



Optimization and Kinetic Studies on Endo-1,4-β-glucanase (endoglucanase) Production by *Aspergillus oryzae* PTCC 5164 Grown on Wheat Straw under Solid-State Fermentation

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Article Info

Document Type:
Research Paper

Received 13/04/2026
Received in revised form
31/07/2026
Accepted 15/08/2026

Published 14/10/2026

Keywords:

Solid-state fermentation,
Endo-1,4-β-glucanase (endoglucanase),
Aspergillus oryzae PTCC 5164,
Wheat straw

Abstract

This study aimed to optimize endo-1,4-β-glucanase (endoglucanase) production by *Aspergillus oryzae* PTCC 5164 growing on wheat straw under solid-state fermentation (SSF), investigate fungal growth and enzyme production kinetics, and evaluate enzymatic hydrolysis of wheat straw for glucose release using concentrated cell-free enzyme solutions. A 2⁴ full factorial design was employed to assess the effects of particle size (0.3–2.0 cm), moisture content (65–85%), ammonium sulfate (0.04–0.12 g/g straw), and carboxymethyl cellulose (CMC, 0–0.08 g/g straw) on endoglucanase yield. In a separate experiment, fungal biomass, enzyme activity, and substrate consumption were monitored over seven days, and biomass yield on substrate ($Y_{X/S}$) and enzyme yield on biomass ($Y_{E/X}$) were calculated. Hydrolysis assays were performed on wheat straw using concentrated cell-free enzyme solutions at varying Straw loading (w/v)%. The results showed that moisture and particle size were the dominant factors influencing endoglucanase production, with significant higher-order interactions with CMC and ammonium sulfate. Maximum endoglucanase activity (9.93 IU/g straw) was achieved at the particle size of 0.3 cm, CMC concentration of 0.08 g/g straw, ammonium sulfate concentration of 0.12 g/g straw, and 65% moisture content. During the seven-day monitoring period, peak endoglucanase activity (6.88 IU/g straw, corresponding to a $Y_{E/X}$ of 3.54 IU/mg biomass protein) was observed on day 3, while peak biomass (2.34 mg biomass protein/g straw, corresponding to a $Y_{X/S}$ of 9.58 mg biomass protein/g consumed straw) was observed on day 5. Enzymatic hydrolysis of wheat straw released up to 5.43 mg glucose/mL, with Straw loading (w/v)% of 8%w/v and enzyme concentration of 0.5 IU/mL. Overall, these results establish that *A. oryzae* PTCC 5164 can efficiently convert wheat straw into cellulolytic enzymes under optimized SSF conditions, and the resulting enzyme preparation is effective for saccharification, offering a viable route for valorization of this agricultural residue.

1. Introduction

The sustainable conversion of agricultural residues into high-value bioproducts is increasingly recognized as a critical strategy to address global energy and environmental challenges. Among these residues, wheat straw is one of the most abundant lignocellulosic byproducts of wheat production, often underutilized or subjected to open-field burning, which contributes to greenhouse gas emissions and air pollution. Its high cellulose and hemicellulose content makes it a promising feedstock for biorefineries, particularly for enzymatic hydrolysis and biofuel production (Bian *et al.*, 2022).

Recent advances have demonstrated that solid-state fermentation (SSF) offers distinct advantages for microbial enzyme production compared to submerged fermentation. SSF provides a low-energy process with high volumetric productivity, reduced risk of bacterial

contamination, and conditions that more closely simulate the natural growth environment of filamentous fungi (Oiza *et al.*, 2022).

Several studies have highlighted that *Aspergillus spp.* and *Trichoderma spp.* are efficient cellulase producers under SSF, achieving higher yields when using agricultural residues such as wheat straw, rice straw, and sugarcane bagasse (Rosas-Vega *et al.*, 2025; Thakur *et al.*, 2024; Borthakur *et al.*, 2024). The choice of particle size, nitrogen and carbon supplementation, and substrate pretreatment significantly influence cellulase productivity (Aggarwal *et al.*, 2017; Rai *et al.*, 2012; El-Shishtawy *et al.*, 2015).

Techno-economic analyses suggest that localized cellulase production through SSF may reduce enzyme costs in small- and medium-scale biorefineries (Gupta *et al.*, 2018; Olofsson *et al.*, 2017). Optimization of process variables through statistical designs continues to be a

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DOI: 10.22104/mmb.2026.8195.1200

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powerful approach for maximizing enzyme yields (Ergun *et al.*, 2025). Kinetic studies have revealed a strong correlation between fungal biomass accumulation and cellulase secretion, with maximum activity typically observed in the late exponential or early stationary phase of growth (Lynd *et al.*, 2002).

Recent investigations into bioreactor design and SSF scale-up further highlight the need for precise control of moisture, aeration, and particle size distribution to maintain enzyme productivity at larger scales (Bamidele *et al.*, 2025). Enzyme extraction methods have also been refined, allowing for recovery of high-activity cellulase cocktails suitable for direct application in biomass hydrolysis (Meas *et al.*, 2015).

