# Enhancement of Endoglucanase Activity through Incorporation of Physical, Chemical, and Combined Mutagenesis in Thermophilic Strain of *Aspergillus fumigatus*



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An indigenous *Aspergillus fumigatus* strain was subjected to physical, chemical, and combined mutagenesis to enhance endoglucanase production. Using rice straw as a greater carbon source, maximum activity (84.94 U $mL^{-1}$) was achieved via 10-minute UV exposure, while 150 μg $mL^{-1}$ ethyl methane sulphonate (EMS) yielded 62.89 U $mL^{-1}$. Combined mutagenesis (10 min UV+ 150 μg $mL^{-1}$ EMS) significantly improved activity to 123.32 U $mL^{-1}$, a 2.2-fold increase over the wild type. Optimization through response surface methodology (RSM) identified optimal conditions: pH 5.0, 55 °C, 5-day incubation, and 5 mL inoculum. Purified endoglucanase exhibited high affinity for CMC and was further activated by 300 mM $CaCl_2$ to 176.92 U $mL^{-1}$. Kinetic and characterization studies confirm that this thermophilic, genetically improved (physical, chemical, and combined mutagenesis) strain is highly suitable for industrial applications in textiles and biofuels.

*Keywords*

cellulase, bioenergy, rice straw, lignocellulosic biomass, response surface methodology, solid state fermentation

## Introduction

Growing environmental concerns and the rising costs of organic materials have led to increased exploration of alternative energy sources[1–3]. Biological conversion of lignocellulosic biomass, which is both abundant and renewable[4,5], represents one of the most promising alternatives in this regard. Agricultural byproducts like rice straw are key sources for biomass valorization[6,7]. *Aspergillus fumigatus*, a thermophilic fungus known for producing a variety of carbohydrate-active enzymes (CAZymes) during biomass degradation[8–11] has attracted considerable attention. Recent genomic research, including whole-genome sequencing and annotation of *Trametes sanguinea* ZHSJ, has provided insights into the genetics of lignocellulolytic enzymes[12]. To convert cellulose into fermentable sugars, a complex of three enzyme classes is required: endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21)[13–17]. Endoglucanases break down the β-1,4-glycosidic bonds in the less structured areas of the cellulose chain[8,18,19]. Their non-specific action makes endo-1,4-β-glucanases potential candidates for industrial scale[20]. Fungal strains often need genetic improvements to increase yield and thermal stability[21,22]. Techniques like random mutagenesis using ultraviolet (UV) radiation and chemicals such as ethyl methane sulfonate (EMS) have significantly increased enzymatic activity[23–26].

Generally, lignocellulose includes 35–50 % cellulose, 20–30 % hemicellulose, and 20–30 % lignin subunits[19,27,28]. Cellulase enzyme is utilized for the degradation of cellulose into fermentable sugars and is produced by bacteria and fungi[15,17,21,29]. Cellulase enzymes are of major industrial importance because they can degrade cellulose into simple sugars that can be further fermented into bioethanol and other value-added products[29,30]. Cellobiohydrolase is specific against reducing and non-reducing ends, while β-glucosidase releases glucose from cellobiose[13,16]. Endo-1,4-β-glucanases are prevalent and are categorized into 16 glycosyl hydrolase (GH) families[8,19]. Enzymes belonging to the glycosyl hydrolase family lack specificity and act on various substrates like cellulose, β-glucan, xylan, lichen, etc[18]. This lack of specificity makes endo-1,4-β-glucanases very advantageous for diverse industrial applications[20,29].

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The demand for endoglucanase is rapidly increasing due to second-generation advanced biofuel production, which requires substantial quantities of enzymes[30]. Consequently, there is a growing need to produce, purify, characterize, and screen novel enzymes possessing enhanced stability and specificity[2]. Biostoning represents one of the most promising applications of endoglucanase in the textile industry. In denim processing, these enzymes are used to provide fabric finishing and create a faded appearance by removing unevenly distributed indigo dye entrapped within the fiber. Treatment of denim with endoglucanase is more environmentally friendly than conventional stone washing. In addition, alkaline cellulases can also be utilized in various industries, e.g., textile, paper and pulp, food, detergent, and biofuel industries[31]. Various *Aspergillus* species have been identified as producers of endoglucanases with significant biotechnological potential[3,8,10]. However, enzymes derived from these strains often exhibit limitations such as low activity, reduced yield, or insufficient thermal stability[8,21,32]. Strain improvement is possible through random mutagenesis, and has proven effective in enhancing microbial productivity during fermentation[23,24,33]. Fungal strains often require genetic improvement to overcome limitations in yield and thermal stability[21,22]. Random mutagenesis via ultraviolet (UV) radiation and chemical agents like ethyl methanesulfonate (EMS) has proven effective in augmenting enzymatic activity multiple-fold[16,23–26]. Repeated or sequential UV treatment of *Aspergillus* species has been reported to increase cellulase yield up to 2.2 fold[22,34,35].

Similarly, EMS-induced mutagenesis of a soil fungal strain was reported to enhance the production of lipase with 365 % enhanced enzyme activity compared to its wild strain[36,37]. This study aims to optimize endoglucanase production from an indigenous *A. fumigatus* strain using rice straw and to investigate the synergistic effects of combined physical and chemical mutagenesis.

## Materials and methods

### Microorganism and biomass feedstocks

A pure culture of *A. fumigatus* was obtained from the Department of Biochemistry, University of Gujrat. Biomass feedstocks (rice straw, wheat straw, corn cobs, sugarcane bagasse, sawdust) were collected locally. Sawdust was obtained from the workshop of the School of Arts and Designing, University of Gujrat, and all were sieved (0.5 mm mesh). Substrate characterization showed that rice straw contained approximately 38 % cellulose and 26 % hemicellulose. Chemicals including ethyl methane sulphonate (Cat# M0880) and Congo Red (Cat# C6277) were purchased from Sigma-Aldrich (USA).

### Screening of lignocellulosic substrates

Initial screening of all substrates (rice straw, wheat straw, corn cobs, sugarcane bagasse, sawdust) was performed. *A. fumigatus* was cultured in a fermented medium containing 5 g of each substrate per flask and incubated for seven days. After fermentation, the culture was harvested and a clear supernatant was collected. The supernatant was subjected to enzyme activity measurement. The most promising substrate was selected for subsequent experiments.

### Cultivation and medium

Fungal cultivation was performed on potato dextrose agar (PDA) (potato infusion 200 g $L^{-1}$; glucose 20 g $L^{-1}$; agar 15 g $L^{-1}$). Spore suspensions were incubated in 90 mm Petri plates at 37 °C for 5 days. After solidification of media, streaking was performed with a sterilized loop. The plates were placed in an incubator at 37 °C for 5 days to obtain maximum growth. After growth, plates containing fungal colonies were preserved in a refrigerator at 4 °C.

### Inoculum preparation

The *A. fumigatus* strain was cultivated in liquid media by adding a loop full of fungal spores on potato dextrose agar (PDA) at 37 °C for 5 days. Spores were harvested using sterile 0.1 % Tween-80. The inoculum was standardized to a concentration of $1.0 \cdot 10^7$ spores $mL^{-1}$ using a Neubauer hemocytometer to ensure consistency across experimental runs.

### Solid-state fermentation (SSF)

SSF was conducted in individual 250-mL Erlenmeyer flasks containing 5 g of individual substrates. Moisture content was adjusted to 60 % (v/w) using sodium citrate buffer (pH 5.0). Each flask was diluted with 5 mL distilled water and sterilized at 121 °C for 15 min. Flasks were autoclaved at 121 °C for 15 min. After cooling, each flask was inoculated with the standardized spore suspension (varied by design) and incubated at temperatures ranging from 20–55 °C to determine the thermophilic potential of the strain.

### Enzyme extraction

The culture extract was filtered through dampened cheesecloth to remove residues of fungus and substrate. The pooled extracts were centrifuged at 8000 rpm, at 4 °C for 10 min. The pellet was dis-

Table 1 – *Coded and uncoded values of physicochemical parameters*

| Factor | Name | −α (−2) | Low (−1) | Center (0) | High (+1) | +α (+2) |
|---|---|---|---|---|---|---|
| A | pH | 2 | 3 | 5 | 8 | 10 |
| B | Incubation (days) | 1 | 3 | 5 | 7 | 9 |
| C | Temperature (°C) | 20 | 25 | 35 | 50 | 55 |
| D | Inoculum size (mL) | 1 | 2 | 4 | 5 | 8 |

carded and the clear supernatant was used as the source of extracellular enzyme for further experimentation[38].

## Experimental design and statistical analysis

To maximize endoglucanase production using solid-state fermentation (SSF), a four-factor central composite design (CCD) was used. This approach helped determine the effects of pH (ranging from 3.0 to 8.0), incubation period (1 to 9 days), temperature (20 to 55 °C), and inoculum size (1 to 8 mL), respectively, on enzyme yield, both independently and in combination. The CCD included 30 experimental runs containing 16 factorial points, 8 axial points (with $\alpha = 2$), and 6 center points to help estimate pure error. The generated coded and uncoded values are given in Table 1. All experiments were performed in triplicate to increase reliability. The flasks were kept at temperatures between 20 °C and 55 °C for the specified time (1 to 9 days). The relationship generated between the variables and endoglucanase activity used a second-order polynomial equation:

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

The terms are defined as follows: $Y$ (Predicted response): The final yield of endoglucanase activity (U mL$^{-1}$) expected under specific conditions. $\beta_0$ (Intercept): The baseline enzyme yield produced when all factors are set at their center levels. $\beta_i X_i$ (Linear terms): The direct impact of each individual factor; shows how much the yield increases or decreases as one variable changes. $\beta_{ii} X_i^2$ (Quadratic terms): The curvature in the data; identifies the peak (optimum) point where yield is highest before declining. $\beta_{ij} X_i X_j$ (Interaction terms): The synergy between factors; shows how the effect of one variable changes depending on another. ε (Residual error): The random variation in the experiment that cannot be explained by the model.

## Fermentation setup and buffer systems

The fermentation experiment was performed in triplicate with five sets of flasks. Five grams of rice straw were added to each 250-mL Erlenmeyer flask. To maintain the pH level generated through CCD, 5.0 mL of specified buffer was added to the substrate before sterilization: Sodium citrate buffer was used for experimental runs requiring pH 3.0 to 5.0, and sodium phosphate buffer was used for experimental runs requiring pH 5.5 to 8.0. The moisture content and initial pH were standardized and the flasks were autoclaved at 121 °C (15 psi) for 15 min.

## Inoculum preparation and inoculation

For the inoculum, the fungal strain was grown on PDA for approximately 120 hours to ensure actively growing cells. The inoculum volume was varied between 1 mL and 8 mL (representing $1 \cdot 10^7$ spores mL$^{-1}$) according to the CCD matrix.

## Spore suspension

The spore suspension was obtained by mixing pure spores of *A. fumigatus* with distilled water. The suspension was spread on different Petri plates already containing PDA medium. This spore suspension was further utilized for the incorporation of physical (UV), chemical (EMS), and combined (UV + EMS) mutation to enhance endoglucanase enzyme production and activity.

## Incorporation of mutagenesis

### *Physical mutagenesis*

For physical mutagenesis, 1 mL of the standardized spore suspension in a 90-mm Petri dish was exposed to UV irradiation at 254 nm. The Petri dish lid was removed during treatment, and a distance of 15 cm was maintained from the UV source[39]. UV treatment was carried out at time intervals of 10, 20, 25, and 30 min. It was determined that 30 min of exposure resulted in 100 % spore mortality, while 10 minutes yielded the optimal mutation frequency. During UV treatment, a distance of 15 cm was maintained between Petri plates and the UV lamp. After UV irradiation of the spore suspension, the mutant culture was spread onto four separate plates containing carboxymethyl cellulose (CMC)-based PDA media along with Congo Red dye for screening. The control plate was not subjected to physical mutation.

*Chemical mutagenesis*

Chemical mutagenesis was conducted by treating the fungal suspension with different concentrations of ethyl methane sulphonate (EMS) as mutagen. A stock solution of 300 μg $mL^{-1}$ was prepared and used to prepare dilutions (100, 150, 200, and 250 μg $mL^{-1}$). EMS solution and spore suspension were added into Eppendorf tubes. For chemical mutagenesis, the suspension was treated with EMS (100–250 μg $mL^{-1}$) in 0.1 M phosphate buffer (pH 7.0) at 37 °C for 24 hours. Combined mutagenesis utilized the optimal UV exposure (10 min) followed by the optimal EMS concentration (150 μg $mL^{-1}$). Finally, 100 μL of EMS-treated spore suspension was spread onto new plates containing CMC-based PDA media[40].

