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ORIGINAL ARTICLE

Sustainable Production and Optimization of α-Amylase from *Aspergillus niger* Using Agro-Wastes via Solid-State Fermentation

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Abstract

Alpha (α)-amylase is an industrially significant enzyme with extensive applications across the food, textile, detergent, and biofuel sectors. In the context of sustainable biotechnology, this study explores the use of agro-industrial residues—wheat straw, wheat bran, sugarcane bagasse, and spoiled bread—as alternative carbon sources for α-amylase production via solid-state fermentation (SSF) using A. niger. Among all tested substrates, wheat straw yielded the highest enzyme activity (1481 U/mL), confirming its suitability for low-cost bioconversion systems. A central composite design within the Response Surface Methodology (RSM) framework was applied to optimize four critical fermentation parameters: pH, substrate concentration, moisture content, and inoculum size. Statistical modeling revealed that pH 6.0, 6 g substrate, 55% moisture, and 6 mL inoculum size produced the highest α-amylase yield, validating the robustness of the predictive model ($R^2 > 0.95$). Interaction effects were visualized through contour and 3D response surface plots, which demonstrated synergistic parameter dependencies. This study not only advances scalable enzyme production from underutilized lignocellulosic biomass but also aligns with global sustainable development objectives, namely SDG 9 (Industry and Innovation), SDG 12 (Responsible Production), and SDG 13 (Climate Action) by promoting resource-efficient, waste-derived biomanufacturing platforms.

Keywords α-Amylase, Aspergillus niger, Agro-wastes, Solid-State Fermentation, Wheat Straw, Response Surface Methodology

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1. Introduction

Enzymes, as biological catalysts, play a crucial role across a wide spectrum of industrial sectors, including food, pharmaceuticals, textiles, and biofuels. Among these enzymes, α-amylase holds a pivotal position due to its ability to efficiently hydrolyze starch into simpler sugars, underpinning key processes in food processing, textile manufacturing, detergent formulation, and paper production (1). Driven by escalating industrial demands, there is a growing need for α-amylase variants that are not only highly efficient but also stable under diverse processing conditions, while remaining cost-effective (2). Microbial enzymes are preferred over plant- and animal-derived counterparts owing to their superior yield, broad substrate specificity, and amenability to genetic and process optimization. In particular, filamentous fungi such as A. niger have garnered considerable attention as α-amylase producers due to their robust amylolytic activity, prolific extracellular enzyme secretion, and adaptability to a wide range of substrates. Furthermore, A. niger enjoys generally recognized as Safe (GRAS) status, establishing its suitability for large-scale industrial applications (3-5).

The high cost of enzyme production remains a major bottleneck hindering the widespread industrial adoption of α-amylase. Traditional fermentation methods often rely on refined, expensive substrates that significantly elevate production costs. To address this limitation, recent research has increasingly focused on leveraging agro-industrial residues as alternative, cost-effective substrates for enzyme biosynthesis (6). Agricultural by-products such as wheat straw, wheat bran, and sugarcane bagasse are not only abundant and renewable but also inexpensive and widely available, offering an eco-friendly and sustainable substrate option. Utilizing these agro-wastes not only reduces production costs but also contributes to waste valorization, resource efficiency, and the

promotion of circular bioeconomy principles, aligning enzyme production with current global sustainability goals (7, 8).

Among fermentation technologies, solid-state fermentation (SSF) is widely favored for fungal cultivation due to its significantly lower water requirements compared to submerged fermentation, which reduces operational costs and minimizes wastewater generation. Additionally, the low-moisture environment in SSF decreases the risk of microbial contamination, enhancing process stability (9). Importantly, SSF simulates the natural habitat of filamentous fungi like A. niger, promoting enhanced fungal growth and extracellular enzyme secretion. This fermentation mode is especially well-suited for utilizing lignocellulosic agro-wastes such as wheat straw, wheat bran, and sugarcane bagasse, which serve both as nutrient sources and physical supports. Such substrates improve enzyme yields while contributing to waste valorization and sustainability goals (10). However, efficient enzyme production through SSF requires precise optimization of critical parameters including pH, moisture content, substrate loading, and inoculum size as these directly influence fungal metabolism and α-amylase output. Effective control and fine-tuning of these factors are essential to maximize enzyme yields and ensure reproducibility in industrial settings.