Enzymatic hydrolysis remains central to the valorization of wheat straw into fermentable sugars for bioethanol and other biochemicals. Advances in enzyme production, co-culture strategies, and substrate loading optimization have improved saccharification yields in both laboratory and pilot-scale studies (Novy *et al.*, 2015; Jahangeer *et al.*, 2025; Vasic *et al.*, 2021). However, despite significant progress, knowledge gaps remain regarding the kinetics of *Aspergillus oryzae* growth, enzyme production, and wheat straw hydrolysis in SSF systems.

Therefore, this study aimed to (i) optimize cellulolytic enzyme production by *A. oryzae* PTCC 5164 grown on wheat straw under SSF using factorial design methodology; (ii) investigate fungal growth kinetics, substrate consumption, and enzyme secretion profiles over a seven-day fermentation period; (iii) establish correlations between biomass yield on substrate ($Y_{X/S}$), enzyme yield on biomass ($Y_{E/X}$), and cellulolytic activity; and (iv) evaluate the hydrolytic performance of the produced cell-free enzyme cocktail on wheat straw at varying straw loadings to assess its potential for glucose release in biorefinery applications.

2. Materials and Methods

2.1. Materials

Carboxymethyl cellulose (CMC), bovine serum albumin (BSA), Coomassie Brilliant Blue G250, 3,5-dinitrosalicylic acid (DNS), sodium potassium tartrate, sodium hydroxide, potassium dihydrogen phosphate, ammonium sulfate, phosphoric acid, glucose, and potato dextrose agar (PDA) (all of analytical grade) were purchased from Merck. Ethanol (96%) was obtained from Shabnam Pharmaceutical and Food Factories, Kerman Co., Iran.

2.2. Fungi and inoculum preparation

Aspergillus oryzae PTCC 5164 (*A. oryzae* PTCC 5164) was obtained from the Iranian Research Organization for Science and Technology (IROST). The fungus was aseptically inoculated on PDA slants and incubated at 30°C for 4 days. Slants were stored at 4°C for later experiments. For inoculum preparation, 5 mL of sterile distilled water was added aseptically to a 4-day-old slant culture of *A. oryzae* PTCC 5164. The tube was gently agitated to dislodge the spores, and 1 mL of this

suspension was then used to inoculate each flask containing the sterilized straw.

2.3. Experimental design

A 2⁴ full factorial design was used to investigate the effects of four factors on endoglucanase production by *A. oryzae* PTCC 5164: (i) wheat straw particle size (0.3 vs. 2.0 cm), (ii) CMC as an inducer (0 vs. 0.08 g/g dry straw), (iii) ammonium sulfate as a nitrogen source (0.04 vs. 0.12 g/g dry straw), and (iv) moisture content (65% vs. 85%). To evaluate experimental error and possible curvature in the response, a central point with four replicates was included. The complete design comprised 20 experimental runs, as summarized in Table 1. The runs were conducted in 500 mL Erlenmeyer flasks containing 5 g of dry wheat straw. The appropriate amounts of CMC, ammonium sulfate, and phosphate buffer (pH 5.5, to adjust moisture content) were added according to the experimental design. Flasks were sterilized by autoclaving at 121°C and 15 psi for 20 min. Each flask was inoculated with 1 mL of fungal spore suspension and incubated at 30°C for 5 days. At the end of the incubation period, 100 mL of phosphate buffer (pH 5.5) was added to each flask to extract the enzyme. The flasks were left at room temperature for at least 30 min, during which they were shaken frequently by hand to ensure adequate endoglucanase extraction. The mixtures were filtered through filter cloth, and 15 mL of the filtrate was centrifuged at 5000 rpm for 20 min at room temperature. The resulting supernatant was collected and used for endoglucanase activity assays.

2.4. Endoglucanase activity assay

Endoglucanase activity was determined by measuring glucose released from carboxymethyl cellulose (CMC) using the DNS method. Briefly, 0.5 mL of enzyme supernatant was mixed with 0.5 mL of 1% (w/v) solution of CMC in phosphate buffer (pH 5.5) and incubated for 30 min at 30°C. Subsequently, 3 mL of DNS reagent was added, and the mixture was boiled for 20 min. Absorbance was then measured at 540 nm using an Optizen 320 UV-Vis spectrometer (Mecasys, Daejeon, Korea). Appropriate blanks were prepared to account for the medium and inactivated enzyme. The amount of glucose produced was calculated from the standard calibration curve, and enzyme activity (IU/mL) was determined accordingly. One unit (IU) of endoglucanase activity was defined as the amount of enzyme required to release 1 μ mol of glucose per minute under the assay conditions.

2.5. Fungal growth and biomass and endoglucanase production as a function of time

To investigate the growth kinetics of *A. oryzae* PTCC 5164 under optimized enzyme production conditions, solid-state cultures were prepared using 5 g of wheat straw with an average particle size of 0.3 cm, supplemented with 0.4 g CMC and 0.6 g ammonium sulfate. The substrate moisture was adjusted to 65% with phosphate buffer (pH 5.5) before sterilization. After inoculation with 1 mL of spore suspension, the flasks were incubated at 30°C.

