doi.org/10.36721/PJPS.2025.38.3.REG.13954.1

Enzymatic disruption of pathogenic biofilms by thermostable endoglucanase from *Bacillus cereus* ARA-12: Insights for pharmaceutical and industrial use

Ayesha Siddiqui[1], Ayisha Aman[2], Rida-e-Noor Azam[2], Safia Mukhtar[2], Aliya Riaz[3], Raheela Rahmat Zohra[1] and Shah Ali ul Qadar[4]*

[1]Department of Biotechnology, University of Karachi, Karachi, Pakistan
[2]Department of Biotechnology, Jinnah University for Women, Karachi, Pakistan
[3]Department of Biochemistry, Jinnah University for Women, Karachi, Pakistan
[4]Department of Biochemistry, University of Karachi, Karachi, Pakistan

Abstract: This study focused on the partial purification and characterization of endoglucanase produced by *Bacillus cereus* ARA-12, with the special emphasis on its anti-biofilm potential against pathogenic bacteria. Partial purification was achieved through ammonium sulfate precipitation, resulting in a 6.97 purification fold with a specific activity of 91.519 U/mg. The optimum temperature and pH of the enzyme were found to be 50°C and 8.0 respectively. The enzyme retained over 80% of its residual activity after 30 minutes of incubation at high temperatures. The kinetic studies revealed a K_m of 3.25 mg/mL and a V_{max} of 2500 µmol/min, indicating high substrate affinity and catalytic efficiency. The enhanced enzyme activity was observed with metal ions and the enzyme also demonstrated stability in the presence of detergents as well. The partially purified endoglucanase showed significant anti-biofilm potential. Inhibition studies revealed a maximum biofilm reduction of 72% for *Pseudomonas aeruginosa* after 36 hours of incubation. Eradication studies demonstrated biofilm detachment rates of 87% for *Pseudomonas aeruginosa*, 72% for *Staphylococcus aureus*, and 56% for *Escherichia coli* after 36 hours. Scanning electron microscopy confirmed structural degradation of biofilms. These findings underscore the enzyme's dual potential for industrial biocatalysis and therapeutic applications, particularly in combating biofilm-associated infections.

Keywords: Anti-biofilm, *Bacillus cereus*, biocatalyst, endoglucanase, pathogenic microorganisms

Submitted on 03-01-2025 – Revised on 28-01-2025 – Accepted on 27-02-2025

INTRODUCTION

Endoglucanases (EC 3.2.1.4), a class of cellulase enzymes, play a critical role in the biotechnological transformation of cellulose, a renewable and abundant natural polymer. Their ability to hydrolyze internal β-1,4-glycosidic bonds in cellulose makes them indispensable in industries focused on sustainable production. The complete hydrolysis of cellulose into glucose requires the action of two more enzymes: exoglucanase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). Endoglucanases initiate random cleavage at internal sites within the cellulose structure, exposing the polymer to further hydrolysis by other cellohydrolases (Sherief *et al.,* 2010). Endoglucanases are produced by a wide variety of microorganisms growing on cellulolytic substrates. Microbial enzymes are preferred over those derived from animal or plant sources due to their lower cost and the well-characterized systems for enzyme production and secretion (Riaz *et al.,* 2023). Bacterial endoglucanases, in particular, have garnered significant interest for their biodiversity, ease of product recovery, and the ability to produce enzymes that withstand extreme environmental conditions (Ladeira *et al.,* 2015). Among bacterial species, *Bacillus cereus* remains a notable workhorse due to its high secretion of cellulolytic enzymes and exceptional adaptability to fluctuating environmental conditions (Nema *et al.,* 2015; Ugras *et al.,* 2024).

Endoglucanases are versatile enzymes with extensive industrial applications including cellulosic biomass conversion to biofuels, pulp and paper manufacturing, textile bio-polishing, food processing and pharmaceutical industries (Pal and Chakraborty, 2021).In the food industry; they improve product quality and extraction processes, such as enhancing juice clarity and reducing bitterness in citrus foods. In pulp and paper manufacturing, they enhance fiber properties, drainage, and brightness while lowering energy consumption. The textile sector utilizes them for bio-stoning denim, refining fabric texture, and improving garment appearance (Grata, 2020).In the conversion of cellulose into simple sugar, maintaining precise control of pH and temperature is crucial, as even minor environmental fluctuations can lead to enzyme denaturation and a consequent loss of enzymatic activity. The stability of endoglucanase under extreme conditions, such as high temperatures and alkaline pH, makes endoglucanases particularly valuable in industries (Yennamalli *et al.,* 2013; Nagl *et al.,* 2021).