*Combined (physical and chemical) mutagenesis*

Combined mutagenesis utilized the optimal UV exposure (10 min) followed by the optimal EMS concentration (150 μg $mL^{-1}$). One hundred microliters of culture treated with both physical and chemical mutagenesis was spread onto separate Petri plates containing CMC-based PDA media.

*Screening mutant colonies of A. fumigatus*

The mutant colonies were further screened by the Congo Red method[39]. For this purpose, spore suspensions treated with combined mutagenesis were spread onto separate Petri plates containing PDA media along with 1 % CMC as substrate and 0.1 % Congo Red dye. The control plate contained spore suspension which was not exposed to mutagenesis. The plates were sealed with parafilm and incubated at 37 °C for 5 days. The colonies showing clear zones of substrate hydrolysis were selected and further subjected to fermentation for endoglucanase production. For this purpose, fresh growth media containing CMC as substrate was inoculated with mutant colonies. After inoculation, the flasks were placed in an orbital shaker (150 rpm) and incubated at 37 °C for 5 days. Subsequently, an enzyme assay was conducted to observe endoglucanase activity in cultures inoculated with different mutant colonies. Accordingly, the culture showing maximum endoglucanase production was screened and used for further experimentation.

*Enzyme assay*

The activity of endoglucanase was determined[41]. For this purpose, 200 μL of diluted enzyme were added to 1.8 mL of CMC (1 %) and the mixture was incubated at 50 °C for one hour. To stop the reaction, 3 mL of dinitrosalicylic acid (DNS) reagent was added to the mixture and boiled vigorously for 5 min, followed by cooling and the addition of 15 mL of distilled water. The release of reducing sugar was estimated by the DNS method and absorbance was noted at 540 nm using a UV/Visible spectrophotometer[42]. One unit of enzyme activity was defined as the amount of enzyme required to release one micromole of reducing sugar per minute. Protein concentration was estimated following the method of Bradford[43].

*Enzyme purification*

The endoglucanase was initially purified using ammonium sulfate precipitation. The crude enzyme was centrifuged at 8000 rpm at 4 °C for 15 min. The supernatants were further subjected to different concentrations (10–90 %) of ammonium sulfate for salting out the enzyme. The debris was dialyzed overnight using a laboratory-grade dialysis membrane by precipitating into sodium citrate buffer (pH 5). The dialysate was collected and stored until further use.

*Gel filtration chromatography*

The endoglucanase active fractions were further purified using gel filtration chromatography by dissolution in 100 mL of sodium citrate buffer (pH 5) and loaded on a Sephadex-G100 (25 × 2 cm) column. The enzyme was eluted using citrate buffer at an elution rate of 0.5 mL $min^{-1}$, and 1 mL of 30 fractions were collected from the column for measurement of endoglucanase activity.

## Characterization of endoglucanase

*Effect of temperature*

The activity of purified endoglucanase was observed at different temperatures (20, 25, 35, 50, 55, 60, 70, 75, and 80 °C). The temperature profile and thermal stability of the enzyme were determined by incubating native and mutant enzymes at different temperature ranges for 3 hours, followed by CMCase assay to measure residual activity[44].

*Effect of pH*

For the determination of optimum pH, enzyme activity was measured under standard conditions at different pH values (5, 6, 7, and 8) in a reaction mixture containing phosphate buffer (pH 5–8)[45].

*Effect of metal ions*

The effects of $Mg^{2+}$, $Zn^{2+}$, and $Ca^{2+}$ as metal ions on endoglucanase activity were studied. For this purpose, different concentrations of metal ions (100, 150, 200, 250, 300, 350, and 400 mM) were prepared and added to the standard reaction mixture. Absorbance was noted to measure enzyme activity after treatment with various activators and inhibitors[45].

## Results and discussion

### Vegetative growth of *A. fumigatus*

The pure culture of *A. fumigatus* was grown on PDA slants containing nutritional medium at 37 °C. The colonies of *A. fumigatus* were observed after 5 days of incubation, and plates were refrigerated at 4 °C until further use.

### Screening of lignocellulosic substrates

*A. fumigatus* was cultured in a fermented medium containing different substrates like rice straw, wheat straw, sugarcane bagasse, sawdust, and corn cobs. After seven days of incubation, fermentation flasks were harvested and a clear supernatant was collected. The supernatant was subjected to enzyme activity measurement. The maximum activity of endoglucanase was observed (9.74 U $mL^{-1}$) when rice straw was used as substrate (Fig. 1).

Rice straw fibers were used as substrate for enzyme production through microorganisms. RSM analysis with Minitab v21.2 showed that both temperature and inoculum size had a significant impact, with p-values below 0.05. The second-order polynomial model fit well with an $R^2$ value of 0.9703. With the combined mutagenesis approach (10 minutes of UV exposure plus 150 μg $mL^{-1}$ EMS) a yield of 123.32 U $mL^{-1}$ was achieved, a notable increase compared to the native strain's activity of 49.75 U $mL^{-1}$. *Aspergillus glaucus* has been reported to hydrolyze cellulase with pretreated rice straw as substrate at 55 °C and pH 5.0 [46]. Endoglucanase was produced using rice straw as a carbon source and the results showed that it was a suitable low-cost material[47]. One other study showed a striking increase in saccharification yield after endoglucanase treatment of three lignocellulosic substrates including rice straw[16]. Their experiment validates our results on rice straw as the better cellulose substrate.

### Response surface methodology

The parameters of inoculum size, temperature, pH, and incubation days were optimized through response surface methodology using Minitab® 21.2 (64-bit). The fitted model was determined through ANOVA (Table 2) and regression table. The 3D diagrams were generated through Design-Expert® version 13.

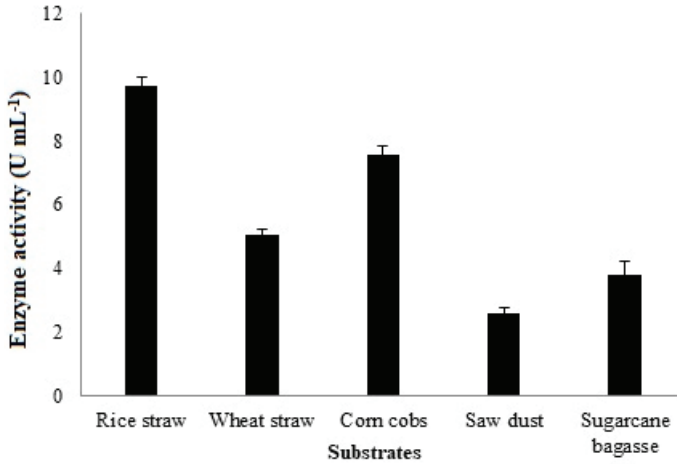


Fig. 1 – *Endoglucanase activity using different lignocellulosic substrates. The maximum activity (9.74 U $mL^{-1}$) was observed using rice straw as substrate.*

### Regression equation in uncoded units

Enzyme activity (U $mL^{-1}$) = –34.8 + 19.22 pH + 1.97 Incubation (days)
– 0.833 Temperature (°C) + 11.73 Inoculum (mL)
– 1.640 pH × pH
– 0.457 Incubation × Incubation
+ 0.01815 Temperature × Temperature
– 1.618 Inoculum × Inoculum
+ 0.387 pH × Incubation
+ 0.0041 pH × Temperature
– 0.374 pH × Inoculum
– 0.0522 Incubation × Temperature
+ 0.564 Incubation × Inoculum
– 0.0027 Temperature × Inoculum

Table 2 – *Analysis of Variance (ANOVA)*

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|
| Model | 14 | 2508.81 | 179.20 | 34.96 | 0.000 |
| Linear | 4 | 452.98 | 113.25 | 22.09 | 0.000 |
| pH | 1 | 0.27 | 0.27 | 0.05 | 0.823 |
| Incubation period (days) | 1 | 4.50 | 4.50 | 0.88 | 0.363 |
| Temperature (°C) | 1 | 164.70 | 164.70 | 32.13 | 0.000 |
| Inoculum size (mL) | 1 | 245.71 | 245.71 | 47.94 | 0.000 |
| Square | 4 | 1886.68 | 471.67 | 92.02 | 0.000 |
| pH × pH | 1 | 1349.24 | 1349.24 | 263.24 | 0.000 |
| Incubation × Incubation | 1 | 92.41 | 92.41 | 18.03 | 0.001 |
| Temperature × Temperature | 1 | 81.54 | 81.54 | 15.91 | 0.001 |
| Inoculum × Inoculum | 1 | 787.94 | 787.94 | 153.73 | 0.000 |
| 2-Way Interaction | 6 | 150.83 | 25.14 | 4.90 | 0.006 |
| pH × Incubation | 1 | 53.31 | 53.31 | 10.40 | 0.006 |
| pH × Temperature | 1 | 0.28 | 0.28 | 0.05 | 0.819 |
| pH × Inoculum | 1 | 31.94 | 31.94 | 6.23 | 0.025 |
| Incubation × Temperature | 1 | 25.71 | 25.71 | 5.02 | 0.041 |
| Incubation × Inoculum | 1 | 43.07 | 43.07 | 8.40 | 0.011 |
| Temperature × Inoculum | 1 | 0.05 | 0.05 | 0.01 | 0.925 |
| Error | 15 | 76.88 | 5.13 | | |
| Lack-of-Fit | 12 | 76.88 | 6.41 | 5229.94 | 0.000 |
| Pure Error | 3 | 0.00 | 0.00 | | |
| Total | 29 | 2585.70 | | | |

$R^2$ = 97.03 %  $R^2$(adj) = 94.26 %

The response surface methodology (RSM) model demonstrated a strong ability to explain outcomes, with an $R^2$ of 97.03 %, F-value of 34.96, and p-value < 0.001, when calculating endoglucanase activity (U mL$^{-1}$). ANOVA results indicated that enzyme production was mainly influenced by quadratic effects, as opposed to just simple linear trends, reflecting an F-value of 92.02 and p-value < 0.001. While temperature ($F = 32.13$, $p < 0.001$) and inoculum size ($F = 47.94$, $p < 0.001$) arose as significant positive linear effects, pH and incubation period exhibited non-significant linear effects ($p > 0.05$) but showed strong quadratic effects—particularly pH ($F = 263.24$, $p < 0.001$), which functioned as a sharp threshold variable with sharp activity declines beyond its optimum, and inoculum size ($F = 153.73$, $p < 0.001$), confirming "overcrowding" inhibition at extreme densities. Significant two-way interactions between pH and incubation period ($p = 0.006$), pH and inoculum size ($p = 0.025$), incubation period and temperature ($p = 0.041$), and incubation period and inoculum size ($p = 0.011$) indicated conditional process dependencies, though the temperature vs. inoculum interaction was non-significant ($p = 0.925$), suggesting additive rather than synergistic effects. The significant lack-of-fit ($F = 5229.94$, $p < 0.001$) indicates modeled higher-order interactions despite high predictive accuracy, though the model successfully identifies that maximum enzyme yields (~49.75 U mL$^{-1}$) require precise thermophilic conditions (~50 °C), optimal biomass loading (5–6 mL), and stringent pH control, with temporal optimization being context-dependent on these physiological parameters.