Table 1. Endoglucanase production on wheat straw by *Aspergillus oryzae* PTCC 5164.

Run	Particle size (cm)	CMC (g/g straw)	(NH ₄) ₂ SO ₄ (g/g straw)	%Moisture	Cellulase (IU/g straw)
1	0.3	0	0.04	65	8.649
2	2	0	0.04	65	6.316
3	0.3	0.08	0.04	65	9.089
4	2	0.08	0.04	65	8.036
5	0.3	0	0.12	65	8.590
6	2	0	0.12	65	7.238
7	0.3	0.08	0.12	65	9.928
8	2	0.08	0.12	65	8.316
9	0.3	0	0.04	85	7.236
10	2	0	0.04	85	5.119
11	0.3	0.08	0.04	85	7.517
12	2	0.08	0.04	85	6.592
13	0.3	0	0.12	85	6.402
14	2	0	0.12	85	6.937
15	0.3	0.08	0.12	85	7.409
16	2	0.08	0.12	85	6.801
17	1.15	0.04	0.08	75	8.243
18	1.15	0.04	0.08	75	8.186
19	1.15	0.04	0.08	75	8.237
20	1.15	0.04	0.08	75	8.492

At 24-hour intervals, one flask was removed and stored at 4°C to stop growth. Following seven days of cultivation, extracellular enzymes were extracted, and the fungal biomass was disrupted by adding 30 mL of 0.25 M phosphoric acid (Cordova-Lopez *et al.*, 1996). The suspension was clarified by filtration and centrifugation (6000 rpm, 20 min, room temperature), and the supernatant was evaluated for the intracellular protein fraction (intracellular protein was used as a proxy for biomass).

The protein concentration was quantified using the Bradford method, based on a standard calibration curve prepared with BSA. The solid residues were dried in an oven (SHIMAZCO, Iran) at a temperature of approximately 50 °C until a constant weight was achieved. The substrate consumption was determined by subtracting the weight of the solid residue from the initial straw weight (5 g). Biomass yield on substrate ($Y_{X/S}$) and enzyme yield coefficient ($Y_{E/X}$) were calculated from the experimental data using Equation (1) and Equation (2):

$$Y_{X/S} = \frac{\text{Biomass protein (mg)}}{\text{Initial straw weight (g)} - \text{Dried solid residue (g)}} \quad (1)$$

$$Y_{E/X} = \frac{\text{Enzyme produced (IU)}}{\text{Biomass protein produced (mg)}} \quad (2)$$

2.6. Wheat straw hydrolysis using the produced enzyme

A concentrated enzyme solution was prepared from the initial enzyme stock (0.27 IU/mL) through ethanol precipitation followed by sterile filtration (0.2 µm) (Marino *et al.*, 2015). The resulting precipitate was resuspended in buffer to obtain enzyme solutions at the desired concentrations. To investigate straw hydrolysis using these concentrated enzyme preparations, two sets of experiments were carried out. In the first experiment, 2 mL of enzyme solution at a concentration of 0.6 IU/mL was combined with 0.3 g of straw in a series of test tubes and incubated at room temperature. Samples were collected

every three hours, and the glucose concentration in the supernatant was determined.

In the second experiment, an enzyme solution with a concentration of 0.5 IU/mL was applied to varying ratios of straw to solution volume. Specifically, mixtures were prepared with 2 g of straw in 50 mL of solution, 2 g of straw in 150 mL, 4 g of straw in 50 mL, 4 g of straw in 150 mL, and 3 g of straw in 100 mL. These mixtures were incubated at room temperature for 15 hours, after which the glucose concentration in the resulting solutions was analyzed.

2.7. Statistical analysis

Minitab 18 was used for all statistical analyses. The software was employed to generate the factorial design matrix (section 2.3) and to perform analysis of variance (ANOVA) to identify significant factors, with a significance threshold of p-values < 0.05. Regression analysis was conducted on the time-course data from section 2.5 to model microbial growth, enzyme production, and substrate consumption, as well as to model the kinetics of sugar release from straw (the second experiment in section 2.6). Additionally, a one-way ANOVA was performed to evaluate the effect of the straw loading (w/v)% on sugar release (the first experiment in section 2.6).

3. Results and Discussion

3.1. Effects of particle size, ammonium sulfate, CMC, and moisture on endoglucanase production

The results of the experimental design are presented in Table 1. The results revealed that endoglucanase production by *A. oryzae* PTCC 5164 was strongly influenced by particle size, CMC supplementation, ammonium sulfate, and moisture level. The analysis of variance (ANOVA) (Table 2) demonstrated that the main

effects of particle size ($p < 0.001$), CMC ($p = 0.001$), ammonium sulfate ($p = 0.011$), and moisture ($p < 0.001$) were all statistically significant, with moisture exerting the most pronounced impact ($F = 488.63$). This observation aligns with recent findings that optimal moisture levels are critical in solid-state fermentation, as they regulate oxygen diffusion, substrate swelling, and microbial metabolic activity, all of which directly influence endoglucanase synthesis (Darabzadeh *et al.*, 2019; Legodi *et al.*, 2023).