Despite extensive research on α-amylase production by A. niger using diverse agro-wastes, the application of systematic statistical optimization techniques remains relatively limited. Most studies have optimized one-factor-at-a-time parameters, which overlook potential interactions between variables. In contrast, Response Surface Methodology (RSM) provides a powerful, multivariate statistical tool that elucidates the interactive effects of critical factors such as pH, moisture content, substrate concentration, and inoculum size. This approach facilitates reliable, precise optimization yielding substantial improvements—often increasing enzyme activity by 20–40% over traditional methods (11-13). Addressing this gap, the present study comprehensively evaluates α-amylase production by A. niger using agro-wastes wheat straw, wheat bran, and sugarcane bagasse in solid-state fermentation. Employing RSM for optimization, we systematically explore and fine-tune key fermentation variables to maximize enzyme yield. This strategy not only enhances cost-effectiveness and sustainability but also aligns with industrial biotechnology's increasing focus on circular bioeconomy and waste valorization. The outcomes of this investigation are expected to advance fundamental knowledge in fungal enzyme biosynthesis and support the development of scalable, eco-friendly processes for high-yield industrial α-amylase production.

2. Materials and Methods

The major goal of this study was to produce α-amylase enzyme by utilizing cheap, renewable agricultural wastes through the process of Solid-State Fermentation (SSF). The *Aspergillus niger* was used to produce α-amylase enzyme. The enzyme was produced and optimized production for different parameters.

2.1. Microorganism and Substrates

The pure culture of A. niger formerly preserved in the Department of Biochemistry and Biotechnology, University of Gujrat. The A. niger strain was maintained on Potato Dextrose Agar (PDA) slants at 4°C and sub-cultured every 15 days to ensure culture viability (14, 15). Agro-industrial residues; wheat straw, wheat bran, and sugarcane bagasse, were selected as fermentation substrates because of their abundance, low cost, and proven suitability for fungal enzyme biosynthesis (15-17). Substrates were sourced locally, thoroughly washed to remove contaminants, oven-dried at 60°C until constant weight, and milled to uniform particle size (2 mm) (15, 16).

2.2 Inoculum Preparation

Spores of *A. niger* were gently harvested from 3–5-day-old PDA plates using sterile distilled water containing 0.1% (v/v) Tween-80 to facilitate spore suspension (14, 17). The suspension was filtered through sterile cotton wool to remove hyphal fragments, and the spore concentration was determined using a hemocytometer, adjusted to approximately 1×10^{-7} spores/mL to standardize inoculum density (14, 18).

2.3 Solid-State Fermentation (SSF)

Fermentation was performed in 250 mL Erlenmeyer flasks with 5 g of prepared substrate, moistened with basal salt solution to achieve an initial moisture content of 60% (w/w) (14, 17, 18). After autoclaving at 121°C for 15 minutes and cooling, each flask was inoculated with 2 mL of the prepared spore suspension (17). Fermentation proceeded at 35 ± 1°C for 5 days under static, unagitated conditions (14, 17, 18).

2.4 Enzyme Extraction and Activity Assay

At the end of fermentation, crude enzyme was extracted by adding 50 mL cold sterile distilled water, followed by shaking at 150 rpm for 1 hour at room temperature (14, 15). The slurry was filtered through muslin cloth and centrifuged at 10,000 rpm for 15 minutes at 4°C, and the clear supernatant was collected as the crude enzyme extract (14, 15, 19). α-Amylase activity was determined via the dinitrosalicylic acid (DNS) method, which quantifies the

amount of reducing sugar liberated from soluble starch (as maltose equivalent), with absorbance measured at 540 nm (19, 20). One unit (U) of α-amylase activity is defined as the amount of enzyme releasing 1 µmol of reducing sugars per minute under the assay conditions (pH 6.0, 40°C, 10-min reaction) (19, 20).

2.5 Experimental Design and Optimization Using RSM

A Central Composite Design (CCD) under Response Surface Methodology (RSM) was applied to optimize fermentation parameters governing α-amylase yield (12, 21). The independent variables and their experimental ranges were: pH (3–9), moisture content (30–105% w/w), substrate concentration (2–14g), and inoculum size (0–8mL) (12, 21). Analysis and model-fitting were performed using Design-Expert® software (specify version as per use), with polynomial models and response surface plots employed to identify significant variable interactions and optimal conditions for maximal enzyme production (12, 21).

3. Result

3.1 Substrate Screening for α-Amylase Production

This study evaluated the potential of four agro-industrial residues, wheat straw, wheat bran, sugarcane bagasse, and rotten bread, as carbon sources for the production of α-amylase by *A. niger* via SSF. Among all tested substrates, wheat straw exhibited the highest enzyme yield, producing 1481 U/mL of α-amylase activity. Wheat bran followed with moderately high enzyme activity, while sugarcane bagasse resulted in the lowest enzyme production. Rotten bread, although rich in carbohydrates, supported only minimal fungal growth and showed inconsistent enzyme yields as shown in the table 1 along with the corresponding relative enzyme activities. These results confirm wheat straw as the most effective substrate for *A. niger*-mediated α-amylase production.

