solid-state fermentation

1. Introduction

Laccase is a multicopper oxidase enzyme that is widely applied in bioremediation, pulp processing, biosensing, and lignocellulosic biomass valorization because of its ability to oxidize a broad spectrum of phenolic and non-phenolic compounds (Helianty et al., 2026; Moilanen et al., 2014; Yang et al., 2020). Solid-state fermentation (SSF) has attracted considerable attention owing to its low water requirement, reduced wastewater generation, high product concentration, and effective utilization of lignocellulosic residues as fermentation substrates (Sala et al., 2022; Thomas et al., 2013).

Despite these advantages, scaling up SSF remains difficult because microbial growth occurs within a heterogeneous porous substrate matrix in which oxygen transport, moisture distribution, and heat dissipation are inherently coupled phenomena (He et al., 2019; Helianty et al., 2026; Mitchell et al., 2023; Vauris et al., 2022). In packed-bed systems, fungal colonization continuously modifies the bed structure, thereby altering the porosity, airflow pathways, and local transport conditions (Perez et al., 2021). Reactor performance is governed not only by microbial activity but also by the stability of coupled heat and mass transfer within the substrate bed, which directly affects oxygen availability, moisture retention, temperature distribution,

and overall reactor performance (Carrasco-García et al., 2026; Fanaei & Mahmoodzadeh Vaziri, 2009; Vauris et al., 2022).

Conventional static solid-state fermentation (SSF) conducted in trays or Erlenmeyer flasks remains constrained by simultaneous limitations in oxygen transport, moisture retention, and metabolic heat dissipation. Oxygen diffusion through the packed substrate bed becomes increasingly restricted as fungal metabolism intensifies, while respiratory heat accumulation and evaporative moisture loss generate heterogeneous microenvironments that reduce process stability and enzyme productivity (Mitchell et al., 2023; Vauris et al., 2022). These transport limitations become progressively more severe during scale-up, representing one of the principal engineering bottlenecks in SSF operations.

To improve laccase production, numerous studies have investigated culture media optimization, nutrient supplementation, inducer addition, and statistical optimization using Response Surface Methodology (RSM) (Agrawal & Verma, 2019; Das et al., 2024; Dhokane et al., 2024; Jiao et al., 2025; Ma et al., 2025). However, these studies have primarily focused on biological optimization under static or laboratory-scale systems, and reactor engineering has comparatively limited attention variables such as packed-bed configuration and humidified aeration remain insufficiently investigated despite, their combined influence on oxygen transfer, moisture retention, heat dissipation, and overall process stability during packed-bed reactor operation (Helianty et al., 2026; Vauris et al., 2022; Zacharopoulos et al., 2024).

Because laccase is an extracellular oxidative enzyme whose biosynthesis depends on sustained aerobic metabolism, it is essential to maintain adequate oxygen availability while preserving substrate moisture for continuous enzyme secretion (Gervais & Molin, 2003; Wang et al., 2014). Accordingly, humidified aeration has emerged as an engineering strategy to stabilize the fermentation microenvironment by enhancing oxygen transport while reducing evaporative moisture loss, thereby improving heat removal and maintaining substrate moisture required for sustained enzyme secretion (Fanaei & Mahmoodzadeh Vaziri, 2009; Gervais & Molin, 2003; He et al., 2019; Phillips et al., 2023).

Among the indigenous fungal isolates maintained in the Laboratory of Biochemistry, Universitas Riau Culture Collection (LBKURCC), *Trichoderma asperellum* LBKURCC1 was selected because it has been systematically investigated in a series of previous studies, providing a well-established physiological and biochemical foundation for reactor engineering. Earlier work demonstrated its ability to produce extracellular laccase and decolorize azo dye under submerged fermentation conditions (Nugroho et al., 2018). Subsequent studies optimized laccase production under static solid-state fermentation by evaluating nutrient composition, moisture content, copper supplementation, and fermentation time using rice straw as the substrate (Rahayu et al., 2019; Sellyna et al., 2020). Building upon this established body of knowledge, the present study shifts the research focus from biological optimization to reactor engineering by evaluating laccase production in a humidified packed-bed column bioreactor.

Therefore, this study investigates laccase production by *Trichoderma asperellum* LBKURCC1 in a humidified packed-bed column bioreactor using rice straw as a representative lignocellulosic substrate. Reactor performance depends on complex interactions among bed geometry, humidified aeration, moisture retention, heat dissipation, and fungal metabolism, the one-factor-at-a-time experimentation cannot be used to achieve optimization (Carrasco-García et al., 2026; He et al., 2019; Umar et al., 2023). Therefore, a central composite design (CCD) combined with response surface methodology (RSM) was employed to efficiently quantify linear, interaction, and quadratic effects while minimizing the number of experimental runs and developing a predictive process model. Although RSM has been extensively applied for optimizing culture media, nutrient composition, inducer concentration, and other biological fermentation variables (Das et al., 2024; Dhokane et al., 2024; Elsayed & Ahmed Abdelwahed, 2020; Jiao et al., 2025; Kumar et al., 2020; Ma et al., 2025), its application to engineering-oriented optimization of humidified packed-bed SSF systems remains limited (Helianty et al., 2026; Vauris et al., 2022; Zacharopoulos et al., 2024). Unlike previous studies that primarily emphasized biological

responses, the present study interprets bed height and humidified aeration as coupled engineering variables governing oxygen transport, moisture stability, and transport stability within the packed substrate. Therefore, this study aimed to identify a stability-defined operating window that simultaneously maximizes laccase production while maintaining favorable transport conditions, thereby providing engineering guidance for the design and scale-up of energy-efficient SSF bioreactors. To the best of our knowledge, this is the first study to integrate humidified aeration, packed-bed reactor configuration, and multivariable statistical optimization to establish a stability-defined engineering operating window for solid-state fermentation of laccase by *Trichoderma asperellum* LBKURCC1.

2. Methods

2.1 Chemicals and reagents used

All the chemicals and reagents used in this study were of analytical or molecular biology grade and were used without further purification. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, $\geq 98\%$, HPLC), Tween[®] 20, soybean flour (Type I), and magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium acetate ($\geq 99\%$) and glycerol ($\geq 99\%$) were obtained from Sigma-Aldrich (Tokyo, Japan) and Sigma-Aldrich (Kuala Lumpur, Malaysia), respectively. Potato Dextrose Agar (PDA), potassium dihydrogen phosphate (KH_2PO_4), ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$), glacial acetic acid, hydrochloric acid (37%), and copper (II) sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) were supplied by Merck KGaA (Darmstadt, Germany). Dextrose monohydrate, 70% ethanol, and demineralized water (Aqua DM) were procured from PT Brataco (Cikarang and Pekanbaru, Indonesia). The agar powder was obtained from Kwong Chee Lan Sdn. (Seremban, Malaysia). All aqueous solutions were freshly prepared using demineralized water before use.