**Corresponding author:* e-mail: saqader@uok.edu.pk

Apart from industrial applications, the endoglucanase gains wide importance in the inhibition of biofilms produced by pathogenic microorganisms. Endoglucanase enzymes have emerged as promising agents for disrupting bacterial biofilms, particularly those formed by highly pathogenic microorganisms such as *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Biofilms, protected by an extracellular polymeric substance (EPS) matrix, provide these bacteria with resilience against environmental stressors and antimicrobial agents, often leading to chronic infections and increased resistance. *P. aeruginosa* biofilms, for instance, are major contributors to persistent infections in burn wounds and other clinical settings, significantly delaying healing and increasing morbidity (Kalia *et al.,* 2023). Endoglucanase specifically targets the polysaccharide component of the EPS, breaking it down and reducing the molecular weight while increasing reducing sugar production.

This enzymatic action promotes biofilm detachment and inhibits biomass formation. Studies have shown that endoglucanase effectively disrupts biofilms on abiotic surfaces, such as glass, and significantly reduces biofilm formation by these pathogenic cultures. Its targeted activity and environmentally friendly nature make endoglucanase a valuable tool in biofilm control, offering a novel approach to combating biofilm-associated infections and enhancing clinical treatment strategies (Ibrahim *et al.,* 2021).

Despite the increasing demand for cellulases, including endoglucanases, Pakistan remains heavily reliant on imports to fulfill industrial requirements. The reliance on foreign sources not only escalates production costs but also imposes a significant burden on the national economy. To address this issue, it is imperative to develop indigenous solutions by exploring local microbial strains capable of producing thermostable and alkaline-active as well as pathogenic biofilm degrading endoglucanases. This approach will not only reduce dependence on imports but also promote research and development, fostering growth in Pakistan's biotechnology and industrial sectors (Riaz *et al.,* 2014).

In the previous study, endoglucanase producing *Bacillus cereus* ARA-12 was isolated, the conditions for the production of enzyme were optimized and the potential of endoglucanse in the production of second generation biofuel was evaluated (Siddiqui *et al.,* 2024). The present study addresses the need for locally produced endoglucanases by investigating the production and partial purification of a thermostable and alkaline-active endoglucanase from *Bacillus cereus* ARA-12. This study aimed to produce and characterize endoglucanase from a novel *Bacillus cereus* strain ARA-12 and evaluate its potential application in disrupting bacterial biofilms. The investigation focused on determining the kinetic properties of the enzyme, and assessing its potential against biofilms, which are the major challenges in clinical and industrial settings due to their resistance to conventional treatments.

MATERIALS AND METHODS

Materials

Bacterial cultures of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were collected from Dr. Essa Laboratory, Karachi, Pakistan. All chemicals, reagents and media used in the study were of analytical grade.

Partial purification of endoglucanase

The isolated *Bacillus cereus* ARA-12 was cultivated in optimized culture media and batch fermentation was done as described previously (Siddiqui *et al.,* 2024). After fermentation, the broth was centrifuged at 10,000 rpm for 20 minutes at 4°C to separate the biomass. The crude enzyme was precipitated with 60% ammonium sulfate using salt fractionation method and the precipitates were dialyzed for 24 hours against Tris-HCl buffer of 1mM ionic strength at 4°C to remove salt molecules from the precipitated enzyme (Zumstein, 1976).The total protein content of the precipitates was determined using the Bradford dye-binding method (Bradford, 1976). The endoglucanase units in the partially purified samples were quantified by spectrophotometric analysis of substrate hydrolysis. One unit of enzyme activity was defined as "the amount of endoglucanase enzyme liberates one µmol of reducing sugar per minute from 1% substrate (carboxymethyl cellulose) at 50℃ and pH 8" (Rahman *et al.,* 2018).