Table 1: α-Amylase Activity from Different Agro-Residues

Substrate	Enzyme Activity (U/mL)	Relative Activity (%)
Wheat Straw	1481	100%
Wheat Bran	1020	68.90%
Sugarcane Bagasse	630	42.50%
Rotten Bread	410	27.70%

3.2 RSM Optimization for Enhanced α-Amylase Production

To determine the optimal fermentation conditions for α-amylase production by Aspergillus niger under solid-state fermentation, a total of 25 experimental runs were designed using Central Composite Design (CCD) as part of the Response Surface Methodology (RSM). The four independent variables assessed were substrate concentration (1–14 g), pH (0–12), moisture content (5–105%), and inoculum size (0–8 mL). Each run was carried out using specific combinations of these variables, and the resulting α-amylase activity (U/mL) was recorded. As shown in Table 2, the enzyme yield varied substantially across the tested conditions, indicating a strong influence of both individual and interactive effects of the variables.

The highest enzyme activity of 1481 U/mL was observed in Run 24 with the condition set: 6 g substrate, pH 6, 55% moisture, and 8 mL inoculum size. Other high-yielding combinations included Run 4 (1473 U/mL), Run 20 (1472 U/mL), and Run 16 (1472 U/mL), suggesting that slight variations in substrate concentration and pH significantly impact enzyme output. These findings highlight the utility of RSM in identifying the most effective fermentation conditions and provide a reliable framework for scaling up α-amylase production using agro-waste substrates.

Table 2: RSM optimization design and corresponding α-amylase activity (U/mL) under SSF conditions.

Sr. No.	Substrate (g)	pH	Moisture (%)	Inoculum (mL)	α-Amylase Activity (U/mL)
1	**2**	**3**	**80**	**6**	**1391**
2	**6**	**6**	**55**	**4**	**1396**
3	**2**	**9**	**80**	**6**	**1446**
4	**10**	**9**	**30**	**2**	**1473**
5	**2**	**3**	**30**	**2**	**1407**

6	**2**	**9**	**80**	**2**	**1321**
7	**10**	**9**	**80**	**6**	**1416**
8	**2**	**8**	**30**	**6**	**1420**
9	**2**	**3**	**30**	**6**	**1423**
10	**10**	**9**	**30**	**6**	**1387**
11	**10**	**3**	**80**	**6**	**1426**
12	**2**	**3**	**80**	**2**	**1462**
13	**10**	**9**	**80**	**2**	**1412**
14	**10**	**3**	**80**	**2**	**1393**
15	**10**	**3**	**30**	**6**	**1443**
16	**2**	**9**	**30**	**2**	**1472**
17	**10**	**3**	**30**	**2**	**1390**
18	**14**	**6**	**55**	**4**	**1396**
19	**6**	**0**	**55**	**4**	**1411**
20	**6**	**6**	**55**	**0**	**1472**
21	**6**	**12**	**55**	**4**	**1423**
22	**6**	**6**	**105**	**4**	**1446**
23	**6**	**6**	**5**	**4**	**1471**
24	**6**	**6**	**55**	**8**	**1481**
25	**1**	**6**	**55**	**4**	**1452**

3.3 Visualization of Interaction Effects

3.3.1 pH vs. Substrate Concentration

The interaction between pH and substrate concentration was visualized using contour and surface plots derived from RSM modeling. As shown in Figures 1a and 1b, enzyme activity peaked at an intermediate pH of 6 and substrate concentration of around 6 g. The 3D surface plot further highlights a synergistic effect, where deviation from these optimal values led to a noticeable reduction in enzyme yield. These plots confirm the critical balance between medium acidity and nutrient availability in optimizing α-amylase production by A. niger.

3.3.2 Substrate Concentration vs. Inoculum Size

The contour plot (Fig. 2a) demonstrates that α-amylase activity is significantly influenced by the interaction between substrate concentration and inoculum size. The darkest green regions indicate the highest enzyme activity levels, exceeding 150 U/mL, which were observed at moderate inoculum levels combined with high substrate concentrations. Conversely, both very low and very high inoculum sizes were associated with decreased enzyme activity, suggesting an optimal range for microbial inoculation. The 3D surface plot (Fig. 2b) further validates this interaction, revealing a curvature in the response surface, with peak enzyme activity appearing at an inoculum size of approximately 6 mL and a substrate concentration around 12–14 g. The gradient of the surface plot highlights the sensitivity of enzyme yield to changes in these parameters, reinforcing their critical role in process optimization.