2.2 Preparation of microorganism and substrates

Trichoderma asperellum LBKURCC1 was obtained from the Culture Collection of the Enzyme, Fermentation and Biomolecular Laboratory, Universitas Riau, and was used as the production strain throughout this study. The strain identity was previously confirmed by rDNA sequencing using internal transcribed spacer (ITS) (Nugroho et al., 2018; Rahayu et al., 2019; Sellyna et al., 2020).

Locally collected rice straw as an agricultural residue was selected as the solid substrate. Its choice was guided by its high lignocellulosic surface area, innate porosity, and structural rigidity (Chen et al., 2021; Dhokane et al., 2024; Wang et al., 2024). These characteristics are advantageous for packed-bed solid-state fermentation (SSF) because they facilitate gas transport while maintaining bed permeability during fungal colonization (Mitchell et al., 2023; Xia & Lin, 2022; Yan et al., 2024). The rice straw was air-dried, cut into approximately 1-cm pieces, and sterilized before inoculation. The substrate was thoroughly mixed with the nutrient solution and sterilized by autoclaving at 121 °C for 20 minutes. Under aseptic conditions, the sterilized substrate was inoculated with the prepared spore suspension at a substrate:nutrient solution:inoculum mass ratio of 4:3:3 (w/w/w), resulting in an initial moisture content of approximately 60% (w/w). This moisture level provides sufficient water activity to support fungal metabolism while preserving interparticle void spaces necessary for effective airflow through packed bed (Gervais & Molin, 2003).

2.3 Custom-design humidified packed-bed column bioreactor

All solid-state fermentation (SSF) experiments were conducted using a custom-designed humidified packed-bed column bioreactor fabricated from borosilicate glass. The bioreactor was designed with a height-to-diameter (H/D) ratio of approximately 3 to minimize wall effects while

maintaining sufficient bed depth for fungal cultivation (Mitchell et al., 2023). A sintered glass frit was installed at the base of the column to function simultaneously as a substrate support and a gas distributor, promoting uniform airflow distribution across the packed-bed cross-section.

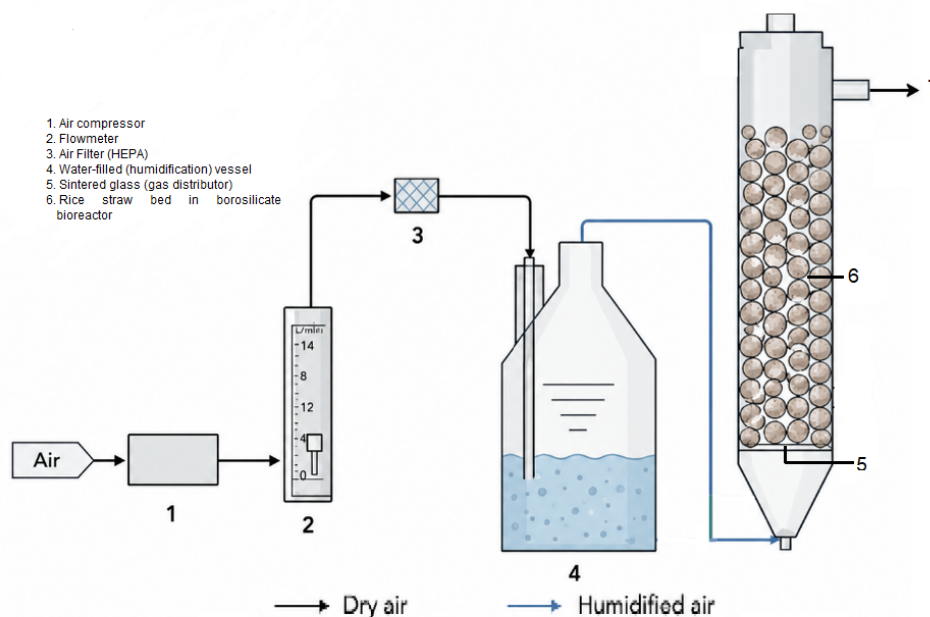


Figure 1 Schematic diagram of the custom-designed humidified packed-bed column bioreactor system used for solid-state fermentation, illustrating the airflow pathway, humidification unit, and packed substrate bed

The complete experimental setup is illustrated schematically in Figure 1. The numbered components include (1) an air compressor, (2) a flowmeter (rotameter), (3) a water-filled humidification vessel, (4) a sintered glass gas distributor, (5) a packed rice straw substrate bed, (6) a borosilicate column bioreactor, and (7) an exhaust gas outlet. Compressed air was continuously bubbled through the humidification vessel before entering the bottom of the reactor to provide humidified aeration throughout the fermentation process. Based on psychrometric calculation at the operating temperature of 30 °C–33 °C (Figure 2), the inlet air approached saturation with an estimated absolute humidity of approximately $2.8 \times 10^4 \text{ mg} \cdot \text{Nm}^{-3}$ (Manan & Webb, 2024; Roosta et al., 2024; Vauris et al., 2022). Statistical error bars are not applicable because Figure 2 is derived from deterministic psychrometric relationships rather than experimental measurements. The complete mathematical derivation is provided in Supplementary Information S1.

The transparent borosilicate column enabled the qualitative observation of substrate morphology, fungal colonization, and moisture-related phenomena throughout the fermentation process. However, direct measurements of the internal relative humidity, oxygen concentration, and temperature profile within the packed bed were beyond the scope of this study. Accordingly, reactor performance was evaluated quantitatively using laccase activity as the primary response variable, while qualitative observations were used solely to support the interpretation of transport phenomena and process stability discussed in the Results and Discussion section.

2.4 Experimental design and operating parameters

A three-factor Central Composite Design (CCD) was employed to investigate the combined effects of bed height (X_1), fermentation time (X_2), and humidified airflow rate (X_3) on laccase production. These variables were selected because they collectively govern oxygen transport, moisture retention, metabolic heat dissipation, and overall transport stability within packed-bed

solid-state fermentation systems (He et al., 2019; Mitchell et al., 2023).