## EG activity between pH and incubation period

Response surface methodology analysis (Fig. 2a) of the pH and incubation period interaction revealed a quadratic relationship with enzyme activity ranging from 11.19 to 49.75 U mL$^{-1}$, where endoglucanase production increased with rising pH from 3.0 to an optimum in the slightly acidic to neutral range (pH 5.5–6.5) before declining toward alkaline conditions, while temporally, yields improved significantly from 3 to 5 days before plateauing or

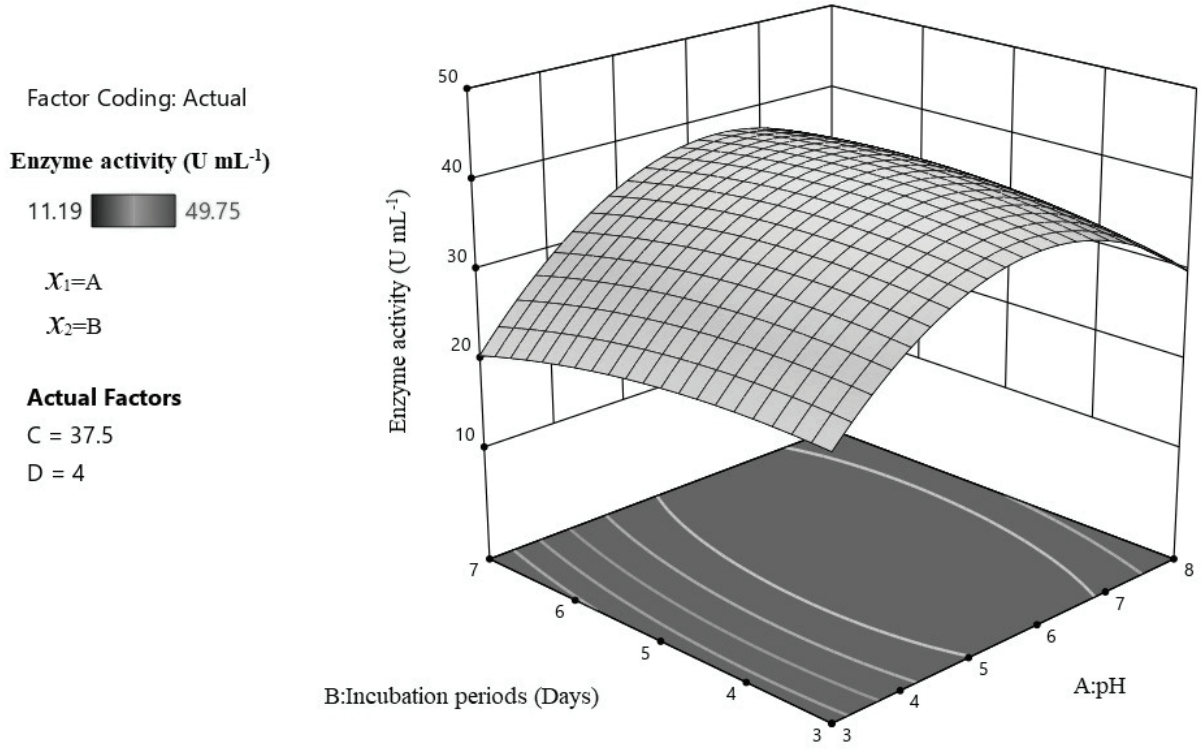


Fig. 2a – *3D Surface plot of EG activity between pH and incubation period (days)*

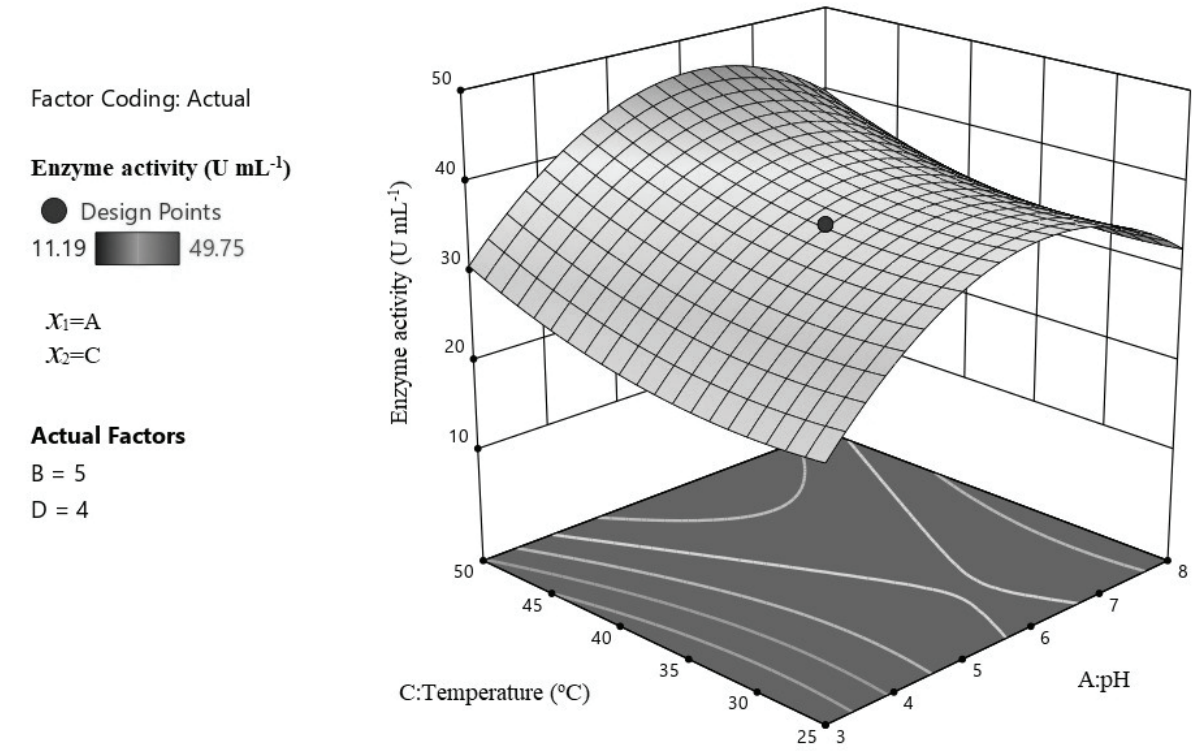


Fig. 2b – *3D Surface plot of EG activity between pH and temperature (°C)*

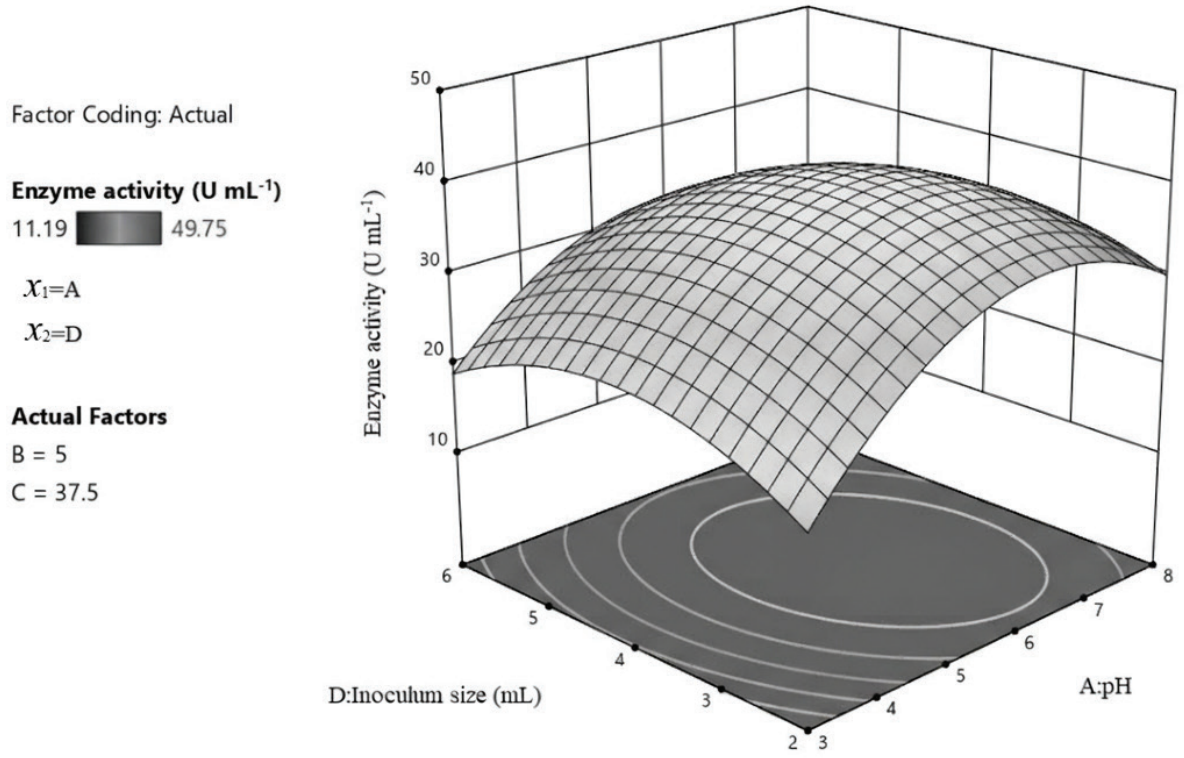


Fig. 2c – *3D Surface plot of EG activity between inoculum size (mL) and pH*

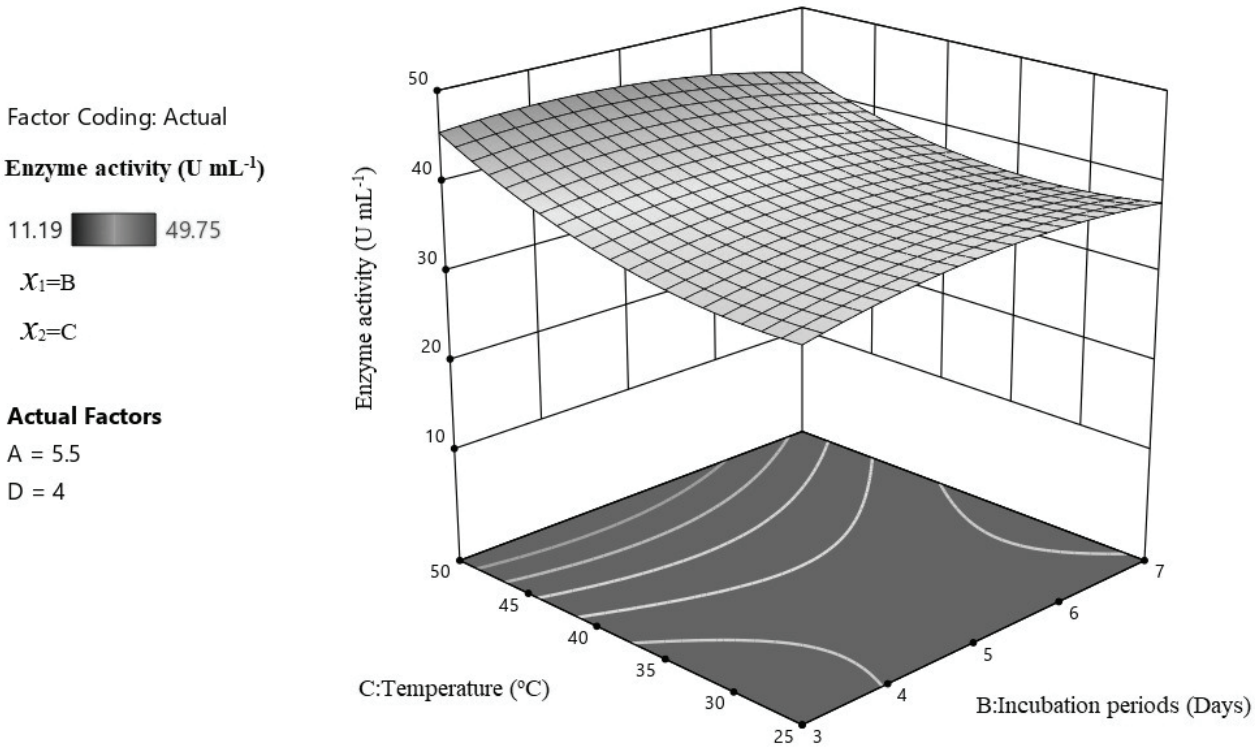


Fig. 2d – *3D Surface plot of EG activity between temperature and incubation period (days)*

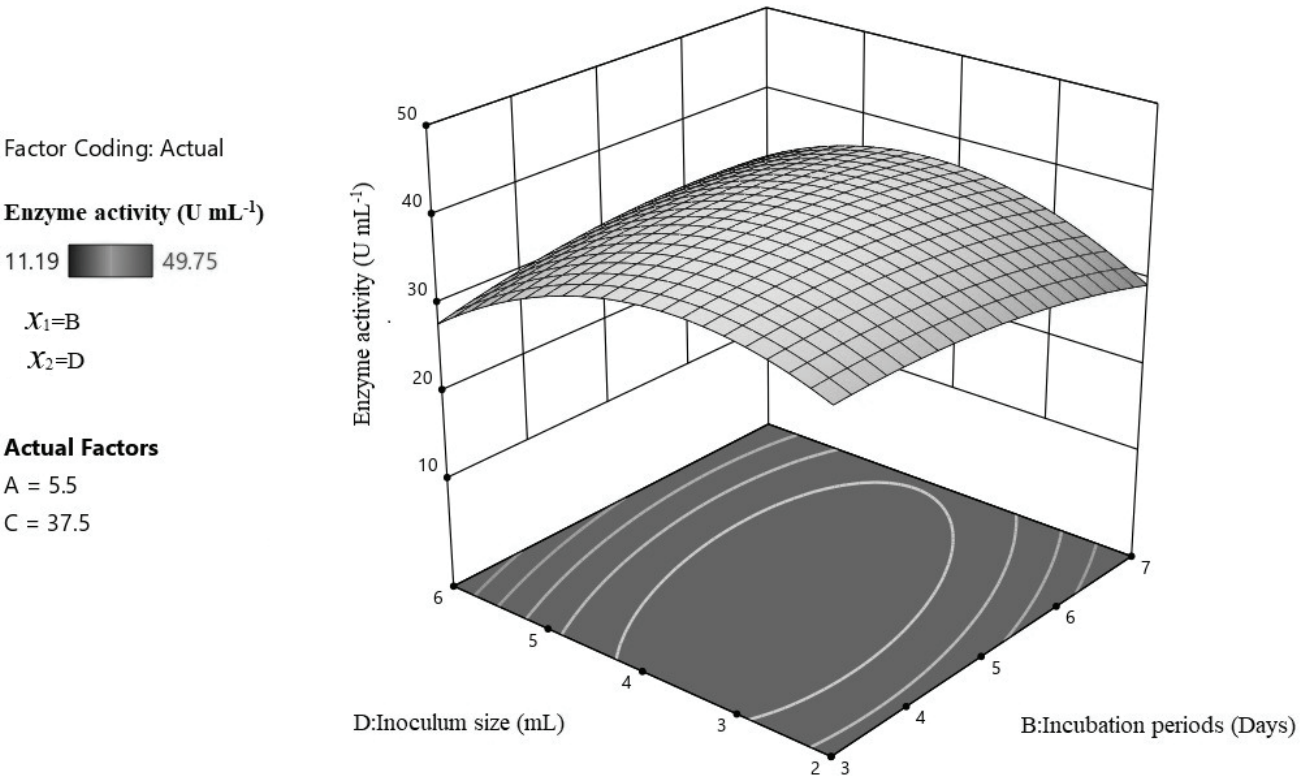


Fig. 2e – *3D Surface plot of EG activity between inoculum size and incubation period (days)*