Characterization of partially purified enzyme

Following parameters were analyzed for the applications of partially purified endoglucanase enzyme. In all steps of the endoglucanseenzyme assay was performed as described in the previous isolation studies of *Bacillus cereus* ARA-12. (Siddiqui *et al.,* 2024).

Effect of Substrate Concentration

The enzyme activity was assessed by incubating the enzyme with carboxymethyl cellulose at concentrations ranging from 1–20 mg/mL.Kinetic parameters, including the Michaelis-Menten constant (Km) and maximum velocity (Vmax), were determined using the Lineweaver-Burk plot (Lineweaver and Burk, 1934).

Effect of Temperature and Thermostability

Enzyme activity was measured at temperatures ranging from 30–100°C by endoglucanase assay to determine the optimal temperature. Thermostability was evaluated by pre-incubating the enzyme at 50, 60, and 70°C for varying time intervals (0, 0.5, 1, 1.5, and 2 hours). Residual activity was measured with 0-hour incubation as the control.

Table 1: Purification profile of endoglucanase produced by *Bacillus cereus* ARA-12

Steps of Purification	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification fold	Yield (%)
Crude extract	1873.81	120	13.115	1	100
$(NH_4)_2SO_4$ precipitates	1272.55	18	70.697	5.39	67.91
Desalted precipitates	1098.23	12	91.519	6.97	58.60

Effect of pH

The effect of pH on enzyme activity was examined using buffers with pH ranges: Acetate buffer for pH 3–5, phosphate buffer for pH 6–7, Tris-HCl buffer for pH 8–9, and glycine-NaOH buffer for pH 10–12 were used. Endoglucanase assay protocol was followed to determine catalytic activity.

Effect of Reaction Time

The reaction time for enzymatic activity was optimized. The partially purified endoglucanase was incubated with carboxymethyl cellulose for 5, 10, 15, 30, 45, and 60 minutes. Activity was measured by endoglucanase assay.

Effect of Metal Ions

The influence of metal ions (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Cr^{3+}, and Pb^{2+}) on enzyme activity was assessed by pre-incubating the enzyme with each ion at concentrations of 1 mM and 5 mM for 30 minutes. Residual activity was determined by endoglucanse assay.

Effect of Inhibitors

The effect of inhibitors, including ethylenediaminetetraacetic acid (EDTA), indole-3-acetic acid (IAA), and urea, was evaluated by pre-incubating the enzyme with each inhibitor at a 1 mM concentration for 30 minutes. Residual activity was calculated, with untreated enzyme activity set as 100%.

Effect of Detergents

The enzyme activity in the presence of detergents (SDS, Tween 80, and Triton X-100) was determined by pre-incubating the enzyme with each detergent separately. Enzyme activity was measured using endoglucanase assay, with untreated enzyme activity considered as the control (100%).

Anti-biofim efficacy of partially purified endoglucanase

Biofilm formation assay

Biofilm formation assay was performed by the colorimetric microtiter plate assay. The overnight cultures of 1 x 10^6 CFU/200 µl of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* grown in nutrient broth was added to each well separately and incubated for 12, 24 and 36 hours. After incubation, the supernatant were aspired from the wells followed by washing with 200 µl of phosphate-buffered saline pH 7.3. To fix the formed biofilm, 99% methanol was added for 15 minutes in each well. The methanol was removed and wells were stained by 200 µl 0.1% crystal violet for 5 minutes followed by rinsing with distilled deionized water. The OD was recorded at 595 nm in a microtiter plate reader (Kamali *et al.,* 2021).

Inhibition of biofilm by endoglucanase enzyme

The inhibition of Biofilm by endoglucanase was performed with a slight modification. 100 µl of each culture was mixed with 100 µl of partially purified endoglucanase and incubated for 12, 24 and 36 hours in the microtiter plate. The same procedure was followed as describe above and the biofilm percentage of inhibition was calculated as described by Li *et al.* (2022). The samples without the addition of enzyme were considered as control for each strain separately. The inhibition rate of control is considered as 0% (Kamali *et al.,* 2021).