Among the two-way interactions, particle size $\times$ ammonium sulfate ($p = 0.009$), particle size $\times$ moisture ($p = 0.010$), and CMC $\times$ moisture ($p = 0.038$) were significant. This suggests that the effect of particle size on endoglucanase activity depends on the availability of nitrogen and substrate moisture. Smaller particles provide a larger surface area for fungal colonization, but when combined with optimal nitrogen supplementation, enzyme yields are further enhanced (Membrillo *et al.*, 2008). Likewise, the synergy between CMC and moisture indicates that substrate supplementation with an easily metabolizable carbon source enhances induction of endoglucanase only when the water activity supports fungal metabolism.

The presence of significant three-way interactions, particularly particle size $\times$ CMC $\times$ ammonium sulfate ($p = 0.006$), underscores the complex, non-linear influence of these parameters, highlighting the necessity of multifactorial optimization.

The half-normal probability plot (Figure 1) further confirmed the significance of particle size (A), CMC (B), and moisture (D) as the dominant factors affecting endoglucanase yield, with their standardized effects lying far from the normal distribution line. Moisture (D) showed the largest absolute effect, followed by particle size (A), reinforcing the ANOVA results. This observation is consistent with previous reports indicating that substrate

particle size influences fungal penetration and aeration (Borkertas *et al.*, 2025), while moisture strongly governed cellulolytic activity in wheat straw-based fermentations (Pensupa *et al.*, 2013).

Overall, the statistical analysis demonstrates that endoglucanase production is primarily driven by moisture and particle size, with supplementary contributions from CMC and ammonium sulfate.

The significantly higher-order interactions highlight the necessity of integrated process optimization rather than relying on single-variable trials. These findings corroborate the broader consensus that endoglucanase yields in solid-state fermentation depend on a careful balance of substrate structure, carbon/nitrogen availability, and physicochemical conditions (Talebnia *et al.*, 2010).

3.2. Fungal growth and endoglucanase production as a function of time

Figure 2A shows the growth of the fungus on wheat straw, along with enzyme production and straw utilization. The growth of *A. oryzae* PTCC 5164 on wheat straw followed a typical microbial growth pattern, consisting of an initial lag phase, followed by an exponential growth phase, and finally a stationary phase. Initially, from day 0 to day 1, the protein concentration remains undetectable, indicating that the fungus is in the adaptation phase, adjusting to the wheat straw as a substrate. Between day 2 and day 5, the protein concentration increases significantly, reaching a peak of 2.34 mg/g straw on day 5, which corresponds to the exponential growth phase. During this period, the fungus actively assimilates the available substrate and increases its biomass. Figure 2B shows the visual appearance of the fungal growth on straw on days 1, 3, and 5.

Table 2. Analysis of variance for the experimental data presented in Table 1.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	16	23.8729	1.49206	79.03	0.002
1-Linear	4	18.6528	4.66321	247.01	0.000
Particle size	1	5.5991	5.59914	296.58	0.000
CMC	1	3.2409	3.24090	171.67	0.001
(NH ₄) ₂ SO ₄	1	0.5879	0.58791	31.14	0.011
Moisture	1	9.2249	9.22489	488.63	0.000
2-Way Interactions	6	1.7569	0.29282	15.51	0.023
Particle size*CMC	1	0.0714	0.07142	3.78	0.147
Particle size*(NH ₄) ₂ SO ₄	1	0.7187	0.71868	38.07	0.009
Particle size*Moisture	1	0.6541	0.65408	34.65	0.010
CMC*(NH ₄) ₂ SO ₄	1	0.0246	0.02457	1.30	0.337
CMC*Moisture	1	0.2379	0.23790	12.60	0.038
(NH ₄) ₂ SO ₄ *Moisture	1	0.0503	0.05029	2.66	0.201
3-Way Interactions	4	1.4839	0.37098	19.65	0.017
Particle size*CMC*(NH ₄) ₂ SO ₄	1	0.9385	0.93848	49.71	0.006
Particle size*CMC*Moisture	1	0.0589	0.05893	3.12	0.175
Particle size*(NH ₄) ₂ SO ₄ *Moisture	1	0.4055	0.40545	21.48	0.019
CMC*(NH ₄) ₂ SO ₄ *Moisture	1	0.0811	0.08108	4.29	0.130
4-Way Interactions	1	0.0395	0.03950	2.09	0.244
Particle size*CMC*(NH ₄) ₂ SO ₄ *Moisture	1	0.0395	0.03950	2.09	0.244
Curvature	1	1.9397	1.93971	102.74	0.002
Error	3	0.0566	0.01888		
Total	19	23.9296			