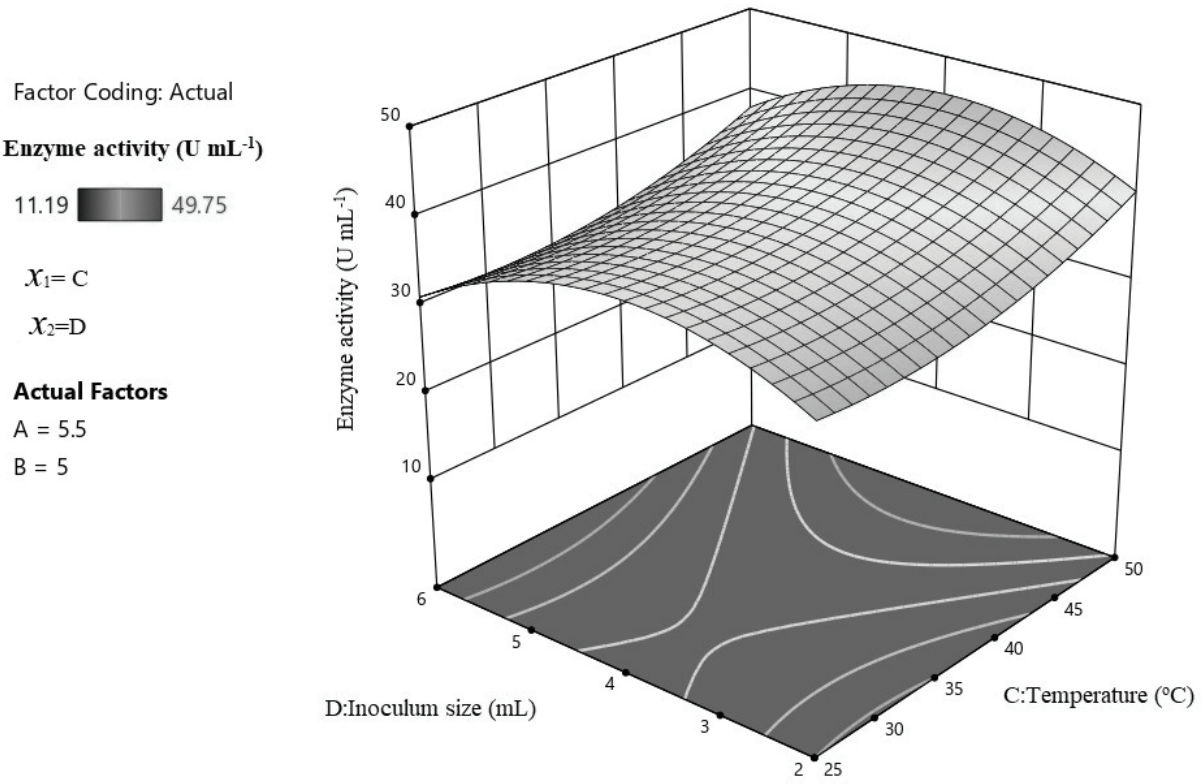


Fig. 2f – *3D Surface plot of EG activity between inoculum size and temperature (°C)*

decreasing slightly beyond 6 days. The elliptical contour plot shape indicates significant mutual interaction between these variables, with maximal activity (>45 U $mL^{-1}$) predicted at the synergistic combination of pH 6.0 and 5.5 days incubation. The observed optimum pH of 5.0 relates to other reports. *Bacillus amyloliquefaciens* was also found to give maximum yield of cellulase enzyme at pH 5.0 and demonstrated activity of 14.04 U $mL^{-1}$ via RSM[48]. Similarly, *Thermobifida fusca* strain showed optimum endoglucanase activity at pH 5 [49]. Response surface analysis revealed significant mutual interactions between pH and incubation period, with 3D surface plot indicating that endoglucanase production depends on the synergistic optimization of both variables[50]. The study identified optimal conditions at pH 6.0 and 5.5 days' incubation, yielding maximum activity beyond 45 U $mL^{-1}$ – an increase of 4.4-fold from minimal production levels[51]. Both factors exhibited quadratic relationships, with enzyme activity peaking in the slightly acidic range (pH 5.5–6.5) and plateauing beyond 6 days due to metabolic shifts and potential proteolytic degradation[52]. The strong narrow optimal region suggests that industrial processes require strict pH and temporal control to avoid low yield[51]. These findings confirm RSM as advantageous over traditional one-factor-at-a-time (OFAT) approaches which fail to detect interaction effects[50].

### EG activity between pH and temperature (°C)

The endoglucanase showed typical bell-shaped activity 3D curves (Fig. 2b) regarding both pH and temperature, peaking at pH 5.5 and between 40 and 45 °C, which aligns with mesophilic microbes. Activity drops due to suboptimal pH can interact with the ionization of active-site amino acids, disrupting substrate binding and catalysis, or high temperatures can destabilize the enzyme's structure by breaking hydrogen bonds and hydrophobic interactions. These interactions are crucial for industrial applications like breaking down lignocellulosic biomass efficiently. RSM effectively modeled the interaction between pH and temperature, showing that temperature's effect on endoglucanase production largely depends on the pH level ($p < 0.05$), and the reverse is also true[53]. The dome-shaped response surface indicates a clear quadratic relationship, with enzyme activity ranging from 11.19 U $mL^{-1}$ up to a predicted maximum of 49.75 U $mL^{-1}$, representing another 4.4-fold increase[54]. Endoglucanase works best at pH 5.5 and temperatures between 40 and 45 °C, typical for cellulolytic organisms like *Trichoderma*, where keeping the active-site carboxyl groups protonated also helps speed up reactions[55]. Dropping activity at extreme pH levels shows that ionic bonding and substrate affinity are disrupted[56], and thermal denaturation above 45 °C compromises structural integrity[54]. The interactions we found suggest enzymes have a broader pH tolerance at moderate temperatures (35–40 °C) but show sensitivity to acid-base changes under thermal stress[53]. For industrial lignocellulosic saccharification, working at pH 6.0 and 40 °C showed highest activity (~38 U $mL^{-1}$), and slightly higher yields at pH 5.5 with temperatures around 42–45 °C [55,56].

### EG activity between inoculum size (mL) and pH

Response surface analysis 3D graph (Fig. 2c) showed significant interactions between inoculum size and pH, with the model predicting highest endoglucanase activity of 49.75 U $mL^{-1}$ under the ideal conditions of pH 5.5–6.0, 35–40 °C, and an inoculum size of 4.0–5.0 mL. The oval contour plots confirmed that there is a meaningful dependency ($p < 0.05$) between these factors. It shows that achieving maximum enzyme yields relies on finding a balance rather than optimizing each factor in isolation[50]. The interaction between pH and temperature displayed the typical 3D curves seen in mesophilic biocatalysts[54]. The enzyme's activity peaked at pH 5.5 but fell sharply below 5.0 and above 6.5 due to changes in the ionization states of active-site amino acids, which disrupted substrate binding[55]. Extreme pH values can also lead to irreversible denaturation by breaking the ionic bonds that keep the enzyme structured[56]. These important interactions are necessary to keep catalytic efficiency up during industrial work. The findings regarding pH and inoculum size also show how critical they are for productivity. Using an inoculum size of 4.0–5.0 mL led to higher yields by shortening lag phases and speeding up biomass growth, while going above 5.0 mL caused activity to drop due to early nutrient depletion, oxygen limits, and the buildup of harmful by-products[51]. The pH trend (optimal range 5.5–6.5) combined with the inoculum size effect indicated the best metabolic efficiency when the active site's protonation was kept optimal alongside moderate cell densities to avoid overstressing the system[56]. Bezerra[50] and Singhania[51] performed RSM and found that the best endoglucanase production for industrial biomass saccharification relies on slightly acidic pH levels, mild temperatures, and a moderate amount of inoculum. This balance is essential to avoid the negative impacts of pH changes, thermal degradation, and stress from too many cells.

### EG activity between temperature and incubation period

RSM 3D graph (Fig. 2d) highlighted a clear optimal range for producing extracellular enzymes at the intersection of thermophilic temperatures (~50 °C) and shorter incubation times (3–4 days)

reaching a predicted maximum activity of 49.75 U mL$^{-1}$. The elliptical 3D and contour lines reflect a significant interaction between temperature and incubation time ($p < 0.05$), with the optimal incubation length strictly depending on temperature conditions[57]. The optimum at 50 °C matches the characteristics of thermophilic or thermotolerant enzymes according to Shah[58], while a drop in activity over longer incubation times highlights the thermal inactivation and degradation phases noted by Udayan[59] and Ameri[57].

### EG activity between inoculum size and incubation period

The RSM 3D Fig. 2e showed a strong interaction between inoculum size and incubation period, with the highest endoglucanase activity of 49.75 U mL$^{-1}$ and inoculum size of 3.5 mL were found to be optimum for maximum endoglucanase activity. The highest activity level at 3.5 mL formed the shift from active growth to a stationary phase, after which a decline was observed. This decline was due to nutrient depletion, the build-up of inhibitory by-products, and possible breakdown of the target enzyme if growth continued too long. The oval 3D contour plots showed significant interaction effects ($p < 0.05$), indicating that higher inoculum levels reach their highest activity faster than lower densities. This showed a complex relationship between biomass buildup and metabolic trade-offs[61]. At first, a positive correlation was observed where higher inoculum density led to better enzyme yield (up to 5 mL). This effect is likely due to more biomass being formed faster, shortening the lag times and facilitating rapid transition into exponential growth and enzyme production[61]. These findings indicate that one-factor-at-a-time approaches are insufficient to address variable interdependencies, thereby necessitating the use of response surface methodology (RSM)[62,63]. This observation is consistent with the findings of Assega[62] who reported that optimization using central composite design (CCD) increased enzyme activity 3.1-fold through various modifications in inoculum concentration. Aslam[63] also demonstrated that the interaction between inoculum size and incubation period plays a critical role in increasing production efficiency, regardless of adjustments to individual timing. Collectively, these findings are valuable in establishing operational limits for industrial bioprocessing. It is crucial to avoid both under-inoculation, which drags out lag phases and cuts down on productivity, and over-inoculation, which leads to early nutrient competition. This balance is essential for producing enzymes in a cost-effective and pilot-scale manner.

### EG activity between inoculum size and temperature

After applying RSM, the 3D graph (Fig. 2f) showed significant interaction between inoculum size and temperature. An inoculum size of 4.0–5.0 mL at 50 °C yielded the highest activity. The oval-shaped 3D and contour plots showed mutual dependency ($p < 0.05$). The optimal activity at these higher temperatures suggests boosted catalytic efficiency and faster kinetic response, consistent with reports on thermotolerant *Bacillus* species[58]. Conversely, the reduced activity observed at 25 °C reflects decreased metabolism and enzyme turnover under suboptimal conditions. The thermal optimum observed at 40–45 °C reflects a balance between increased molecular collisions at moderate temperatures and thermal instability beyond 45 °C when hydrogen bonds and hydrophobic interactions start to fail[53]. The optimal incubation period of 3–4 days followed by a decline at continued cultivation (7 days) corresponds with classic fermentation kinetics, where further growth leads to nutrient depletion and increases in harmful by-products, particularly under heat stress[59]. Previous studies have also reported that an inoculum size of 4.0–5.0 mL led to higher yields by shortening lag phases and exponential biomass production, while exceeding 5.0 mL caused a decline in activity due to early nutrient depletion, oxygen limits, and the buildup of harmful by-products[51]. The key interactions shown in the 3D oval contour confirm that RSM is essential compared to traditional single-variable approaches as the effect of incubation time is strongly dependent on the prevailing thermal conditions[57,60].