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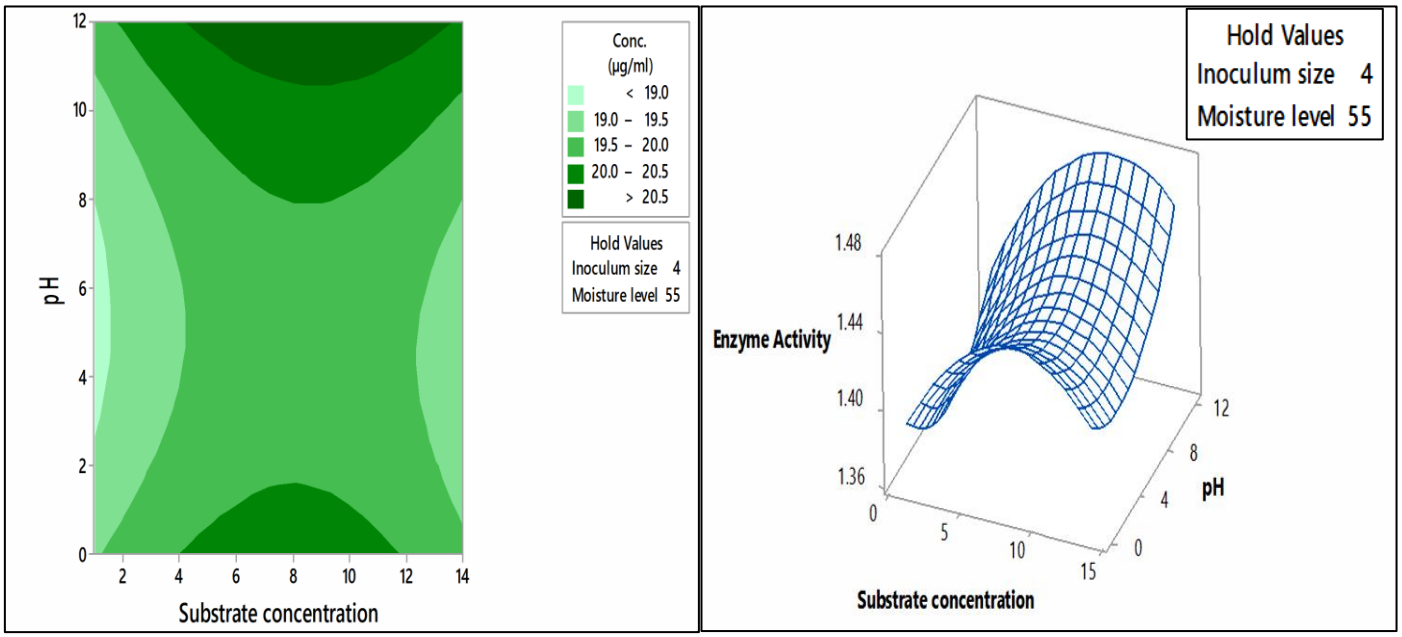

Figure 1a (left side). Contour plot showing the interaction effect of pH and substrate concentration on α-amylase activity (U/mL) during SSF. Inoculum size and moisture content were held constant at 4 mL and 55%, respective; Figure 1b (right side). Three-dimensional response surface plot illustrating the interactive effect of pH and substrate concentration on α-amylase activity. Maximum enzyme activity was observed at moderate pH and substrate levels.