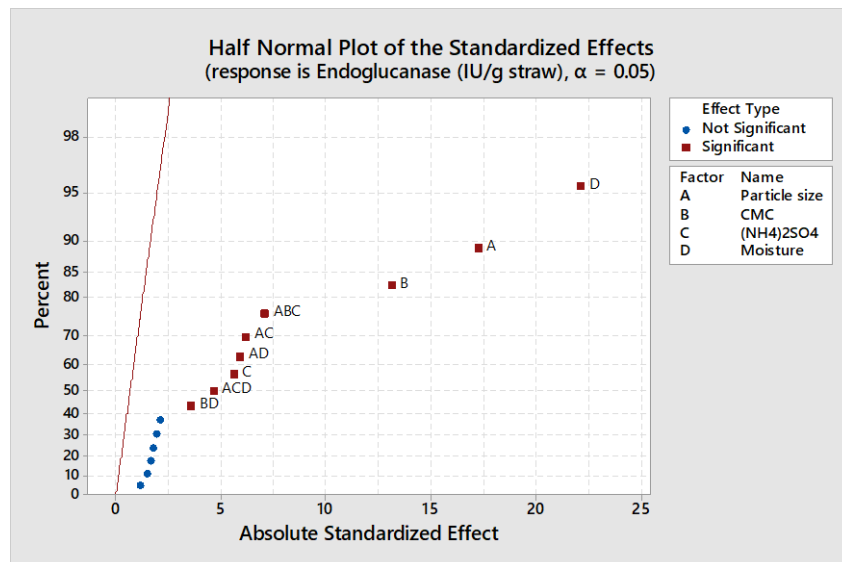
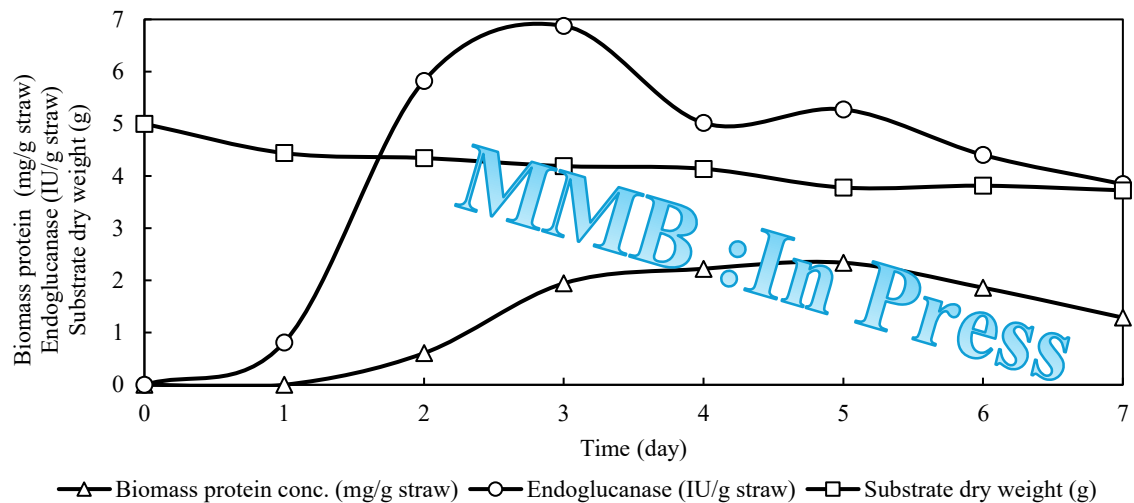


Figure 1. Normal probability plot of effects depicting the relative importance of the effective factors on endoglucanase production by *A. oryzae* PTCC 5164.

(A)



(B)

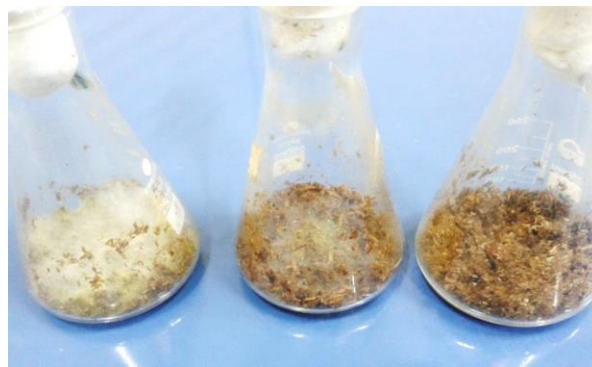


Figure 2. (A) Time course of *A. oryzae* PTCC 5164 growth, enzyme production, and straw utilization in solid-state fermentation. (B) *A. oryzae* PTCC 5164 growth on straw particles after 1, 3, and 5 days (right to left).

Endoglucanase production showed a similar trend to biomass accumulation, increasing rapidly from day 1 (0.813 IU/g straw) to a peak value of 6.877 IU/g straw on day 3. This indicates a direct correlation between the production of endoglucanase and biomass, suggesting that the fungus is producing more enzymes to break down the

cellulose in the wheat straw for growth. However, after day 3, endoglucanase production begins to decrease, possibly due to a reduction in substrate availability, enzyme production suppression due to the presence of simple sugars (a phenomenon known as carbon catabolite repression), and enzyme degradation. Carbon catabolite

repression is a well-documented regulatory mechanism in which the presence of easily metabolizable carbon sources, such as glucose, suppresses the expression of genes encoding plant cell wall-degrading enzymes, including cellulases (Wang *et al.*, 2024).