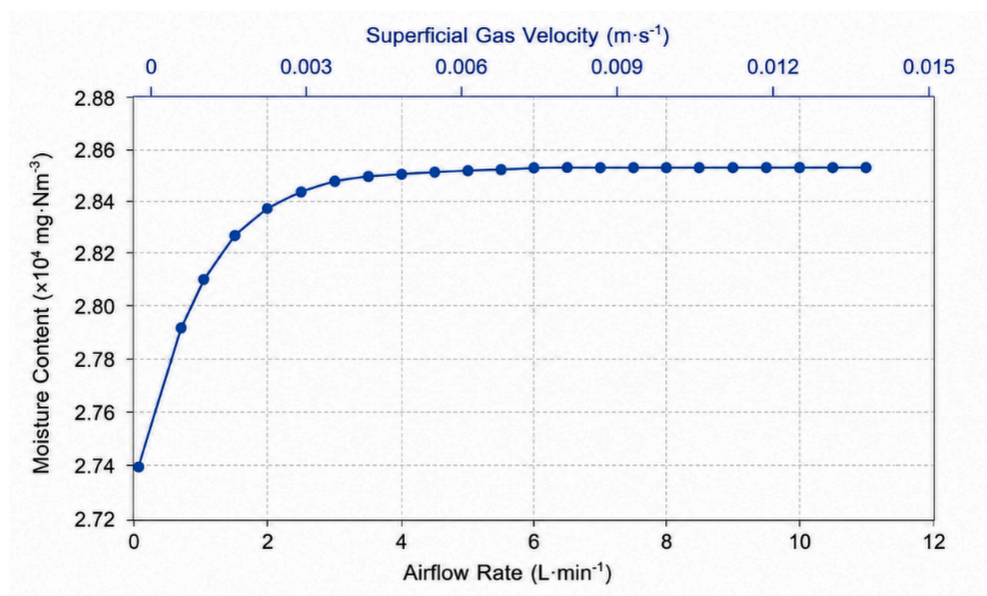


Figure 2 Psychrometric estimation of the absolute moisture content of humidified inlet air as a function of airflow rate under the operating temperature range of 30 °C–33 °C, illustrating the approach toward near-saturation conditions

Humidified air was continuously supplied from the bottom of the bioreactor at controlled flow rates ranging from 0.3 to 3.7 L · min⁻¹, corresponding to superficial gas velocities of 9.94×10^{-4} to 1.23×10^{-2} m · s⁻¹. Moisture regulation relies exclusively on humidified aeration without auxiliary humidity-control devices, thereby providing a simple and practically scalable reactor configuration while minimizing substrate dehydration during continuous aeration (Cerrone & O'Connor, 2025; Das et al., 2024; Gervais & Molin, 2003; Phillips et al., 2023).

Bed height was varied according to the experimental design to evaluate its influence on gas diffusion resistance, airflow distribution, and gas residence time within the packed substrate. Fermentation time was included to capture the temporal progression of fungal colonization and extracellular laccase production. All experiments were conducted in batch mode under ambient laboratory conditions (Vauris et al., 2022).

The investigated operating ranges were selected to represent practically feasible conditions for humidified packed-bed SSF systems while avoiding excessive bed compaction and substrate dehydration. Experimental data were analyzed using RSM to develop a predictive quadratic model describing the linearity, interactions, and quadratic effects of the selected engineering variables. The resulting model was subsequently used to identify a stability-defined operating window that simultaneously maximized laccase production while maintaining favorable transport conditions within the bioreactor.

2.5 Crude extracellular laccase extraction

Crude extracellular laccase was extracted by adding chilled sodium acetate buffer (pH 5.5) to the fermented solid substrate at a solid-to-liquid ratio of 1:10 (w/v). The addition of acetate buffer serves as a biological mimic solution to preserve enzyme stability during extraction (Brooke et al., 2015). The mixture was pre-filtered through a glass wool plug to remove fungal spores and coarse substrate debris.

The primary filtrate was further clarified by filtering through Whatman GF/C glass microfiber filter paper and transferred into Corning centrifuge tubes for a second chilled centrifugation. Separation of the resulting biphasic mixture—comprising a clear upper supernatant and a solid pellet—was separated.

2.6 Determination of laccase activity

The laccase activity was determined spectrophotometrically using ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] as the substrate (Fu et al., 2025). ABTS oxidation was monitored by measuring the increase in absorbance at 420 nm ($\epsilon_{420} = 36,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$) using a microplate reader under standard assay conditions. One unit (U) of laccase activity was defined as the enzyme amount required to oxidise 1 μmol of ABTS per minute. Enzyme activity was expressed as $\text{U} \cdot \text{L}^{-1}$ of crude extract and used as the primary response variable for statistical modeling and process optimization (Ma et al., 2025). The measured activity reflects the coupled influence of oxygen availability, moisture retention, and fungal metabolic performance within the packed-bed fermentation system (Ávila et al., 2019; Moilanen et al., 2014; Yang et al., 2020).

2.7 Experimental Design and Analysis of Data

Response Surface Methodology (RSM) was employed to develop a second-order polynomial model to characterize the relationship between process variables and laccase activity (Equation 1):

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad (1)$$

where Y is the predicted laccase activity ($\text{U} \cdot \text{L}^{-1}$), β_0 is the intercept, X_i and X_j are the coded independent variables, and β_i , β_{ij} , and β_{ii} are regression coefficients corresponding to linear, interaction, and quadratic terms, respectively.

The RSM model was used to evaluate interaction effects, characterize nonlinear system behavior, quantify the individual and interactive effects of the operating variables, characterize nonlinear process behavior, and predict laccase production within the investigated experimental domain (Sharma & Murty, 2021). Model adequacy was evaluated using analysis of variance (ANOVA), the coefficient of determination (R^2), Adjusted R^2 , Predicted R^2 , Adeq Precision, residual diagnostics to verify the underlying regression assumptions and assess predictive reliability (Amdoun et al., 2018; Zewide et al., 2025). Finally, numerical optimization based on the desirability function was performed to identify the operating conditions that simultaneously maximized laccase activity within the experimental design constraint (Ma et al., 2025).

3. Results and Discussion

3.1 Development of Empirical Models and Statistical Validation

Response Surface Methodology (RSM) based on a Central Composite Design (CCD) was employed to establish the empirical relationship between bed height (X_1), fermentation time (X_2), airflow rate (X_3), and laccase activity. The CCD consisted of factorial, axial, and center-point experiments, enabling efficient linear, interaction, and quadratic effects estimation while minimizing the total number of experimental runs. Supplementary Table S1 provided the complete experimental data. The observed variation in laccase activity across the experimental domain indicates the presence of nonlinear interactions among the investigated variables, suggesting that simple linear relationships cannot adequately describe reactor performance.