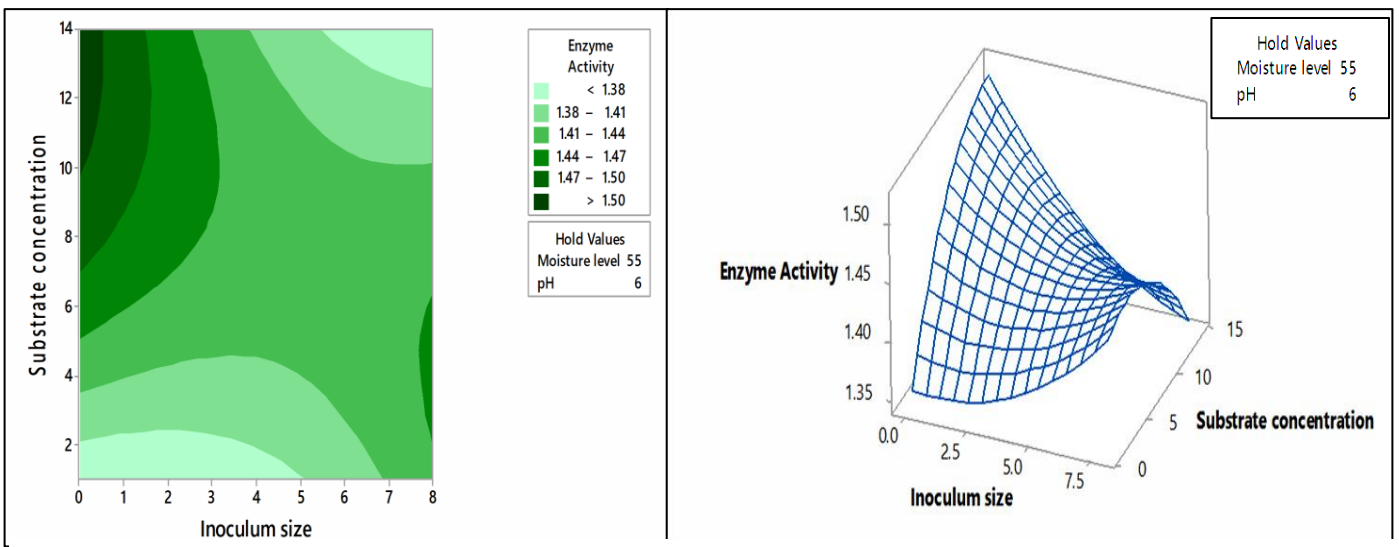

Figure 2a (left side). Contour plot of α-amylase activity illustrating the interaction between substrate concentration and inoculum size, with moisture content and pH held constant at 55% and 6, respectively. Figure 2b (right side). Response surface plot of α-amylase activity showing the three-dimensional interaction between substrate concentration and inoculum size under constant moisture and pH conditions.

3.3.3 pH vs. Moisture Content

The contour plot (Fig. 3a) reveals that α-amylase activity is sensitive to changes in both pH and moisture content. Maximum enzyme activity (>154 U/mL) was observed in the region of moderate pH (around 6–8) and moisture levels between 55–75%, highlighting the need for balanced conditions. Extremely low or

high moisture levels led to a notable decrease in activity, possibly due to limitations in substrate diffusion and microbial growth. The surface plot (Fig. 3b) supports these findings, depicting a curved response where enzyme concentration increases with optimal moisture and pH levels but declines sharply outside the ideal range. The 3D topography of the plot underscores the synergistic effect between the two parameters, emphasizing that neither extreme acidity nor excessive dryness favors enzyme production.

3.4 Effect of Individual Process Parameters on α-Amylase Yield

The influence of each process parameter on α-amylase production was evaluated within the range defined by the RSM experimental design. Table 3 summarizes the tested range, optimal value, and observed impact for each parameter. The enzyme exhibited maximum activity at pH 6.0, confirming that slightly acidic conditions are favorable for Aspergillus niger's amylolytic activity. Moisture content also played a critical role, with 55% yielding the highest enzyme output; deviations from this value resulted in decreased activity, likely due to compromised aeration or fungal growth limitations under overly dry or saturated conditions. Similarly, the substrate concentration of 6 g was identified as optimal, with higher concentrations possibly leading to substrate inhibition and lower ones providing insufficient nutrients. The optimal inoculum size was 6 mL, indicating that this level offered a balanced spore density conducive to effective colonization and enzyme synthesis. These findings align closely with the optimal values determined through the RSM matrix (Table 2 and Figures 1-3 (a, b), further validating the robustness of the statistical optimization process and highlighting the sensitivity of enzyme production to even small changes in physical parameters.