A. oryzae, the strain used in this study, is well documented as a co-producer of both cellulases and proteases, particularly under SSF conditions. Protease secretion is often upregulated during the later stages of fermentation, especially when the primary carbon source (cellulose) becomes limiting. Under such nutrient-depleted conditions, the fungus may initiate a metabolic shift toward the utilization of its own secreted proteins as a secondary nutrient source (Panchanawaporn *et al.*, 2025).

It is noteworthy that the maximum enzyme yield obtained in the kinetic experiment (6.877 IU/g) was approximately 43% lower than that observed under the same nominal conditions in the factorial design experiment (9.93 IU/g, Run 7). This discrepancy may be attributed to two factors. First, in the factorial experiment, all flasks were extracted immediately after 5 days of incubation, whereas in the kinetic study, flasks were removed daily and stored at 4 °C until day 7, when all samples were extracted together. This extended cold storage may have resulted in partial enzyme degradation prior to analysis. Second, the entire study was conducted over a period of approximately one year, and the kinetic experiment was performed a few months after the factorial design.

During this interval, the microbial culture was maintained through repeated serial transfers onto fresh agar slants, which may have led to gradual phenotypic degeneration or reduced metabolic capacity for enzyme production—a well-recognized phenomenon in long-term culture maintenance. Although the strain was not systematically characterized between the two experimental phases, the temporal gap remains a plausible contributor to the observed reduction in yield.

The substrate dry weight decreases over the course of the experiment, from 5 g at the start to 3.725 g by day 7, indicating that the fungus is consuming the wheat straw. This decrease in substrate is more rapid during the early growth stages (days 0–5) but slows down towards the end of the experiment, suggesting a reduction in available cellulose for degradation as the fungus approaches the stationary phase. To quantify the efficiency of biomass and

enzyme production, the biomass yield on substrate ($Y_{X/S}$) and enzyme yield on biomass ($Y_{E/X}$) were estimated over the period of active growth (days 2–5). The biomass yield on substrate ($Y_{X/S}$), which represents the amount of biomass produced per unit weight of substrate consumed, was found to be 9.58 mg biomass protein per gram of substrate at its peak value. This value indicates the efficiency with which *A. oryzae* PTCC 5164 converts the wheat straw into biomass protein. The enzyme yield on biomass ($Y_{E/X}$), which indicates the amount of endoglucanase produced per unit of biomass protein, had a peak value of 3.54 IU per milligram of biomass protein during the active growth period. These results confirm that the fungus not only produces biomass but also synthesizes a significant amount of endoglucanase per unit of its own mass.

The experimental data for straw degradation were fitted with a first-order kinetic model given by Equation (3):

$$\frac{S(t)}{S_0} = e^{-0.0484t} \quad (3)$$

where $S(t)$ represents the residual straw weight (g) at time t (day), S_0 is the initial straw weight, and the rate constant $k=0.0484 \text{ day}^{-1}$ quantifies the degradation rate. The goodness-of-fit and statistical significance of the model are summarized in Table 3. The coefficient of determination (R^2) was 96.54%, indicating that the first-order model adequately describes the temporal pattern of straw mass loss. Furthermore, the analysis of variance (ANOVA) revealed a highly significant regression effect ($F=195.41$, $p\text{-value}<0.001$), confirming that the degradation process is statistically significant over time.

The experimental data for biomass protein production were fitted to a second-order polynomial kinetic model given by Equation (4):

$$X(t) = 0.806t - 0.0829t^2 \quad (4)$$

where $X(t)$ represents the biomass protein (mg biomass protein/g straw) at time t (days). The analysis of variance (ANOVA) for the regression model is presented in Table 4. The regression model was highly significant ($F=39.50$, $p\text{-value}<0.001$), with both the linear (t) and quadratic (t^2) terms contributing significantly to the model ($p\text{-value}=0.003$ and $p\text{-value}=0.027$, respectively). The coefficient of determination (R^2) was 92.94%, indicating that the model adequately describes the experimental data over the time period investigated.