The sum-of-squares analysis of the sequential model demonstrated that the linear and two-factor interaction (2FI) models were insufficient to represent the experimental response. The inclusion of quadratic terms significantly improved model performance and resulted in the selection of a second-order polynomial model as the most appropriate representation of the system behavior (Al-Sareji et al., 2023; Kumar et al., 2020; Ma et al., 2025; Salem et al., 2024; Vauris et al., 2022). The final regression equation in terms of actual factors is expressed as Equation (2):

$$\begin{aligned} \text{Laccase Activity (U} \cdot \text{L}^{-1}) = & 587.53 - 6.40X_1 - 50.79X_2 - 403.82X_3 - 1.88X_1X_2 \\ & - 3.67X_1X_3 + 15.76X_2X_3 + 2.28X_1^2 + 2.74X_2^2 + 72.25X_3^2 \end{aligned} \quad (2)$$

The developed model exhibited a high coefficient of determination ($R^2 = 0.9642$), indicating that the selected operating variables could explain 96.42% of the variability in laccase activity. The model's adequacy was further supported by an Adjusted R^2 value of 0.9321, demonstrating strong agreement between the predicted and observed responses. Although the Predicted R^2 value (0.7065) was lower than the Adjusted R^2 , indicating reduced predictive capability outside the experimental domain, the model remained acceptable for empirical bioprocess modeling because the Predicted R^2 exceeded 0.7 and the regression was experimentally validated under the optimum operating conditions. The observed discrepancy is likely attributable to the inclusion of quadratic and interaction terms required to describe the nonlinear behavior of the humidified packed-bed SSF system, where substrate heterogeneity and biological variability increase model complexity. Furthermore, the Adeq Precision value of 15.30 substantially exceeded the minimum recommended value of 4.0, confirming an adequate signal-to-noise ratio and the suitability of the model for navigating the experimental design space.

The standard deviation (25.74) and coefficient of variation (24.66%) are consistent with the variability level commonly observed in solid-state fermentation processes, where substrate heterogeneity and fungal growth dynamics contribute to experimental uncertainty (Carrasco-García et al., 2026; Dhokane et al., 2024). Overall, the statistical indicators and subsequent experimental validation demonstrate that the quadratic model provides a reliable basis for evaluating the effects of bed height, airflow rate, and fermentation time on laccase production.

Model validation was further confirmed through experimental verification at the optimum operating conditions (11 cm bed height, 4 days fermentation time, and $1 \text{ L} \cdot \text{min}^{-1}$ airflow rate). Experimental laccase activity of $253.8 \text{ U} \cdot \text{L}^{-1}$ showed close agreement with the prediction of $242.1 \text{ U} \cdot \text{L}^{-1}$, corresponding to a deviation of 4.8%. The predicted laccase activities also agreed well with the experimental observations across the investigation design space, with most deviations remaining below 5%. This level of agreement confirms that the developed quadratic model possesses satisfactory predictive capability and provides a reliable basis for process optimization within the investigated operating range.

3.2 Diagnostic evaluation and model reliability

The adequacy of the developed quadratic model was further evaluated using diagnostic plots and residual analysis. As shown in Figure 3, the predicted versus actual plot demonstrates excellent agreement between the experimental and model-predicted laccase activities. Most data points are tightly distributed along the 45° diagonal line, indicating that the empirical framework successfully captured the overall response behavior within the investigated experimental domain without introducing severe systematic or structural errors.

The regression model's reliability was further assessed through the distribution of externally studentized residuals (Figure 3b). The residuals were randomly scattered around zero without any obvious trend, indicating that the statistical assumptions of independence and constant variance (homoscedasticity) were satisfied. Furthermore, all residual values remained within the acceptable confidence limits, indicating the absence of significant outliers or systematic model bias.

A small deviation was observed for one experimental point located near the design space. However, this deviation was isolated and did not exhibit any systematic pattern. Such variations are commonly encountered in solid-state fermentation systems due to substrate heterogeneity, local moisture gradients, and differences in fungal colonization within the porous matrix (Carrasco-García et al., 2026; Mitchell et al., 2023). Therefore, the observed deviation was considered a normal manifestation of biological variability rather than evidence of model inadequacy.

The diagnostic results are consistent with the statistical indicators presented in Section 3.1, including a high coefficient of determination ($R^2 = 0.9642$), acceptable Predicted R^2 (0.7065), Adeq precision value of 15.30, and coefficient of variation of 24.66%. Collectively, these results confirm that the developed quadratic model provides a reliable representation of the relationship between bed height, airflow rate, fermentation time, and laccase activity (Elsayed & Ahmed

Abdelwahed, 2020; Hawashi et al., 2019).

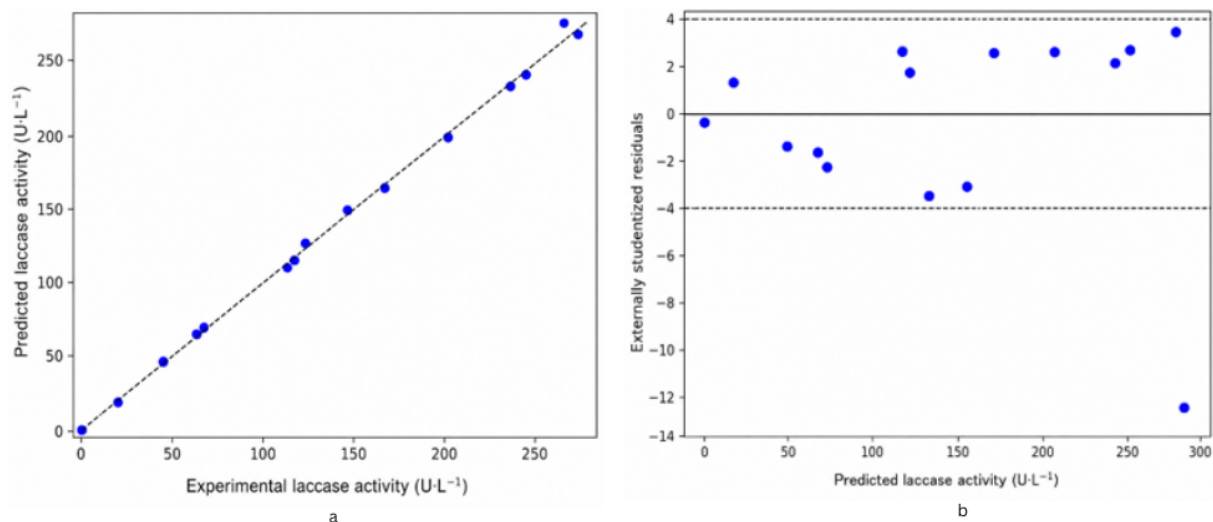


Figure 3 Diagnostic plots for the developed quadratic regression model: (a) predicted vs. experimental laccase activity demonstrating good agreement between model predictions and experimental observations; (b) externally studentized residuals plotted against the predicted response, showing random scatter around zero without systematic patterns, indicating satisfactory model adequacy

3.3 Analysis of variance and parameter interactions

The ANOVA results confirmed that the quadratic model was highly significant ($F = 29.97$, $p < 0.0001$), demonstrating its suitability for describing laccase production within the investigated operating domain (Table 3). Among the linear effects, fermentation time (X_2) significantly influenced enzyme activity ($p < 0.001$), whereas bed height (X_1) and airflow rate (X_3) were not significant independent factors. This finding suggests that the response was governed predominantly by nonlinear and interaction effects rather than by isolated changes in individual variables (Elsayed & Ahmed Abdelwahed, 2020).