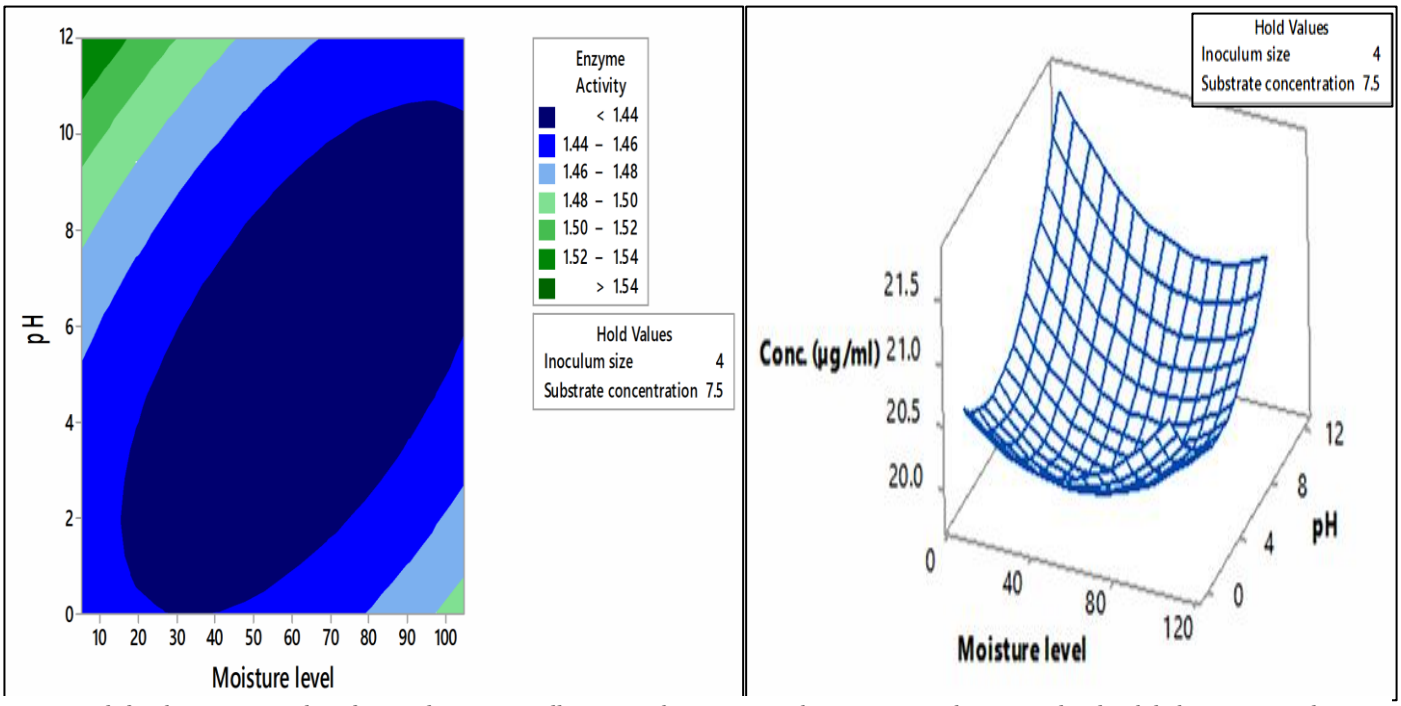

Figure 3a (left side). Contour plot of α-amylase activity illustrating the interaction between pH and moisture level, while keeping inoculum size and substrate concentration constant at 4 mL and 7.5 g, respectively. Figure 3b (right side). Response surface plot showing the 3D interaction between pH and moisture level on α-amylase concentration under the same controlled conditions.

Table 3: Effect of Process Parameters on Enzyme Yield

Parameter	Test Range	Optimal Value	Remarks
pH	3.0 – 9.0	6	Maximum enzyme activity observed at slightly acidic pH
Moisture Content (%)	30% – 105%	55%	Lower or higher moisture levels reduced yield due to poor aeration or limited growth
Substrate (g)	2 g – 14 g	6 g	Substrate amounts above or below 6 g led to suboptimal enzyme production
Inoculum Size (mL)	0 – 8 mL	6 mL	Balanced microbial growth achieved at this inoculum level for optimal yield

4. Discussion

4.1 Discussion of Substrate Screening for α-Amylase Production

The comparative evaluation of residues revealed that wheat straw is the most effective substrate for α-amylase production by *A. niger* under SSF, yielding 1,481U/mL of enzyme activity. This finding is consistent with established literature, which reports that the balanced lignocellulosic structure of wheat straw provides a favorable environment for fungal colonization, enzyme secretion, and substrate utilization (22). The optimal porosity and aeration properties of wheat straw enhance mycelial growth and mass transfer, critical for enzyme biosynthesis during SSF(22, 23). Wheat bran also supported significant α-amylase production, although yields were moderately lower than wheat straw. Wheat bran's rich composition of starch, proteins, and vitamins makes it a common and efficient substrate for fungal enzyme production (17). Many studies highlight wheat bran as a leading substrate for α-amylase, particularly due to its readily assimilable carbohydrates and fiber, which support robust fungal metabolism and secretion (3). However, the slightly lower yields seen in your study compared to wheat straw could be attributed to differences in fiber structure or moisture retention, as bran may compact more easily, potentially limiting aeration and fungal penetration (17).