Table 3. Regression analysis for straw degradation as a function of time.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Regression	1	0.32768	0.327684	195.41	0.000
t(day)	1	0.32768	0.327684	195.41	0.000
Error	7	0.01174	0.001677		
$R^2 = 96.54\%$					

Table 4. Regression analysis for biomass protein production as a function of time.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Regression	2	18.283	9.1413	39.50	0.000
t(day)	1	5.553	5.5528	24.00	0.003
t(day)*t(day)	1	1.964	1.9644	8.49	0.027
Error	6	1.388	0.2314		
Total	8	19.671			
$R^2=92.94\%$					

The time-course profile of endoglucanase production was also modeled using a second-order polynomial given by Equation (5):

$$E(t) = 2.89t - 0.3466t^2 \quad (5)$$

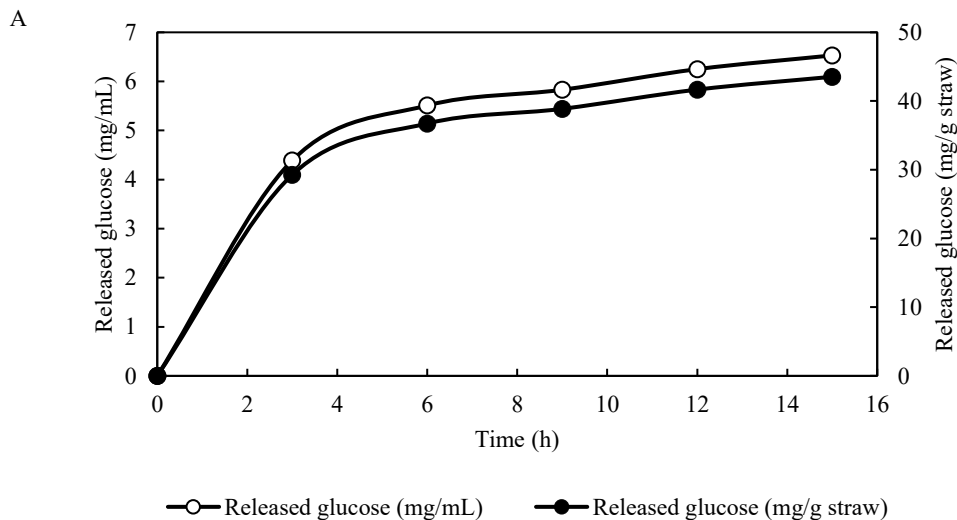
where $E(t)$ denotes endoglucanase activity (IU/g straw) at time t (days). Table 5 summarizes the ANOVA results for this regression model. The overall model fit was statistically robust ($F=55.83$, $p\text{-value}<0.001$), accounting for 94.9% of the total variance in the response ($R^2=94.9\%$). Examination of the individual regression coefficients revealed that both the primary (t) and secondary (t^2) kinetic terms exerted a statistically significant influence on endoglucanase accumulation, with p -values of less than 0.001 and 0.003, respectively. These statistical indicators confirm that the chosen quadratic function adequately captures the rising and subsequently declining trend in enzymatic activity observed over the incubation period.

3.3. Wheat straw hydrolysis using the produced enzyme

The enzymatic hydrolysis experiments were conducted to evaluate the performance of the produced enzyme on wheat straw. The time-course assay (Figure 4A) showed that glucose release increased rapidly during the first 6 h, reaching approximately 5.5 mg/mL, after which the rate of hydrolysis slowed considerably. A near-plateau was reached at ~6.5 mg/mL after 15 h, indicating that most of the easily accessible cellulose had been hydrolyzed within the early phase of the reaction. This behavior is typical of enzymatic saccharification processes, where rapid initial conversion is followed by a reduced rate due to decreased substrate accessibility and potential product inhibition (Alias *et al.*, 2021; Kristensen *et al.*, 2009). Figure 4B visually shows the disintegration of wheat straw after 15 h of incubation in a cell-free enzyme solution obtained from *A. oryzae* PTCC 5164.

Table 5. Regression analysis for endoglucanase production as a function of time.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Regression	2	160.410	80.205	55.83	0.000
t(d)	1	71.425	71.425	49.72	0.000
t(d)*t(d)	1	34.314	34.314	23.89	0.003
Error	6	8.620	1.437		
Total	8	169.029			
$R^2 = 94.9\%$					



B



Figure 4. (A) Glucose release from straw as a function of time in a mixture containing 2 mL enzyme solution at a concentration of 0.6 IU/mL and 0.3 g straw. Visual comparison of wheat straw hydrolysis in a cell-free solution by the cellulolytic enzyme from *A. oryzae* PTCC 5164, at 0 h (left) and after 15 h of incubation (right). (B) The clear disintegration and liquefaction of straw are evident after 15 h, indicating effective enzymatic degradation.

The kinetic profile of glucose release was evaluated by regression analysis, in which a second-order polynomial function was fitted to the experimental data, as described by Equation (6):

$$G(t) = 1.208t - 0.0537t^2 \quad (6)$$

where $G(t)$ represents glucose concentration (mg/L) at time (h). The corresponding ANOVA results are compiled in Table 6. The regression model demonstrated exceptional significance ($F=125.96$, $p\text{-value}<0.001$), with a coefficient of determination (R^2) of 98.44%, indicating that nearly all the variability in glucose release was accounted for by the fitted equation. Both the linear and quadratic terms were statistically significant predictors, yielding p -values of 0.001 and 0.010, respectively. These statistical results validate the adequacy of the quadratic model for describing the time-course pattern of glucose liberation under the specified hydrolysis conditions.