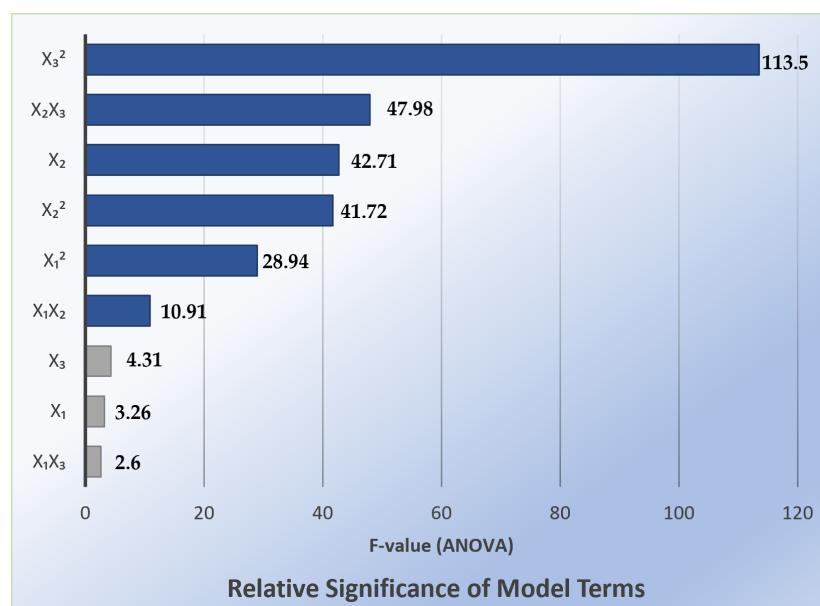


Figure 4 Pareto chart of standardized effects showing the relative significance of linear, interaction, and quadratic terms affecting laccase activity

Significant interaction effects were observed between bed height and fermentation time (X_1X_2 , $p = 0.0080$) and between fermentation time and airflow rate (X_2X_3 , $p < 0.001$). These results indicate that the effect of one variable is dependent on the level of another variable, confirming the presence of coupled relationships within the experimental domain.

All quadratic terms (X_1^2 , X_2^2 , and X_3^2) were highly significant, with the airflow-rate quadratic term (X_3^2) exhibiting the highest F-value (113.50). This result identifies aeration as the dominant factor contributing to the response surface's curvature. The significance of the quadratic terms further confirms the nonlinear behavior of the system and justifies the use of a second-order polynomial model to describe laccase production (De Castro et al., 2011; Salem et al., 2024). The pure error was negligible due to identical responses at the replicated center points, which limited the statistical assessment of lack of fit but did not affect the regression model's overall significance (Ridder et al., 1999). Overall, the ANOVA results demonstrate that the developed quadratic model adequately captured the main sources of variation in laccase activity and provided a reliable basis for subsequent response surface analysis.

3.4 Response surface and contour analysis

The interaction effects of bed height (X_1), fermentation time (X_2), and airflow rate (X_3) were further evaluated using contour plots and three-dimensional response surface visualizations (Figures 5 and 6). These graphical analyses provide a visual interpretation of the quadratic regression model and facilitate the identification of the response trends within the experimental domain under investigation.

The contour plots (Figure 5) exhibited predominantly elliptical patterns, indicating the presence of significant interactions among the variables under investigation. Such contour configurations suggest that laccase activity could not be adequately explained by the independent effects of each factor alone but was influenced by their combined behavior. This observation is consistent with the significant interaction terms identified in the analysis of variance.

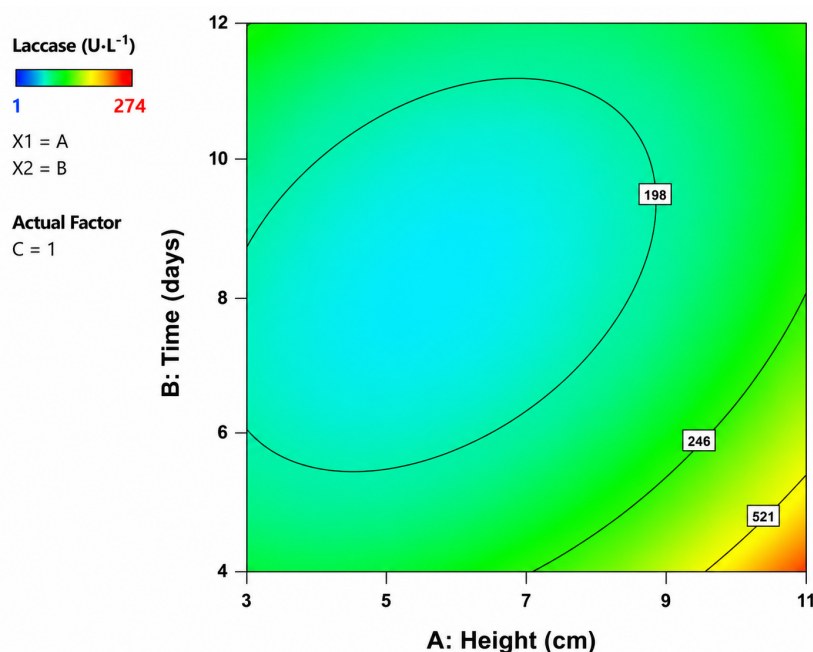


Figure 5 Contour plot of predicted laccase activity as a function of bed height (X_1) and fermentation time (X_2) at an airflow rate of $1 \text{ L} \cdot \text{min}^{-1}$

The response surface visualization (Figure 6) revealed a distinct region of elevated laccase activity at intermediate factor levels. The highest predicted response was observed at a bed height of approximately 11 cm, a fermentation time of 4 days, and an airflow rate of $1 \text{ L} \cdot \text{min}^{-1}$. Around this region, the contour lines were relatively smooth and widely spaced, indicating lower

response sensitivity to small variations in operating conditions. In contrast, regions located near the boundaries of the design space exhibited steeper gradients and more densely packed contour lines, reflecting the increased sensitivity of the response to factor variation. This behavior is characteristic of systems governed by significant quadratic effects and confirms the regression model's nonlinear nature.