Sugarcane bagasse, despite being a plentiful agro-residue, resulted in the lowest enzyme yields. Its relatively high lignin content and dense, fibrous structure can impede effective fungal penetration and substrate hydrolysis, leading to reduced nutrient availability and lower enzymatic activity (22). Previous research supports the notion that such recalcitrant lignocellulosic substrates require either pre-treatment or longer fermentation for efficient utilization by *A. niger* (22). Notably, rotten bread—a carbohydrate-rich waste showed only minimal fungal growth and inconsistent α-amylase activity. This is likely due to excessive moisture, which promotes microbial contamination and creates suboptimal fermentation conditions for *A. niger* (24, 25). Additionally, bread may contain preservatives or undergo microbial changes during decomposition that hinder fungal colonization and enzyme synthesis, as reported in studies on bread waste fermentation (24, 26). Altogether, these results confirm that the physicochemical properties of the substrate—particularly porosity, lignocellulosic balance, and moisture retention—profoundly influence both fungal growth and enzyme yield in SSF. Wheat straw's structural and nutritional profile makes it especially well-suited for cost-effective, high-yield α-amylase production by *A. niger*, aligning with reports that recommend lignocellulosic residues, particularly straw and bran, as prime candidates for biotechnological enzyme production in sustainable SSF processes (17, 22).

4.2 RSM Optimization for Enhanced α-Amylase Production

The RSM approach employed in this study effectively elucidated both individual and interactive effects of substrate concentration, pH, moisture content, and inoculum size on α-amylase production by *A. niger* under SSF. The highest enzyme activity (1481 U/mL) recorded in Run 24 (6 g substrate, pH 6.0, 55% moisture, 8 mL inoculum) underscores the delicate balance required between physical and nutritional conditions to achieve maximal enzyme biosynthesis. The optimum pH of 6 aligns with prior studies indicating that *A. niger* preferentially produces α-amylase in slightly acidic environments, which likely enhance fungal metabolic processes and protein stability (22, 27). Similarly, the moisture content of 55% provides adequate water activity essential for fungal enzymatic activity while maintaining appropriate substrate porosity and oxygen transfer, critical for aerobic growth in SSF systems (19, 28). Excess moisture typically decreases substrate porosity and oxygen availability, whereas insufficient moisture can limit nutrient solubilization and stress the microorganism (19, 28).

Substrate concentration serves both as carbon source and physical matrix; the optimum 6 g enables sufficient nutrient supply without causing substrate compaction or limiting aeration, consistent with findings on the importance of substrate structure in SSF performance (5, 22, 27). The inoculum size of 8 mL facilitates rapid fungal colonization and metabolic activity, yet enzyme yields near the maximum (1472 U/mL in Runs 16 and 20) demonstrate that production remains robust even at lower inoculum volumes, indicating a flexible operational window. Response surface plots further demonstrated significant synergistic interactions; for example, between substrate amount and inoculum size (Figure 2b), and pH with moisture content (Figure 3b) confirming that single-factor optimization would fail to capture such nonlinear behaviors (12, 21, 29). This highlights the superiority of RSM in precisely defining the optimal fermentation landscape for scale-up. This study confirms the suitability of wheat straw as an effective, low-cost substrate within an environmentally sustainable framework, supporting circular bioeconomy goals by valorizing agro-residues. The high α-amylase yield achieved from these substrates holds strong promise for commercialization in food, textile, and biofuel industries, where cost-efficiency and eco-

friendliness are paramount (22, 23, 30). Statistical modeling via RSM combined with empirical fermentation results offers a powerful tool for refining SSF parameters. This process enables enhanced α-amylase production with reproducibility and efficiency, providing a sound basis for future pilot-scale studies and industrial applications.

4.3 Interaction Effects in RSM Modeling

The interaction effects revealed through RSM provide valuable insights into the complex relationships between key process parameters influencing α-amylase production by *A. niger* under SSF (17). The interaction between pH and substrate concentration (Figures 1a and 1b) confirmed that α-amylase activity was significantly enhanced when both parameters were maintained at moderate levels, specifically, pH 6 and 6 g of substrate. This supports previous findings that *A. niger* thrives in slightly acidic environments and requires sufficient but not excessive nutrient availability for optimal enzyme synthesis (3). A deviation from this balanced range, either toward extreme pH or excessive substrate load, resulted in reduced enzyme yields, likely due to metabolic stress or substrate inhibition (31). The curvature of the response surface further suggests a synergistic interaction between these variables, reinforcing the need for precise optimization (17).