### Physical mutagenesis UV treatment

Among UV irradiation durations of 10, 20, 25, and 30 min, the 10-min exposure produced maximum endoglucanase with an activity of 84.94 U mL$^{-1}$ (Fig. 3) compared to the untreated control (49.46 U mL$^{-1}$). Spore suspensions exposed to UV for 25 min showed decreased microbial growth due to 80 % spore damage. Similarly, spore suspension after 30 min of continuous UV exposure showed no microbial growth due to 100 % spore damage. Enzyme activities following 10- and 20-min treatments exceeded the control value (49.46 U mL$^{-1}$), indicating successful incorporation of positive mutagenesis in *A. fumigatus* up to 20 min of continuous UV exposure. However, 25- and 30-min treatments resulted in enzyme activities below the control (49.46 U mL$^{-1}$), indicating incorporation of negative mutagenesis leading to decrease in enzyme production due to maximum damage of fungal spores.

At the optimal UV exposure time of 10 min, endoglucanase activity reached a maximum of

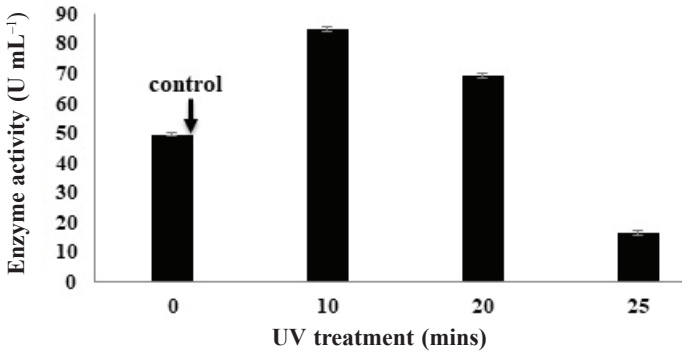


Fig. 3 – *Comparison of endoglucanase (EG) activity after UV exposure. The maximum enzyme activity (84.94 U mL$^{-1}$) was observed at 10 min UV exposure as compared to native (49.46 U mL$^{-1}$).*

84.94 U mL$^{-1}$. These findings are consistent with reports that *Aspergillus niger* exhibited optimum cellulase activity of 322 μmol mL$^{-1}$ min$^{-1}$ following 10 min of UV irradiation compared to a control value of 94 μmol mL$^{-1}$ min$^{-1}$ [35]. During incorporation of mutagenesis in *Aspergillus flavus*, it was also found that physical mutagenesis is efficient in enriching fungal cellulase activity in the presence of UV as mutagenic agent[64]. Our findings are comparable with other research on mutagenesis. Gene editing of *A. fumigatus* LMB-35Aa with CRISPR/Cas9 resulted in a 40 % increase in endoglucanase activity, albeit by an alternative strategy[65]. The positive rates of enhancement obtained by our standard mutagenesis methodology proved similar to those previously obtained with classical mutation systems, making it clear that classical mutagenesis remains significant.

## Chemical mutagenesis

Treatment with ethyl methyl sulfonate (EMS) at 100 μg mL$^{-1}$ concentration did not alter fungal morphology. However, spore suspension treated with 150 μg mL$^{-1}$ EMS concentration showed significant morphological changes in growth pattern and exhibited maximum enzyme activity (Fig. 4) among all treatments. The spore suspension treated with 200 and 250 μg mL$^{-1}$ EMS concentration showed decreased fungal growth due to maximum killing of spores, as these concentrations were found toxic to normal growth. EMS treatments at 100 and 150 μg mL$^{-1}$ concentrations exhibited 55.06 U mL$^{-1}$ and 62.89 U mL$^{-1}$ endoglucanase activities, respectively, showing incorporation of positive mutagenesis as these activities both exceeded the untreated control (49.46 U mL$^{-1}$). However, EMS treatments of 200 and 250 μg mL$^{-1}$ concentrations exhibited 37.71 and 19.03 U mL$^{-1}$ endoglucanase activities, respectively, showing incorporation of negative mutagenesis compared to the control (49.46 U mL$^{-1}$). Our strategy of using EMS concentration of 150 μg mL$^{-1}$ successfully enhanced enzyme activity (62.89 U mL$^{-1}$) compared to the control. This concentration was most feasible since the higher concentrations (200–250 μg mL$^{-1}$) caused reduction in enzyme activity because they were too toxic to the cells. In another research, *A. niger* treatment with 150 μg mL$^{-1}$ of EMS yielded best outcomes, exhibiting 390 μmol mL$^{-1}$ min$^{-1}$ enzyme activity, which is in agreement with our findings[35]. Higher EMS concentrations have been repeatedly found to possess cytotoxic effects, and hence an optimization of mutagen dosage is required. Combined mutagenic treatments using chemical mutagenesis resulted in an increase in cellulase activity compared to wild-type *Aspergillus flavus*[64]. These findings are in accordance with our observation that when used at an optimized concentration, chemical mutagens can significantly increase the production levels and activities of microbial enzymes.

## Combined mutagenesis

UV exposure of 10 min and EMS treatment of 150 μg mL$^{-1}$ concentration maximally enhanced endoglucanase activity during incorporation of physical and chemical mutagenesis, respectively. Therefore, these optimized conditions of both physical and chemical mutagenesis were followed to incorporate combined mutation into the thermophilic strain of *A. fumigatus*. For this purpose, spores were exposed to UV for 10 min followed by their chemical treatment with 150 μg mL$^{-1}$ EMS concentration. The combined treatment of physical and chemical mutagenesis showed dense growth of fungal spores as well as dense mycelium growth during submerged fermentation. Interestingly, the strategy of combined mutation yielded promising results towards enhancement of endoglucanase activity of

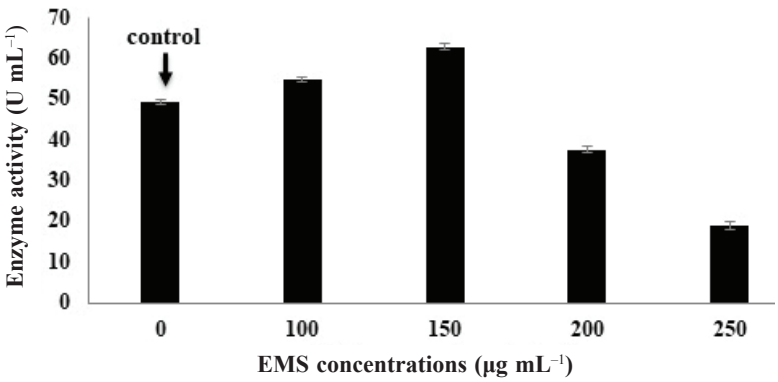


Fig. 4 – *Comparison of EG activity after chemical mutagenesis (EMS treatment). The maximum activity of EG (62.89 U mL$^{-1}$) from A. fumigatus was found at 150 μg mL$^{-1}$ EMS concentration.*

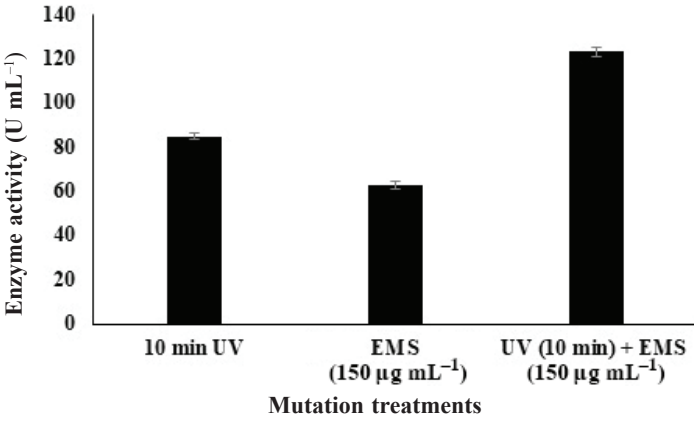


Fig. 5 – *Comparison of EG activity after physical, chemical, and combined (both physical and chemical) mutagenesis*

123.32 U $mL^{-1}$ compared to the previous enzyme activities of individual UV and EMS treatments (Fig. 5). The enhanced endoglucanase production achieved through combined mutagenesis (123.32 U $mL^{-1}$) places this enzyme as an important source for many industrial applications. Recent innovations in cellulase technology have placed relevance on economical methods of production. Even an increase of 40 % in enzyme activity can produce significant biotechnological outcomes[65]. In accordance with our strategy to induce mutation in fungi, literature has also reported enhancement of endoglucanase activity from fungus *Trichoderma citrinoviride* through incorporation of chemical mutagenesis using ethidium bromide and EMS[66]. Research on *Talaromyces pinophilus* has also demonstrated the increase in the yield of cellulase enzyme as a result of combined mutagenesis[67].

## Partial purification of endoglucanase

### *Ammonium sulfate precipitation*

Crude endoglucanase was precipitated with different saturations of ammonium sulfate followed by dialysis. The results showed that the maximum amount of native enzyme was precipitated at 30 % saturation of ammonium sulfate with a protein content of 312.72 mg, while mutant endoglucanase was precipitated at 20 % saturation with a protein content of 557.84 mg. Our purification strategy utilizing ammonium sulfate precipitation obtained maximum precipitation at 30 % saturation for native enzyme and 20 % for mutant enzyme. Similarly, ammonium sulfate precipitation has been used for purification of endoglucanase from *A. fumigatus* by different researchers to yield maximum protein output[68]. The purification techniques led to enhanced enzyme activities up to 1.52-fold from various microbes[69]. Most probably, mutation caused the variation in optimal saturation levels between native (30 %) and mutant (20 %) enzymes, potentially affecting protein solubility and precipitation characteristics.

### *Chromatographic purification*

The dialyzed enzyme solution was further purified by gel filtration chromatography. The dialyzed fractions of native and mutant enzyme were applied to a Sephadex G-100 column and enzyme activity was noted. Native endoglucanase was purified up to 1.46-fold with a specific activity of 0.190 U $mg^{-1}$ (Table 3), while endoglucanase from mutant *A. fumigatus* was purified up to 1.54-fold with a higher specific activity of 0.314 U $mg^{-1}$ (Table 4). Total

Table 3 – *Purification summary of native endoglucanase from A. fumigatus*

| Step | Total activity (U) | Total protein (mg) | Specific activity (U $mg^{-1}$) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 165.50 | 1273.08 | 0.130 | 1.00 | 100.00 |
| Ammonium sulfate (30 %) | 142.33 | 312.72 | 0.455 | 3.50 | 86.00 |
| Dialysis | 128.70 | 215.50 | 0.597 | 4.59 | 77.76 |
| Sephadex G-100 | 59.26 | 312.00 | 0.190 | 1.46 | 35.81 |

Table 4 – *Purification summary of mutant endoglucanase from A. fumigatus*

| Step | Total activity (U) | Total protein (mg) | Specific activity (U $mg^{-1}$) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 383.70 | 1653.88 | 0.232 | 1.00 | 100.00 |
| Ammonium sulfate (20 %) | 308.68 | 557.84 | 0.553 | 2.38 | 80.45 |
| Dialysis | 278.90 | 386.52 | 0.721 | 3.11 | 72.68 |
| Sephadex G-100 | 110.52 | 352.00 | 0.314 | 1.54 | 28.80 |

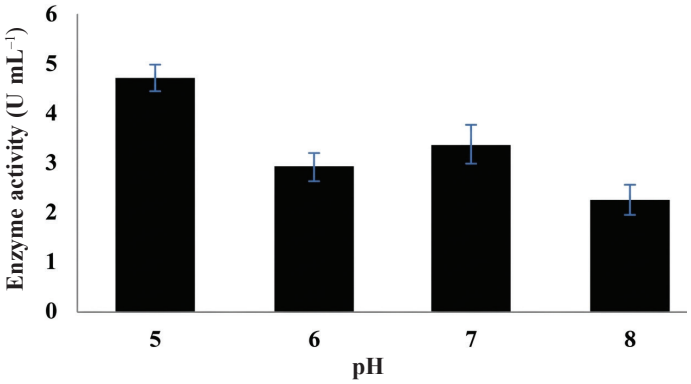


Fig. 6 – *Effect of pH on EG from native A. fumigatus. Maximum activity of EG was observed at pH 5.*

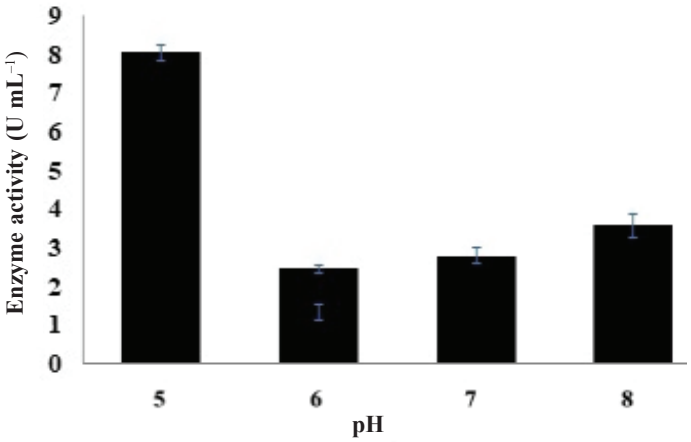


Fig. 7 – *Effect of pH on EG from mutant A. fumigatus. Maximum activity of EG was observed at pH 5.*

protein concentration decreased due to purification of endoglucanase and exclusion of impurities. We obtained approximately 1.5-fold purification of both mutant and wild-type enzymes with our gel filtration chromatography. More recent purification studies have been conducted using the same methods with increasing success. Our 1.5-fold purification corresponds to partial purification strategies frequently used for industrial enzyme preparations.