Eradication of biofilm by endoglucanase enzyme

The biofilm eradication efficacy of endoglucanase enzyme was also performed. The 100 µl of selected strains were allowed to form biofilm as described above. After the fixation of biofilm, the 100 µl of endoglucanase enzyme was added in each well except control and incubated for 12, 24 and 36 hours. The further process was carried out as per standard procedure and the percentage of biofilm eradication was calculated (Li *et al.,* 2024). In the colorimetric microtiter plate assay for biofilm inhibition and eradiction, samples without the addition of the enzyme were used as the negative control for each strain. The inhibition and eradication rate for the control samples was set at 0%, following the method described by Kamali *et al.* (2021). The absorbance values of the test samples were determined by calculating the difference between the absorbance of the control and the test samples, with the control serving as the baseline for comparison. The scanning electron microscopy of control and test was performed with the sample showed best eradication efficiency.

STATISTICAL ANALYSIS

All experiments were conducted in triplicate, and the results are presented as mean values with standard error (SE). Error bars on the bar graphs represent the standard error of the mean, illustrating the variability among replicates.

RESULTS

Partial Purification of Endoglucanase

The endoglucanase enzyme was produced and partially purified from *Bacillus cereus* ARA-12 under batch

fermentation conditions. The enzyme was partially purified, with a yield of 58.6% and a 6.97 purification fold. This purification process significantly enhanced the enzyme's catalytic efficiency, increasing its specific activity from 13.115 U/mg to 91.519 U/mg (table 1).

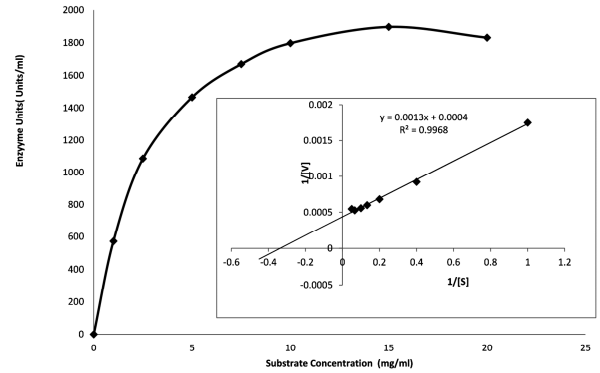

Fig. 1: Enzyme kinetics of endoglucanase (Lineweaver-Burk plot and Michaelis-Menten constant)

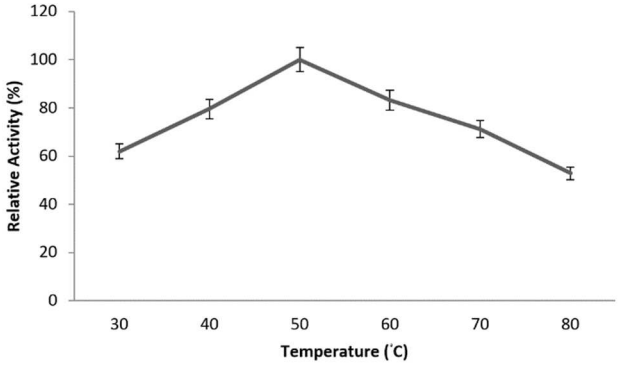

Fig. 2: Optimum temperature for enzyme activity

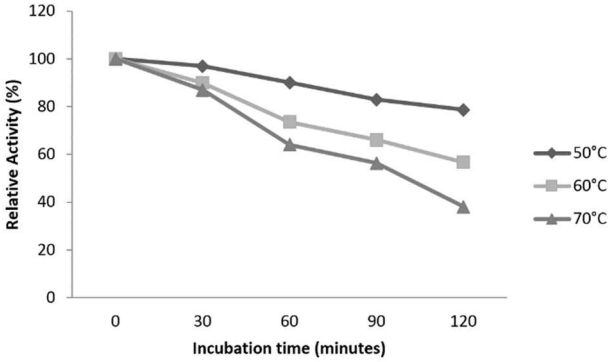

Fig. 3: Thermostability of enzyme at various temperatures

Characterization of Partially Purified Endoglucanase

Following characteristics were analyzed to characterize the partially purified endoglucanase,

Effect of Substrates and Enzyme Kinetics

In this study, values of Vmax and Km were determined by Lineweaver-Burk plot. Results indicated that the partially purified endoglucanase exhibited a K_m of 3.25 mg/ml, indicating its affinity for the substrate, and a V_{max} of 2500 µmol/min, showing its catalytic potential under optimal conditions (fig. 1).