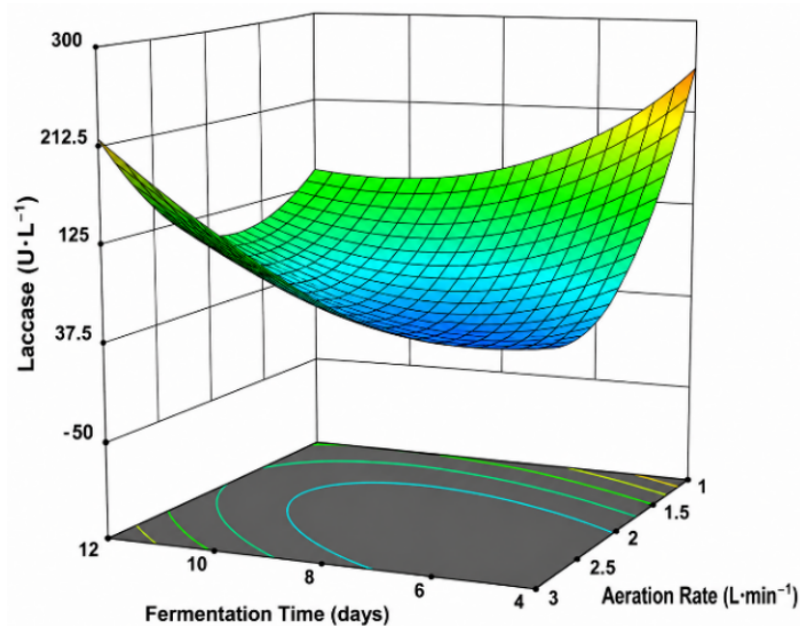


Figure 6 Three-dimensional response surface of predicted laccase activity as a function of fermentation time (X_2) and airflow rate (X_3) at a fixed bed height ($X_1 = 11$ cm)

The desirability analysis (Figure 7) demonstrated strong agreement with the contour and response surface interpretations. The highest desirability values ($D \approx 0.9$) coincided with the region of the maximum predicted laccase activity, confirming the consistency of the optimization results. The optimum condition identified by the model corresponded to a bed height of approximately 11 cm, a fermentation time of 4 days, and an airflow rate of $1 \text{ L} \cdot \text{min}^{-1}$.

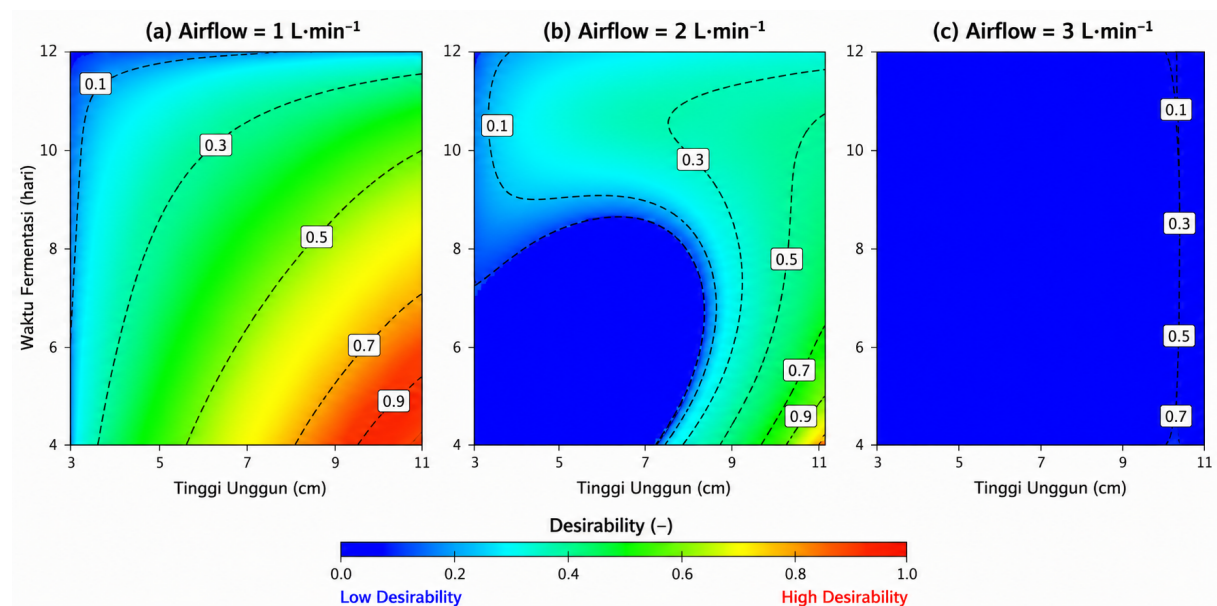


Figure 7 Desirability contour plots showing the combined effects of bed height (X_1) and fermentation time (X_2) at different airflow rates

3.5 Multiphase transport and spatial colonization dynamics

Solid-state fermentation performance is strongly influenced by the coupled transport of oxygen and moisture within the porous substrate matrix. Under the optimum operating conditions identified by the RSM model (11 cm bed height, 1 L · min⁻¹ humidified airflow, and 4 days of fermentation), a visually uniform fungal colonization pattern was observed throughout the RSM bed (Figure 8).

Higher-magnification images revealed extensive hyphal attachment on the rice straw surface and penetration into the substrate particles' tubular lumen. Such penetration is particularly important in lignocellulosic SSF systems because the substrate functions not only as a physical support matrix but also as the primary carbon source for microbial metabolism. Therefore, the observed colonization pattern indicates effective substrate utilization and intimate contact between the fungal biomass and the lignocellulosic structure.

Although direct measurements of internal oxygen concentration, moisture gradients, and temperature profiles were not available, the widespread fungal distribution observed under the optimum condition provides qualitative evidence that the humidified packed-bed configuration successfully maintained environmental conditions conducive to fungal growth and enzyme production. The extensive colonization observed throughout the bed is consistent with the high laccase activity obtained under the same operating conditions, suggesting that the bed height and humidified airflow acted as coupled process variables influencing the overall stability of the fermentation environment.