Combined mutagenesis resulted in the highest endoglucanase yield (123.32 U $mL^{-1}$). Partial purification via ammonium sulfate precipitation (20–30 % saturation) followed by Sephadex G-100 chromatography yielded a 1.54-fold improvement in specific activity. While the improvement is modest, it aligns with requirements for industrial-grade enzyme cocktails where high purity is less critical than volumetric productivity and thermal stability.

## Characterization of purified endoglucanase

### *Effect of pH*

The effect of pH on endoglucanase activity is shown in Fig. 6 for native, and Fig. 7 for mutant endoglucanase. Endoglucanase from native and mutant strains exhibited maximum activity of 4.71 U $mL^{-1}$ and 8.06 U $mL^{-1}$, respectively, at pH 5. These results suggest that endoglucanase can work better in an acidic environment in different applications. These findings are consistent with Pothiraj[70] who determined that *Aspergillus niger*, *Aspergillus terreus*, *Rhizopus stolonifer*, and *Trichoderma* species are capable of producing cellulases. Another study showed that FPase, CMCase, and Avicelase activities exhibit an optimum at pH 4 [71].

### *Effect of temperature and thermal stability*

Temperature has a significant influence on the enzyme. The results of temperature characterization showed that endoglucanase is thermophilic and exhibited maximum activity at 55 °C whether produced from native (Fig. 8) or mutant *A. fumigatus* (Fig. 9). In the case of mutant endoglucanase, maximum activity was found to be 55.28 U $mL^{-1}$. Previous studies have also confirmed that the temperature optima for CMCase existed between 40 to 60 °C synthesized from *Cryptococcus* sp.[72] The optimum temperature for cellulase produced from *Trichoderma viride* was found to be 55 °C [71]. Cellulase 5A produced from *Bacillus subtilis* showed maximum activity at pH 5.0 and 50 °C and maximum stability at pH 5.0–9.0 and 20–60 °C [73]. The stability pattern of our enzyme is in agreement with these results, which suggests that there are no major variations in the optimum conditions based on the source of microbes.

### *Effect of metal ions*

By varying the concentrations of zinc, magnesium, and calcium ions at 100, 150, 200, 250, 300, 350, and 400 mM, their effect was studied on the activity of endoglucanase. It was observed that $CaCl_2$ acted as an activator for native (Fig. 10) and mutant endoglucanase (Fig. 11). In the presence of 300 mM concentration of $CaCl_2$, activity of mutant enzyme increased up to 100.16 U $mL^{-1}$. Similarly, activity of native enzyme increased up to 13.97 U $mL^{-1}$ at 400 mM concentration. Significant activity of endoglucanase was retained in the presence of $ZnSO_4$. These findings suggest that $CaCl_2$ acted as an activator for both native and mutant endoglucanase, with mutant enzyme activity increasing to 100.16 U $mL^{-1}$ at 300 mM $CaCl_2$ and native enzyme reaching 13.97 U $mL^{-1}$ at 400 mM. This calcium ion activation is consistent with structural analysis that identifies calcium ions in the active sites of enzymes[74]. Divalent cations were found to play a critical role in enzyme functions as reviewed by metal ion signaling in biomedicine[75].

The description of various factors described by our endoglucanase fits the numerous industrial applications. Recent studies have demonstrated the

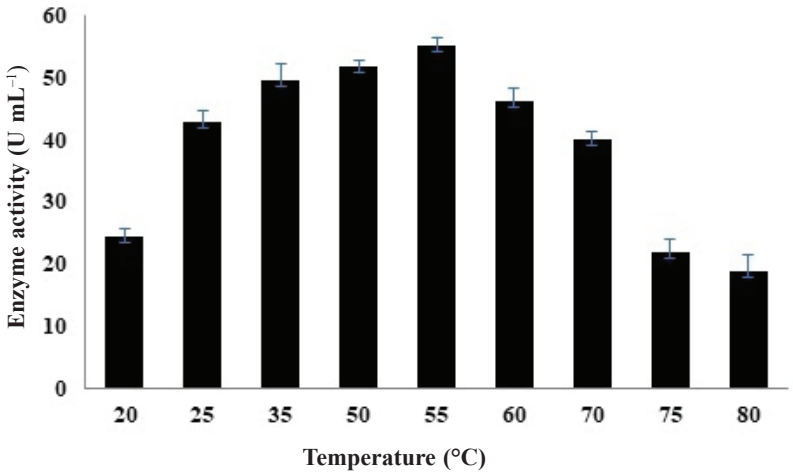


Fig. 8 – *Effect of temperature on stability of EG from native A. fumigatus. Maximum activity was observed at temperature 55 °C.*

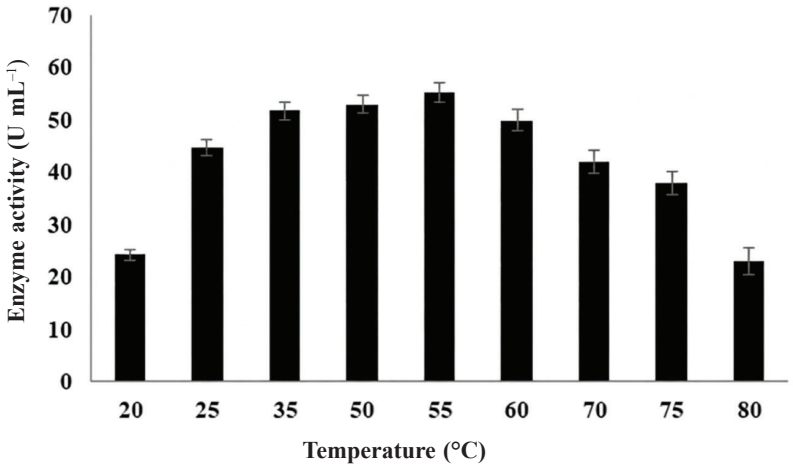


Fig. 9 – *Effect of temperature on stability of EG from mutant A. fumigatus. Maximum activity was observed at temperature 55 °C.*

importance of fast cellulases for processing of lignocellulosic biomass[76]. It has been demonstrated that optimization of physicochemical conditions to achieve enzyme production using lignocellulosic biomass under solid-state fermentation promotes yield and stability to a great extent[77]. Agro-industrial wastes have been shown to be effective as substrates, and statistical optimization of physiochemical parameters to maximize xylanase and other associated enzyme production has been reported[78]. Pectinolytic enzyme cocktails can also be used in the saccharification of lignocellulosic material and the clarification of fruit juice as well, showing their multifaceted use in industry[79].

## Conclusion

Endoglucanase activity was substantially enhanced in a thermophilic mutant strain of *Aspergillus fumigatus* developed through combined mutagenesis (UV + EMS). Enzyme activity increased to 123.32 U $mL^{-1}$ compared to 55.28 U $mL^{-1}$ in the native strain. Response surface methodology (RSM) under SSF revealed that among various lignocellulosic substrates, rice straw exhibited maximum endoglucanase activity at pH 5 after 5 days of incubation. The mutant enzyme was purified 1.54-fold through ammonium sulfate precipitation, dialysis, and gel filtration chromatography compared to 1.46-fold purification of native endoglucanase. Among metal ions, $CaCl_2$ successfully increased enzyme activity (176.92 U $mL^{-1}$) at a concentration of 300 mM. In addition, enzyme kinetics study exhibited more affinity of endoglucanase with CMC as substrate. The reported thermophilic and chemically engineered endoglucanase from *A. fumigatus* showed its maximum activity at a temperature of 55 °C, which recommends its industrial exploitation in the paper, textile, and biofuel industries. This study successfully enhanced endoglucanase activity in an indigenous *A. fumigatus* strain using a synergistic

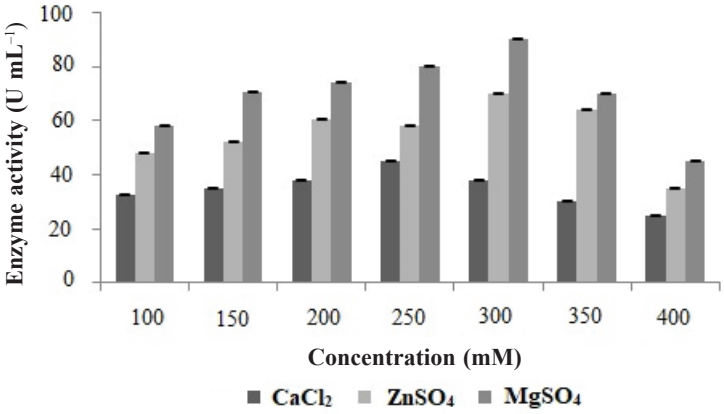


Fig. 10 – *Effect of metal ions on native endoglucanase. $CaCl_2$ (300 mM) was found to be the best activator for native EG (90.58 U mL$^{-1}$).*

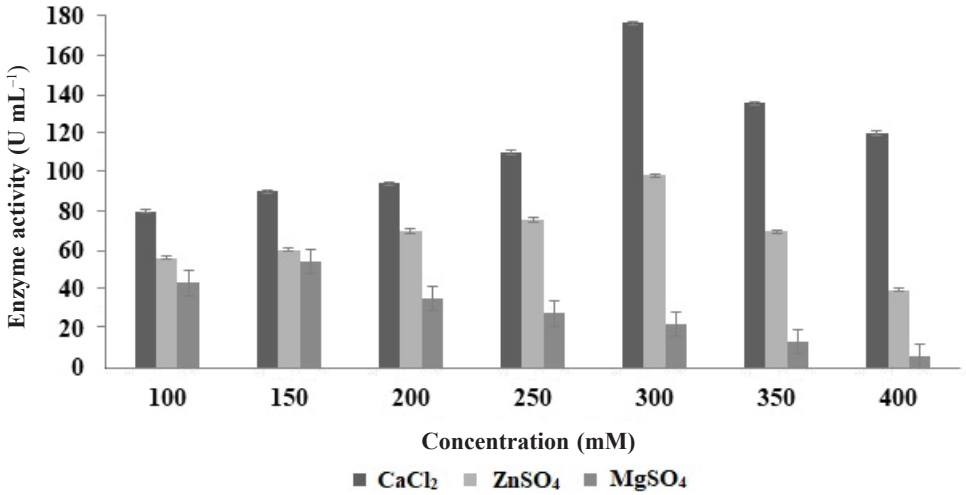


Fig. 11 – *Effect of metal ions on mutant endoglucanase. $CaCl_2$ (300 mM) was found to be the best activator for EG, enhancing activity (176.92 U mL$^{-1}$).*

combination of UV and EMS mutagenesis. Statistical optimization via RSM successfully identified optimal bioprocessing conditions, achieving a 2.5-fold increase in enzyme titers compared to the wild type. The mutant enzyme showed robustness at 55 °C and pH 5.0, with significant activation by calcium ions. These findings suggest the strain is a promising candidate for pilot scale validation to be considered for further biofuel and textile applications.