The influence of straw loading (w/v)% on hydrolysis efficiency is presented in Table 7. The enzymatic hydrolysis of wheat straw under the tested conditions yielded glucose ranging from 6.79 to 23.23 g per 100 g of straw, depending on the straw loading. These yields are modest compared to reports using extensive pretreatment or high enzyme loadings, which can exceed 70% conversion. However, the present experiments were conducted under mild conditions with relatively low enzyme loading, representing a baseline for further optimization rather than a final performance metric. The results demonstrate that even under these suboptimal conditions, enzymatic saccharification is feasible, with the highest yield (23.23%) achieved at the lowest solid loading (1.33% w/v). Future work will focus on optimizing enzyme loading, hydrolysis time, and pretreatment strategies to improve overall sugar yields. Statistical evaluation via one-way ANOVA is presented in Table 8. The results in the table revealed that the straw loading (w/v)% exerted a highly significant effect on glucose release ($F=1798.13$, $p\text{-value}=0.018$), with the treatment accounting for the vast majority of the observed variability. The negligible error variance (Adj MS = 0.00045) further underscores the precision of the measurements and the reliability of the observed treatment

effects. These findings illustrate that increasing substrate loading enhances glucose concentration, whereas excessive dilution of the enzyme system diminishes saccharification efficiency. This behavior is consistent with previously documented observations in lignocellulosic hydrolysis, wherein enzyme accessibility and substrate concentration are pivotal determinants of hydrolytic performance (Kristensen *et al.*, 2009).

4. Conclusion

This study demonstrates the potential of wheat straw as a sustainable substrate for endoglucanase production using *A. oryzae* PTCC 5164 under solid-state fermentation. Factorial design analysis revealed that moisture content and particle size are the primary factors influencing enzyme yield, while supplementary carbon (CMC) and nitrogen (ammonium sulfate) sources further enhance endoglucanase production through synergistic effects. The significantly higher-order interactions observed highlight the necessity of multifactorial optimization rather than single-variable approaches.

Kinetic analysis of fungal growth and enzyme production confirmed a strong correlation between biomass accumulation and endoglucanase synthesis, with maximum enzyme activity occurring during the late exponential growth phase. The estimated biomass and enzyme yield coefficients indicate efficient conversion of wheat straw into both fungal biomass and cellulolytic enzymes. Furthermore, enzymatic hydrolysis assays demonstrated that the produced cellulolytic enzymes effectively released glucose from wheat straw, with hydrolysis efficiency influenced by both substrate loading and enzyme concentration. Overall, the optimization performed here—establishing 65% moisture, 0.3 cm particle size, 0.08 g CMC/g straw, and 0.12 g ammonium sulfate/g straw as the optimal parameters, alongside a day-3 harvest window for peak enzyme activity—provides a practical guide for pilot-scale SSF operations. The promising saccharification results achieved (5.43 mg glucose/mL at 8% w/v loading) demonstrate that the enzyme preparation from *A. oryzae* PTCC 5164 is functionally suitable for bioethanol production.

Table 6. Regression analysis for glucose release from straw in a cell-free endoglucanase solution as a function of time.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Regression	2	162.740	81.3702	125.96	0.000
time (h)	1	43.163	43.1627	66.81	0.001
time (h)*time (h)	1	13.662	13.6622	21.15	0.010
Error	4	2.584	0.6460		
Total	6	165.324			

$R^2 = 98.44\%$

Table 7. Enzymatic wheat straw hydrolysis using an enzyme solution at a concentration of 0.5 IU/mL in 15 hours

Straw (g)	Enzyme solution (mL)	Straw-to-enzyme solution (w/v)%	Released glucose (mg/mL)	Released glucose (w/v)%	Yield (g glucose/100 g straw)
2	50	4	3.62	0.362	9.05
2	150	1.33	3.09	0.309	23.23
4	50	8	5.43	0.543	6.79
4	150	2.66	4.00	0.4	15.04
3	100	3	3.61	0.361	12.03
3	100	3	3.64	0.364	12.13

Conflict of interest

The authors declare no conflict of interest.

Acknowledgment

The authors would like to thank the laboratory staff in the Departments of Biology and Chemical Engineering for their valuable cooperation throughout this study.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Funding

None declared.

Authors' Contributions

Zohreh Noruzi Motlagh conducted all experimental work and collected the data. Mohammad Hassan Fazelipour conceived the research idea, supervised the study, and wrote the manuscript. Arastoo Badoei Dalfard provided some advice during the research. All authors read and approved the final version of the manuscript.

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How to cite this paper:



Noruzi Motlagh, Z., Fazaelpoor, M. H. & Badoei Dalfard, A. (2026). Optimization and Kinetic Studies on Endo-1,4-β-glucanase (endoglucanase) Production by *Aspergillus oryzae* PTCC 5164 Grown on Wheat Straw under Solid-State Fermentation. *Microbiology, Metabolites and Biotechnology*, 9(2), X-XX. DOI: [10.22104/mmb.2026.8195.1200](https://doi.org/10.22104/mmb.2026.8195.1200)