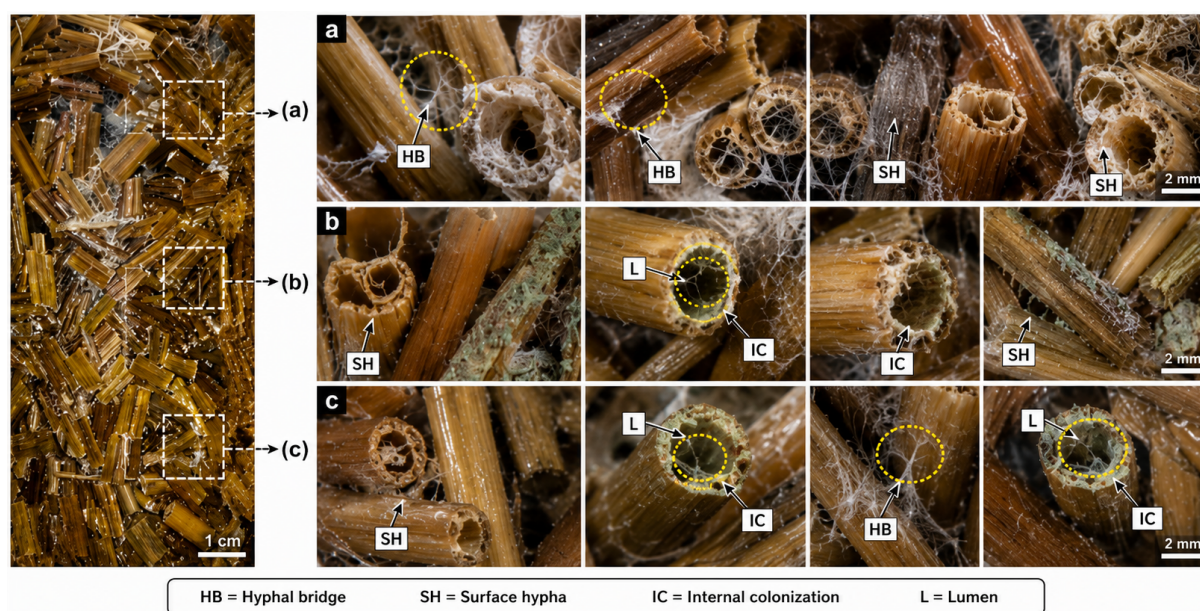


Figure 8 Multiscale visualization of fungal growth on rice straw after fermentation. The left panel shows the overall distribution of fungal colonization, while the enlarged regions (a–c) illustrate representative fungal structures, including surface hyphae (SH), hyphal bridges (HB), internal colonization (IC), and the rice straw lumen (L). Scale bars indicate 1 cm (left panel) and 2 mm (right panels)

3.6 Psychrometric Considerations and Condensation Qualitative Observation

Maintaining a favorable moisture environment is essential in solid-state fermentation systems because water is retained primarily as a thin adsorbed layer on the substrate surface rather than as a free liquid phase. Excessive moisture loss can impair fungal metabolism, enzyme secretion, and overall process stability (Yáñez-Hernández et al., 2023; Zacharopoulos et al., 2024).

Condensation droplets were consistently observed on the inner surface of the transparent reactor lid during fermentation under the optimum operating conditions. Although the inter-

nal relative humidity, dew-point temperature, and gas-phase humidity were not directly measured, the appearance of condensation qualitatively indicates that the headspace atmosphere approached saturation. From a psychrometric perspective, condensation occurs when humid air contacts a surface below its dew-point temperature, suggesting that humidified aeration successfully maintained a highly humid internal environment within the reactor.

The presence of this humid atmosphere is consistent with the improved laccase production observed under the optimized operating conditions. By reducing excessive moisture loss while maintaining continuous airflow, humidified aeration likely supported a more favorable balance between oxygen availability and substrate moisture, thereby promoting stable fungal metabolism and sustained extracellular enzyme production. Although these transport phenomena were not directly measured, the observed process performance supports the proposed engineering role of humidified aeration in maintaining favorable operating conditions within the packed bed.

The repeatable appearance of condensation suggests the potential for developing simple visual indicators of psychrometric stability in packed-bed SSF systems from an engineering perspective. Rather than serving as a quantitative measurement, condensation behavior may provide a practical qualitative indicator of stable humidification during reactor operation. To validate this concept and establish quantitative relationships between psychrometric conditions and bioreactor performance, future studies incorporating measurements of internal humidity, oxygen concentration, and temperature profiles will be required.

3.7 Comparative Performance of Static SSF Systems

The performance of the proposed humidified packed-bed column bioreactor was compared with that of the optimized flask-based solid-state fermentation system previously reported by Rahayu et al. (2019) for *Trichoderma asperellum* LBKURCC1 cultivated on the same rice straw substrate under optimized nutritional conditions. As summarized in Table 1, the reactor achieved a validated laccase activity of $253.8 \text{ U} \cdot \text{L}^{-1}$, representing a 4.40-fold increase (339.9%) over the maximum activity reported for the optimized flask system ($57.7 \text{ U} \cdot \text{L}^{-1}$). Furthermore, the fermentation time required to reach maximum enzyme production was reduced from 8 to 4 days, corresponding to a 50% reduction in production time.

Table 1 Comparison of laccase production performance between the static SSF system and the humidified packed-bed column bioreactor

Parameter	(Rahayu et al., 2019)	Present study	Improvement
Reactor configuration	Flask SSF	Humidified column	–
Aeration mode	Passive diffusion	Continuous humidified aeration	–
Substrate	Rice straw	Rice straw	–
Maximum laccase activity ($\text{U} \cdot \text{L}^{-1}$)	57.70	253.80	+339.9% (4.40-fold)
Time to peak production (day)	8	4	50% shorter
Volumetric productivity ($\text{U} \cdot \text{L}^{-1} \cdot \text{day}^{-1}$)	7.21	63.45	+780.0% (8.80-fold)

In contrast to the previous study, in which process optimization was achieved primarily through nutrient supplementation and medium formulation, the present work focused on reactor engineering by introducing continuous humidified aeration within a packed-bed column. By improving oxygen availability while minimizing moisture loss during fermentation, this modification fundamentally altered the transport environment inside the substrate bed. Consequently, the enhancement in laccase production cannot be attributed solely to biological optimization

but rather to improved control of the fungal metabolism-governing physical transport processes. The observed uniform mycelial colonization and stable moisture conditions under the optimum operating regime further support this interpretation.