## References

1. *Broda, M., Yelle, D. J., Serwańska, K.,* Bioethanol production from lignocellulosic biomass-challenges and solutions, Molecules **27** (2022) 8717. doi: https://doi.org/10.3390/molecules27248717
2. *Dhillon, G. S., Brar, S. K., Kaur, S., Verma, M.,* Green approach for nanoparticle biosynthesis by fungi: Current trends and applications, Crit. Rev. Biotechnol. **32** (2012) 49. doi: https://doi.org/10.3109/07388551.2010.550568
3. *Miao, Y., Liu, D., Li, G., Li, P., Xu, Y., Shen, Q., Zhang, R.,* Genome-wide transcriptomic analysis of a superior biomass-degrading strain of *A. fumigatus* revealed active lignin-degrading genes, BMC Genomics **16** (2019) 424. doi: https://doi.org/10.1186/s12864-015-1658-2
4. *Maitra, M., Sruthi, S., Rao, P. M., Avanthi, V. S., Patra, S.,* In Advances in Sustainable Bioenergy, Springer (2024) 1–15.
5. *Liu, G., Qin, Y., Li, Z., Qu, Y.,* Development of highly efficient, low-cost lignocellulolytic enzyme systems in the post-genomic era, Biotechnol. Adv. **33**(2) (2015) 181. doi: https://doi.org/10.1016/j.biotechadv.2014.11.002
6. *Rathour, R. K., Devi, M., Dahiya, P., Sharma, N., Kaushik, N., Kumari, D., Kumar, P., Baadhe, R. R., Walia, A., Bhatt, A. K., Bhatia, R. K.,* Recent trends, opportunities and challenges in sustainable management of rice straw waste biomass for green biorefinery, Energies **16** (2023) 1429. doi: https://doi.org/10.3390/en16031429
7. *Naik, G. P., Poonia, A. K., Chaudhari, P. K.,* Pretreatment of lignocellulosic agricultural waste for delignification, rapid hydrolysis, and enhanced biogas production: A review, J. Indian Chem. Soc. **98** (2021) 100147. doi: https://doi.org/10.1016/j.jics.2021.100147

8. *Bernardi, A. V., Gerolamo, L. E., Uyemura, S. A., Dinamarco, T. M.,* A thermostable *Aspergillus fumigatus* GH7 endoglucanase over-expressed in *Pichia pastoris* stimulates lignocellulosic biomass hydrolysis, Int. J. Biol. Macromol. **117** (2018) 1074. doi: https://doi.org/10.1016/j.ijbiomac.2018.06.036
9. *Liew, K. J., Chan, C. S., Yusoff, M. S. M., Gan, H. M., Lim, M. G., Chong, C. S.,* Thermophiles and carbohydrate-active enzymes (CAZymes) in biofilm microbial consortia that decompose lignocellulosic plant litters at high temperatures, Sci. Rep. **12** (2022) 2987. doi: https://doi.org/10.1038/s41598-022-06943-9
10. *De Gouvêa, P. F., Bernardi, A. V., Gerolamo, L. E., de Souza Santos, E., Riaño-Pachón, D. M., Uyemura, S. A., Dinamarco, T. M.,* Transcriptome and secretome analysis of *Aspergillus fumigatus* in the presence of sugarcane bagasse, BMC Genomics **19** (2018) 232. doi: https://doi.org/10.1186/s12864-018-4627-8
11. *De Vries, R. P., Mäkelä, M. R.,* Genomic and postgenomic diversity of fungal plant biomass degradation approaches, Trends Microbiol. **28** (2020) 116. doi: https://doi.org/10.1016/j.tim.2020.01.004
12. *Lodi, R. S., Jia, X., Yang, P., Peng, C., Dong, X., Han, J., Liu, X., Wan, L., Peng, L. R. S.,* Whole genome sequencing and annotations of *Trametes sanguinea* ZHSJ, Sci. Data **12** (2025) 1460. doi: https://doi.org/10.1038/s41597-025-05798-9
13. *Ma, L., Li, J., Zhao, W., Wang, J., Li, Y., Xiong, Y., He, Y., Chu, X., Liu, Q., L.,* Key cellulase components synergizing with lactic acid bacteria to degrade alfalfa lignocellulose to improve lactic acid fermentation, Front. Microbiol. **16** (2025) 1566973. doi: https://doi.org/10.3389/fmicb.2025.1566973
14. *Fernandes, C. G., Sawant, S. C., Mule, T. A., Khadye, V. S., Rathod, V. K.,* Enhancing cellulases through synergistic β-glucosidases for intensifying cellulose hydrolysis, Process Biochem. **119** (2022) 177. doi: https://doi.org/10.1016/j.procbio.2022.06.011
15. *Sharma, K. K., Shrivastava, B., Sastry, V. R. B., Sehgal, N., Kuhad, R. C.,* Saccharification of alkali-treated rice straw by cellulase complex from *Trichoderma reesei*: Role of β-glucosidase and xylanase, World J. Microbiol. Biotechnol. **29**(7) (2013) 1239. doi: https://doi.org/10.1007/s11274-013-1282-4
16. *Brunecky, R., Knott, B. C., Subramanian, V., Linger, J. G., Beckham, G. T., Amore, A., Taylor, L. E., Wall, T. A., Lunin, V. V., Zheng, F., Garrido, M., Schuster, L., Fulk, E. M., Farmer, S., Himmel, M. E., Decker, S. R.,* Engineering of glycoside hydrolase family 7 cellobiohydrolases directed by natural diversity screening, J. Biolog. Chem. **300** (3) (2024) 105749. doi: https://doi.org/10.1016/j.jbc.2024.105749
17. *Zhao, L.-C., Guo, L.-Q., Xiao, H., Zheng, L.-J., Wang, Y., Lin, J.-F.,* Purification and characterization of the recombinant multifunctional cellulase from *Volvariella volvacea*, Food Biotechnol. **26**(2) (2012) 164. doi: https://doi.org/10.1080/08905436.2012.670890
18. *Perrot, T., Pauly, M., Ramírez, V.,* Emerging roles of β-glucanases in plant development and adaptive responses, Plants **11** (2022) 1119. doi: https://doi.org/10.3390/plants11091119
19. *Hua, C., Li, W., Han, S., Wang, Q., Nie, M., Jiang, N., Li, Q., Zhao, J., Kang, Z., Huang, J.,* Characterization of a novel thermostable GH7 endoglucanase from *Chaetomium thermophilum* capable of xylan hydrolysis, Carbohydr. Res. **460** (2018) 25. doi: https://doi.org/10.1016/j.carres.2018.03.001
20. *Sahin, S., Ozmen, I., Bıyık, H.,* Industrial applications of endoglucanase obtained from novel and native *Trichoderma atroviride*, Chem. Biochem. Eng. Q. **30**(3) (2016) 265. doi: https://doi.org/10.15255/cabeq.2014.2130
21. *Rigoldi, F., Donini, S., Redaelli, A., Parisini, E., Gautieri, A.,* Review: Engineering of thermostable enzymes for industrial applications, APL Bioeng. **2**(1) (2018) 011501. doi: https://doi.org/10.1063/1.4997367
22. *Wang, H., Zhai, L., Geng, A.,* Enhanced cellulase and reducing sugar production by a new mutant strain *Trichoderma harzianum* EUA20, J. Biosci. Bioeng. **130** (2020) 121. doi: https://doi.org/10.1016/j.jbiosc.2019.08.016
23. *Arora, N., Yen, H. W., Philippidis, G. P.,* Harnessing the power of mutagenesis and adaptive laboratory evolution for high lipid production by oleaginous microalgae and yeasts, Sustainability **12** (2020) 5125. doi: https://doi.org/10.3390/su12125125
24. *Bleisch, R., Freitag, L., Ihadjadene, Y., Sprenger, U.,* Strain development in microalgal biotechnology—random mutagenesis techniques, Life **12** (2022) 96. doi: https://doi.org/10.3390/life12070961
25. *Batayyib, R. S. M., Al-Twaty, N. H., Al-Ghamdi, A. A., Alsharif, S. M.,* Molecular characterization of the superior lignin peroxidase-producing *Streptomyces lavendulae* R-St-1 mutants and fusants, Egypt. J. Bot. **53** (2023) 363. doi: https://doi.org/10.4103/epj.epj_141_22
26. *Haq, I., Saeed, M. B., Nawaz, A., Mustafa, Z., Akram, F.,* Strain improvement of *Aspergillus oryzae* for enhanced biosynthesis of phytase through chemical mutagenesis, Pak. J. Bot. **54** (2022) 1. doi: https://doi.org/10.30848/PJB2022-6(17)
27. *Pang, Y. X., Yan, Y., Tsang, D. C. W., Varbanov, P. S., Ok, Y. S.,* The influence of lignocellulose on biomass pyrolysis product distribution and economics via steady state process simulation, J. Anal. Appl. Pyrolysis **155** (2021) 105050. doi: https://doi.org/10.1016/j.jaap.2020.104968
28. *Sun, D., Lv, Z.-W., Rao, J., Tian, R., Sun, S.-N., Peng, F. D.,* Effects of hydrothermal pretreatment on the dissolution and structural evolution of hemicelluloses and lignin: A review, Carbohydr. Polym. **276** (2022) 11873. doi: https://doi.org/10.1016/j.carbpol.2021.119050
29. *Aich, A., Basu, A., Varma, A., Bhattacharya, A., Goud, V. V.,* Optimization studies for lipase-catalyzed biodiesel production from *Madhuca indica* oil in ionic liquids, Process Biochem. **58** (2017) 318. doi: https://doi.org/10.1016/j.procbio.2017.04.040
30. *Dodd, D., Mackie, R. I., Cann, I. K. O.,* Xylan degradation, a metabolic property shared by rumen and human colonic Bacteroidetes, Mol. Microbiol. **79**(2) (2010) 292. doi: https://doi.org/10.1111/j.1365-2958.2010.07462.x
31. *Sajeevan, T. P., Sajith, S., Sreedevi, S., Ishaque, P. M., Singh, I. S. B.,* Mixed ligand Cu(II) complexes derived from dithiocarbamate and azole as antifouling agents, Chem. Eng. J. **295** (2016) 9. doi: https://doi.org/10.1016/j.cej.2016.02.108
32. *Sales, M. R., Moura, R. B., Porto, A. L. F., Macedo, G. R., Melo, E. S.,* Optimization of *Penicillium roqueforti* cellulase production using waste paper as substrate through central composite rotational design, Enzyme Res. **2011** (2011) 421043. doi: https://doi.org/10.4061/2011/421043
33. *Cyrus, E. T., Juwon, A. D.,* Effects of radiation and chemical mutagenesis on expression of aflatoxigenic traits in *Aspergillus parasiticus* SMS08-C, Res. J. Microbiol. **10**(5) (2015) 205. doi: https://doi.org/10.3923/jm.2015.205.213