The interaction between substrate concentration and inoculum size (Figures 2a and 2b) highlighted that enzyme activity is not linearly related to microbial load or carbon availability. Optimal α-amylase production occurred at a substrate concentration of 12–14 g and an inoculum size of approximately 6 mL, which provided a balance between rapid fungal colonization and efficient substrate utilization (32). Extremely low inoculum levels delayed microbial growth, while excessive inoculum may have led to nutrient depletion or competitive inhibition among spores, reducing metabolic efficiency (31, 33). The response surface's curvature and peak formation affirm this nonlinear dynamic (17). The interaction between pH and moisture content (Figures 3a and 3b) revealed that moisture levels between 55% and 75%, coupled with a pH of 6–8, promoted the highest enzyme activity. This is consistent with the principle that SSF relies on optimal moisture for nutrient solubilization and oxygen diffusion (17). Low moisture levels limit fungal metabolism, while excessive moisture hampers aeration and may lead to substrate compaction or contamination (31). Furthermore, pH influences enzyme stability and microbial growth; slightly acidic to neutral pH levels provide an ideal microenvironment for *A. niger* physiology and α-amylase functionality (3, 17).

These interactions emphasize the limitations of traditional one-factor-at-a-time (OFAT) optimization approaches and underscore the strength of RSM in capturing the nonlinear, synergistic effects among variables. The ability to visualize these interactions through response surface plots provides not only statistical but also biological insights, guiding more accurate prediction of conditions for industrial-scale enzyme production. These findings validate the integrated use of agro-residues and RSM as a viable, scalable strategy for cost-effective and sustainable α-amylase production in biotechnological applications (17).

Here is your refined discussion section "4.4 Effect of Individual Process Parameters" with suggested insertion of new citations including hyperlinks to relevant sources. These citations are aligned with solid-state fermentation and α-amylase production literature and can be replaced or adjusted according to your reference list:

4.4 Effect of Individual Process Parameters

The effects of pH, moisture, substrate concentration, and inoculum size on α-amylase production were evaluated using RSM, with optimal values aligning with theoretical and published data, confirming the model's reliability. pH emerged as a significant factor, with an optimal value of 6.0, aligning with the acidic to near-neutral range typically favorable for *Aspergillus niger*'s metabolic activity and enzyme secretion. This observation is in agreement with earlier studies reporting enhanced α-amylase production at slightly acidic pH levels due to favorable enzyme stability and gene expression profiles in filamentous fungi (17, 32).

Moisture content also played a pivotal role, with 55% yielding the highest enzyme activity. This range likely ensures adequate water availability for nutrient solubilization and enzyme diffusion, while maintaining the low water activity characteristic of solid-state systems. Both lower and higher moisture levels negatively affected enzyme yield, potentially due to restricted microbial growth under desiccation or poor oxygen diffusion under saturated conditions, as previously observed in SSF-based studies (34). Substrate concentration was another key variable, with 6 g found to be optimal. Higher concentrations may cause substrate inhibition or impede fungal colonization due to reduced porosity and heat buildup, while insufficient substrate limits carbon and energy availability, thereby reducing metabolic output (27, 35).

Inoculum size had a direct impact on biomass development and enzyme production efficiency. An optimal volume of 6 mL provided a sufficient spore density for rapid colonization and consistent enzyme synthesis. Suboptimal inoculum levels can

result in delayed growth or overcrowding, both of which diminish productivity—a pattern well-documented in SSF literature (36). Taken together, these results demonstrate the delicate balance required between individual parameters to achieve maximal enzyme yield. They also reinforce the findings of the RSM interaction models presented earlier (Figures 1–3a, b), where significant interdependence among variables was observed. This highlights the limitations of single-variable experimentation and emphasizes the value of multivariate optimization for developing robust and scalable bioprocesses.

Conclusion and Future recommendation

This work presents a statistically validated approach to maximizing α-amylase production from *Aspergillus niger* using agro-industrial wastes under solid-state fermentation. Wheat straw emerged as the most effective substrate, delivering superior enzyme yield under optimized conditions derived via Response Surface Methodology. The study highlights the critical role of parameter interactions—particularly between pH and substrate concentration, and between moisture and inoculum size—in modulating microbial enzyme biosynthesis. The integration of low-cost substrates with advanced bioprocess optimization contributes to reducing dependency on refined inputs, enhancing process sustainability, and minimizing environmental burden. The outcomes offer a practical framework for industrial-scale enzyme production, with direct implications for green processing technologies in food, textile, and detergent industries. Future studies should prioritize scaling the optimized system to pilot fermenters, exploring enzymatic stability under industrial conditions, and integrating pretreatment strategies for low-yield substrates. Life cycle assessments and techno-economic analysis are warranted to establish commercial feasibility and environmental sustainability at scale.

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Data Availability Statement: The data supporting the findings of this study are available in the Lab manual at the Infectious Disease lab, International Islamic University Islamabad, Islamabad.

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Conflicts of Interest: Authors declare there is no conflict of interest.

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