The engineering benefits become even more evident when reactor productivity is considered. The volumetric productivity increased from 7.21 to 63.45 $\text{U} \cdot \text{L}^{-1} \cdot \text{day}^{-1}$, corresponding to an 8.80-fold improvement (780%). This substantial increase reflects the combined effect of higher enzyme yield and a markedly shorter fermentation period, indicating that the process intensity and reactor utilization efficiency are enhanced by the humidified packed-bed configuration. Such improvements are particularly important for industrial solid-state fermentation, where process economics largely depends on productivity rather than final enzyme concentration alone.

Overall, the comparison demonstrates that reactor configuration is a critical engineering variable that complements conventional biological optimization strategies. Although previous studies have successfully improved laccase production through nutritional optimization, the present study shows that controlling airflow and moisture transport within the packed substrate can generate substantially greater performance gains. These findings highlight the potential of humidified packed-bed bioreactors as a practical strategy for overcoming transport limitations in static SSF and provide a scalable engineering framework for enzyme production from lignocellulosic biomass.

3.8 Scale-Up Implications and Visual Soft-Sensor Perspective

The operating window identified in this study (11 cm bed height, 1 $\text{L} \cdot \text{min}^{-1}$ humidified airflow, and 4 days of fermentation) provides engineering-relevant guidance for the scale-up of humidified packed-bed SSF systems. Rather than representing a single optimum point, these conditions define a transport regime in which oxygen supply, moisture retention, and metabolic heat removal remain balanced, indicating that successful scale-up should preserve transport similarity rather than merely increasing reactor dimensions.

The performance of the humidified packed-bed bioreactor was governed by the coupled interaction between bed geometry and humidified aeration, unlike conventional static SSF systems. Bed height determines the resistance to gas transport and heat dissipation, whereas humidified airflow simultaneously regulates oxygen supply and substrate moisture. Consequently, increasing reactor dimensions without preserving these transport characteristics may increase diffusion resistance, moisture heterogeneity, and local heat accumulation. Table 2 summarizes the principal transport similarity criteria that should be preserved during reactor scale-up.

Therefore, future scale-up strategies should prioritize the preservation of transport phenomena over geometric enlargement alone. Maintaining comparable hydrodynamic and moisture-transfer characteristics through modular packed-bed configurations or parallel reactor arrangements may provide a more reliable approach than increasing bed depth within a single reactor because these configurations are expected to preserve oxygen distribution, convective heat removal, and moisture equilibrium throughout the substrate bed. Although internal humidity was not directly measured in this study, the future humidified packed-bed SSF systems may benefit from noninvasive monitoring approaches. Image-based analysis of condensation patterns or substrate surface moisture distribution could serve as soft-sensing variables after calibration against quantitative humidity measurements, thereby supporting digital process monitoring and intelligent bioreactor operation.

Overall, these findings indicate that humidified packed-bed SSF scale-up should focus on preserving transport stability rather than maximizing reactor size. Accordingly, the engineering operating window identified by RSM represents not only the condition associated with maximum laccase production but also a practical design criterion for the development of robust, scalable, and energy-efficient packed-bed bioreactors.

Table 2 Engineering considerations for the scale-up of the humidified packed-bed SSF bioreactor

Engineering aspect	Parameter to preserve	Expected function
Bed geometry	Bed height (≈ 11 cm laboratory scale), H/D ratio	Maintain similar gas distribution and avoid channeling
Gas transport	Superficial gas velocity	Preserving oxygen transfer characteristics
Moisture control	Humidified inlet air ($RH \approx 100\%$)	Preventing substrate drying while avoiding flooding
Thermal management	Continuous upward airflow	Removing metabolic heat and maintaining uniform temperature
Hydrodynamic regime	Uniform upward flow	Minimizing dead zones and preferential flow paths
Scale-up strategy	Modular or parallel packed-bed configuration	Preserving transport similarity rather than increasing the bed depth

4. Conclusions

This study demonstrated that the production of laccase by *Trichoderma asperellum* LBKURCC1 in a humidified column bioreactor is governed by the effects of bed height, fermentation time, and humidified airflow rate. The RSM successfully identified a statistically significant quadratic model ($R^2 = 0.9642$), with airflow exhibiting the strongest nonlinear influence on reactor performance. The optimum operating condition (11 cm bed height, 4 days of fermentation, and $1 \text{ L} \cdot \text{min}^{-1}$ humidified airflow) produced a validated laccase activity of $253.8 \text{ U} \cdot \text{L}^{-1}$. Compared with the previously reported optimized flask-based solid-state fermentation system, the humidified column bioreactor achieved a 4.40-fold increase in laccase activity while reducing the time required to reach peak production by 50%. Beyond statistical optimization, this study demonstrates that humidified airflow functions as a key engineering control variable by simultaneously regulating oxygen transport and substrate moisture within the packed bed. The stability-defined operating window provides practical guidance for the design and scale-up of humidified packed-bed bioreactors and illustrates how statistical optimization can be integrated with transport-based interpretation to support the development of more robust and energy-efficient solid-state fermentation systems for lignocellulosic bioprocesses.

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Author Contributions

Sri Helianty (S.H.): Conceptualization, Methodology, Validation, Data Analysis, Investigation, Sample Preparation, Visualization, Writing—original draft preparation. **Panca Setia Utama (P.S.U.):** Formal Analysis, Software, Data Curation, Experimental Design Review, Writing—review & editing. **Yuana Nurulita (Y.N.):** Supervision, Experimental Design Review, Laboratory Support, Writing—review & editing. **Titania Nugroho (T.N.):** Conceptualization, Supervision, Experimental Design Review, Writing—review & editing, funding acquisition, **Gilang Ramadhan (G.R.):** Investigation, Laboratory Support, Sample Preparation, Project Administration. All authors have read and approved the final manuscript.

Conflict of Interest

The authors declare no conflicts of interest.

Supplementary Materials

Figure S1. Graphical Abstract of Laccase Production in a Humidified Column Bioreactor

Table S1. Coded and actual independent variable levels used in the CCD

Table S2. Experimental design matrix and observed laccase activity

Table S3. Statistical indicators of model adequacy for quadratic regression model

Table S4. Validation of model prediction at optimal operating condition

Table S5. Summary statistics of the developed model for laccase production

Table S6. Performance Comparison between Conventional Static SSF and the Humidified Column Bioreactor (HCB)

S1. Validation of Laccase Activity Improvement

S2. Validation of Fermentation Time Reduction

S3. Validation of Volumetric Productivity Improvement

Supplementary Conclusion

Declaration of AI

The authors acknowledge the use of artificial intelligence (AI)-assisted tools solely for language editing and improvement of manuscript clarity. These tools were utilized to refine the English expression and organization of the text. All scientific content, data analysis, interpretation, and conclusions presented in this study were developed and verified by the authors. The authors take full responsibility for the integrity and accuracy of the work.

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