34. *Saranya, S., Chellapandi, P.,* Comparative evaluation of chemical and UV mutagenesis with post-mutagenesis process optimization for enhanced cellulolytic enzyme production by *Aspergillus oryzae,* Bioresour. Technol. Rep. **26** (2025) 101863. doi: https://doi.org/10.1016/j.biteb.2025.102349
35. *Ahmad, W., Zafar, M., Anwar, Z.,* Enhancement of cellulase activity from indigenous strain of *Aspergillus niger* through incorporation of physical, chemical and combined mutagenesis, J. Xi'an Shiyou University **19**(11) (2023) 224. doi: https://doi.org/10.19811/j.xsyu202311224
36. *Yamada, R., Kashihara, T., Ogino, H.,* Improvement of lipid production by the oleaginous yeast *Rhodosporidium toruloides* through UV mutagenesis, World J. Microbiol. Biotechnol. **33**(5) (2017) 99. doi: https://doi.org/10.1007/s11274-017-2269-7
37. *Aich, A., Basu, A., Varma, A., Bhattacharya, A., Goud, V. V.,* Optimization studies for lipase-catalyzed biodiesel production from *Madhuca indica* oil in ionic liquids, Process Biochem. **58** (2017) 318. doi: https://doi.org/10.1016/j.procbio.2017.04.040
38. *Vu, V. H., Pham, T. A., Kim, K.,* Improvement of fungal cellulase production by mutation and optimization of solid state fermentation, Mycobiology **39**(1) (2011) 20. doi: https://doi.org/10.4489/myco.2011.39.1.020
39. *Shobana, P., Maheswari, N. U.,* Production of cellulase from *Aspergillus fumigatus* under submerged and solid state fermentation using agricultural waste, Int. J. Adv. Pharm. Biol. Chem. **2**(3) (2013) 595.
40. *Rabia, I., Zafar, M., Anwar, Z., Dildar, I., Mustafa, G., Tariq, T., Ghorbanpour, M., Hassan, M.,* Hyper-production and characterization of exoglucanase through physical, chemical, and combined mutagenesis in indigenous strain of thermophilic *Aspergillus fumigatus*, Appl. Biochem. Biotechnol. **197**(6) (2025) 4002. doi: https://doi.org/10.1007/s12010-025-05222-3
41. *Muddassar, Z., Ahmed, S., Khan, M. I. M., Jamil, A.,* Recombinant expression and characterization of a novel endoglucanase from *Bacillus subtilis* in *Escherichia coli*, Mol. Biol. Rep. **41**(5) (2014) 3295. doi: https://doi.org/10.1007/s11033-014-3192-8
42. *Miller, G. L.,* Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal. Chem. **31**(3) (1959) 426. doi: https://doi.org/10.1021/ac60147a030
43. *Bradford, M. M.,* A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. **72** (1976) 248.
44. *Teixeira da Silva, V. d. C., de Souza Coto, A. L., de Carvalho Souza, R., Bertoldi Sanchez Neves, M., Gomes, E., Bonilla-Rodriguez, G. O.,* Effect of pH, temperature, and chemicals on the endoglucanases and β-glucosidases from the thermophilic fungus *Myceliophthora heterothallica* F. 2.1.4. obtained by solid-state and submerged cultivation, Biochem. Res. Int. **2016** (2016) 9781216. doi: https://doi.org/10.1155/2016/9781216
45. *Pol, D., Laxman, R. S., Rao, M.,* Purification and biochemical characterization of endoglucanase from *Penicillium pinophilum* MS 20, Indian J. Biochem. Biophys. **49**(3) (2012) 189.
46. *Tao, Y.-M., Zhu, X.-Z., Huang, J.-Z., Ma, S.-J., Wu, X.-B., Long, M.-N., Chen, Q.-X.,* Purification and properties of endoglucanase from a sugar cane bagasse hydrolyzing strain, *Aspergillus glaucus* XC9, J. Agric. Food Chem. **58**(10) (2010) 6126. doi: https://doi.org/10.1021/jf1003896
47. *Narra, M., Dixit, G., Divecha, J., Madamwar, D., Shah, A. R.,* Production, purification and characterization of a novel GH 12 family endoglucanase from *Aspergillus terreus* and its application in enzymatic degradation of delignified rice straw, Bioresour. Technol. **165** (2014) 277. doi: https://doi.org/10.1016/j.biortech.2014.03.109
48. *El-Khamisi, E. F., Soliman, E. A. M., El-Sayed, G. M., Nour, S. A., Abdel-Monem, M. O., Hassan, M. G.,* Optimization, gene cloning, expression, and molecular docking insights for enhanced cellulase enzyme production by *Bacillus amyloliquefaciens* strain elh1, Microb. Cell Fact. **23** (2024) 191. doi: https://doi.org/10.1186/s12934-024-02454-6
49. *Zainudin, M. H. M., Hassan, M. A., Tokura, M., Shirai, Y.,* A highly thermostable crude endoglucanase produced by a newly isolated *Thermobifida fusca* strain UPMC 901, Sci. Rep. **9**(1) (2019) 13526. doi: https://doi.org/10.1038/s41598-019-49598-2
50. *Bezerra, M. A., Santelli, R. E., Oliveira, E. P., Villar, L. S., Escaleira, L. A.,* Response surface methodology (RSM) as a tool for optimization in analytical chemistry, Talanta **76** (2008) 965. doi: https://doi.org/10.1016/j.talanta.2008.05.019.
51. *Singhania, R. R., Sukumaran, R. K., Patel, A. K., Larroche, C., Pandey, A.,* Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases, Enzyme Microb. Technol. **46** (2010) 541. doi: https://doi.org/10.1016/j.enzmictec.2010.03.010
52. *Li, Y., Irwin, D. C., Wilson, D. B.,* Processivity, substrate binding, and mechanism of cellulose hydrolysis by *Thermobifida fusca* Cel9A, Appl. Environ. Microbiol. **73** (2007) 3165. doi: https://doi.org/10.1128/AEM.02960-06
53. *Verma, N., Kumar, V.,* Application of Box–Behnken design for the optimization of cellulase production under solid-state fermentation, Applied Sci. **1**(12) (2019) 17. doi: https://doi.org/10.1007/s42452-019-1779-3
54. *Ibrahim, A. M., Hamouda, R. A., El-Naggar, N. E., AI-Shakankery, F. M.,* Bioprocess development for enhanced endoglucanase production by newly isolated bacteria, purification, characterization and in-vitro efficacy as anti-biofilm of *Pseudomonas aeruginosa,* Sci. Rep. **11** (2021) 9754. doi: https://doi.org/10.1038/s41598-021-87901-9
55. *Bagewadi, Z. K., Mulla, D. I., Ninnekar, H. Z.,* Optimization of endoglucanase production from *Trichoderma harzianum* strain HZN11 by central composite design under response surface methodology, Biomass Conversion Bioref. **8** (2018) 8. doi: https://doi.org/10.1007/s13399-017-0285-3
56. *Argo, B. D., Rahadi, B., Sari, P. D.,* Effect of pH and incubation time against endoglucanase activity from *Trichoderma reesei* and *Aspergillus niger* with rice straw as substrate, Res. Review Biosci. **9** (2014) 56.
57. *Ameri, A., Shakibaie, M., Ameri, A., Faramarzi, M. A., Forootanfar, H.,* Screening and optimization of process parameters for the production of L-asparaginase by indigenous fungal-type strains, Iran. J. Sci. Technol. **45** (2021) 1. doi: https://doi.org/10.1007/s40995-020-01056-2
58. *Sharif, S., Shah, A. H., Fariq, A., Jannat, S., Rasheed, S., Yasmin, A.,* Optimization of amylase production using response surface methodology from newly isolated thermophilic bacteria, Heliyon. **9**(1) (2023) e12901. doi: https://doi.org/10.1016/j.heliyon.2023.e12901
59. *Udayan, A., Arumugam, M., Kathiresan, K.,* The nutritional and cultural conditions in shake flask culture for improved production of L-asparaginase from endophytic fungus *Fusarium* sp. LCJ324: A sequential statistical method, Ital. J. Mycol. **52** (2023) 62. doi: https://doi.org/10.6092/issn.2531-7342/16067

60. *Smitha, K. V., Pradeep, B. V.,* Application of Box-Behnken design for the optimization of culture conditions for novel fibrinolytic enzyme production by *Bacillus altitudinis* S-CSR 0020, J. Pure Appl. Microbiol. **11** (2017) 1447. doi: https://doi.org/10.22207/JPAM.11.3.28
61. *Zhou, A., Zhang, N., Dong, H., Huang, Y., Xie, L.,* Fermented food-polysaccharides as gut health regulators: Sources, optimization, structural characteristics and mechanism, Foods **14** (2025) 4108. doi: https://doi.org/10.3390/foods14234108
62. *Assega, B. G., Getahun, K. A., Milkessa, T., Yohannes, T. G., Moges, F., Aemro, M., Andualem, B.,* Production of extracellular L-arginase by *Alcaligenes aquatilis* BC2 isolated from soda lakes (Lake Chitu) of Ethiopia, J. Ind. Microbiol. Biotechnol. **52** (2025). doi: https://doi.org/10.1093/jimb/kuaf017
63. *Aslam, S., Asgher, M., Hussain, F., Khan, N. A.,* Statistical optimization of fungal laccase produced by solid state fermentation, Life Sci. Inf. Public. **4** (2018) 180. doi: https://doi.org/10.26479/2018.0405.14
64. *Abo Elsoud, M. M., Abo-Estado, M. A., Saleh, S. A.,* Mutations and statistical modeling for cellulase production by *Aspergillus flavus* using gamma radiation, UV-radiation and ethidium bromide, Egypt. J. Chem. **66**(8) (2023) 419. doi: https://doi.org/10.21608/EJCHEM.2023.157417.8140
65. *Benites-Pariente, J. S., Samolski, I., Ludeña, Y., Villena, G. K.,* CRISPR/Cas9 mediated targeted knock-in of eglA gene to improve endoglucanase activity of *Aspergillus fumigatus* LMB-35Aa, Sci. Rep. **14**(1) (2024) 19468. doi: https://doi.org/10.1038/s41598-024-56417-6
66. *Chandra, M., Kalra, A., Sangwan, N. S., Gaurav, S. S., Darokar, M. P., Sangwan, R. S.,* Development of a mutant of *Trichoderma citrinoviride* for enhanced production of cellulases, Bioresour. Technol. **100**(4) (2009) 1659. doi: https://doi.org/10.1016/j.biortech.2008.09.011
67. *Liu, F., Liang, C., Wang, F., Yao, J., Cai, Z., Yang, Z., Chen, S.,* Enhanced cellulase production through random mutagenesis and adaptive laboratory evolution of *Talaromyces pinophilus*, Bioresour. Technol. **302** (2020) 122800. doi: https://doi.org/10.1016/j.biortech.2020.122800
68. *Abdulmajeed, A. T. A., Şahin, S., Ozmen, I.,* Production and purification of the endoglucanase enzyme from local isolate *Aspergillus fumigatus* HBF356, Biointerface Res. Appl. Chem. **12**(4) (2021) 4337. doi: https://doi.org/10.33263/BRIAC124.43374347
69. *Shyaula, M., Khadayat, K., Gautam, K., Tuladhar, A., Mahato, N. K., Aryal, N., Parajuli, N.,* Characterization of thermostable cellulase from *Bacillus licheniformis* PANG L isolated from the Himalayan soil for potential industrial applications, Int. J. Microbiol. **2023** (2023) 8867674. doi: https://doi.org/10.1155/2023/8867674
70. *Pothiraj, C., Balaji, P., Eyini, M.,* Enhanced production of cellulases by various fungal cultures in solid state fermentation of cassava waste, Afr. J. Biotechnol. **5**(20) (2006) 1882.
71. *Iqbal, H. M. N., Ahmed, I., Zia, M. A., Irfan, M.,* Purification and characterization of the kinetic parameters of cellulase produced from wheat straw by *Trichoderma viride* under SSF and its detergent compatibility, Adv. Biosci. Biotechnol. **2**(3) (2011) 149. doi: https://doi.org/10.4236/abb.2011.23024
72. *Thongekkaew, J., Ikeda, H., Masaki, K., Iefuji, H.,* An acidic and thermostable carboxymethyl cellulase from the yeast *Cryptococcus* sp. S-2: Purification, characterization and improvement of its recombinant enzyme production by high cell-density fermentation of *Pichia pastoris*, Protein Expr. Purif. **60**(2) (2008) 140. doi: https://doi.org/10.1016/j.pep.2008.03.021
73. *Yao, X., Guo, Y., Wang, Y., Adeel, M., Zhang, P., Zhao, R., Jiang, Y.,* Genomic functional analysis and cellulase characterization of halotolerant *Bacillus subtilis* Y4X3 isolated from saline soil in Xinjiang, China, J. Anim. Sci. Biotechnol. **16**(1) (2025) 32. doi: https://doi.org/10.1186/s40104-025-01054-3
74. *Hadden, J. M., Declais, A. C., Phillips, S. E., Lilley, D. M.,* Metal ions bound at the active site of the junction-resolving enzyme T7 endonuclease I, EMBO J. **21**(12) (2002) 3505. doi: https://doi.org/10.1093/emboj/cdf368
75. *Rodriguez, R., Martinez-Martinez, D., Choi, H., Ramirez-Lee, M. A., Contreras-Torres, F. F., Lee, K. T.,* Metal ion signaling in biomedicine, Chem. Rev. **125**(1) (2025) 506. doi: https://doi.org/10.1021/acs.chemrev.3c00728
76. *Ma, X., Liu, Y., Shi, W., Chen, J., Gao, J., Xu, Y., Wei, D.,* An overview on the current status and future prospects in cellulase production from *Aspergillus* species, Environ. Res. **240** (2024) 117552. doi: https://doi.org/10.1016/j.envres.2024.117552
77. *Ayesha, R., Anwar, Z., Zafar, M.,* Optimization of physicochemical parameters and characterization of laccase enzyme produced by a novel strain of *Fomes fomentarius* through solid state fermentation, Biomass Convers. Biorefin. **14** (2022) 3999. doi: https://doi.org/10.1007/s13399-023-04276-3
78. *Ali, M. B., Irshad, M., Anwar, Z., Zafar, M., Imran, M.,* Screening and statistical optimization of physiochemical parameters for the production of xylanases from agro-industrial wastes, Adv. Enzyme Res. **4**(01) (2016) 20. doi: https://doi.org/10.4236/aer.2016.41003
79. *Salim, D., Anwar, Z., Zafar, M., Anjum, A., Bhatti, K. H., Irshad, M.,* Pectinolytic cocktail: Induced yield and its exploitation for lignocellulosic materials saccharification and fruit juice clarification, Food Biosci. **22** (2018) 154. doi: https://doi.org/10.1016/j.fbio.2018.01.006