Effect of Temperature and Thermostability

To determine the optimum temperature for endoglucanase produced by *Bacillus cereus* ARA-12, enzyme activity was assessed across a temperature range of 30–80ºC. Maximum activity was observed at 50ºC, with over 80% activity retained at 60ºC. However, enzyme activity began to decline above 60ºC (fig. 2). Thermostability was tested by pre-incubating the enzyme at 50ºC, 60ºC, and 70ºC for 0-120 minutes, followed by activity analysis. The enzyme showed remarkable thermostability, retaining over 80% activity at 50ºC after 120 minutes of incubation (fig. 3). The enzyme maintained high stability at all tested temperatures up to 30 minutes.

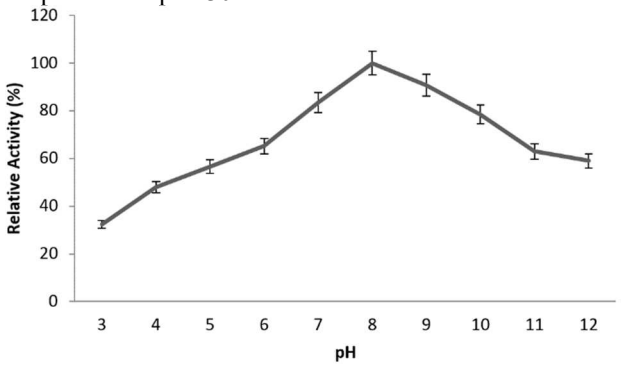

Fig. 4: Optimum pH for enzyme activity

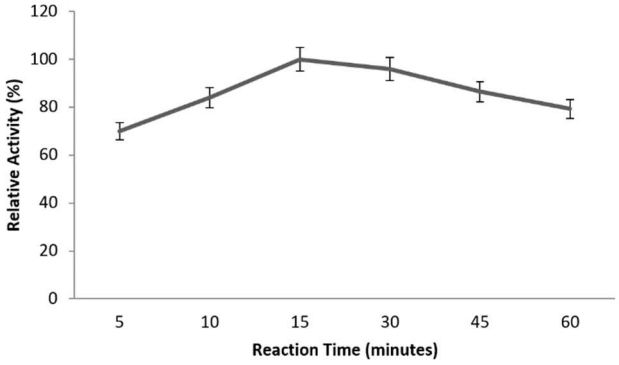

Fig. 5: Effect of reaction time on enzyme activity

Effect of pH on enzyme activity

In this study, endoglucanase activity was assessed in buffers with pH ranging from 3 to 12. The enzyme exhibited the highest activity at pH 8, with substantial activity maintained between pH 7 and pH 10 (fig. 4). Endoglucanase activity decreased at acidic pH but retained about 80% activity at pH 7 and 10.

Effect of reaction time on enzyme activity

Enzyme-substrate reaction time was analyzed by incubating the enzyme with the substrate for various time intervals. The results showed that enzyme activity increased up to 15 minutes, after which it declined with longer incubation times (fig. 5).

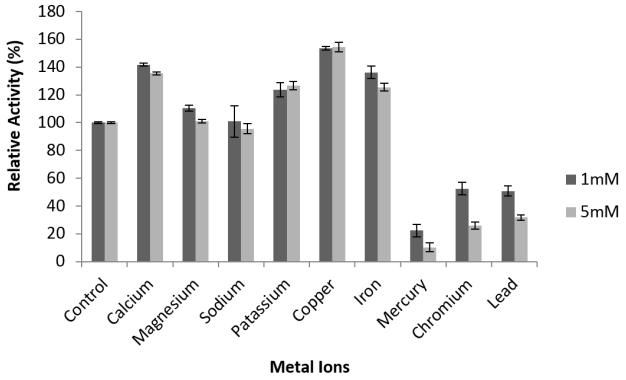

Fig. 6: Effect of metal ions on enzyme activity

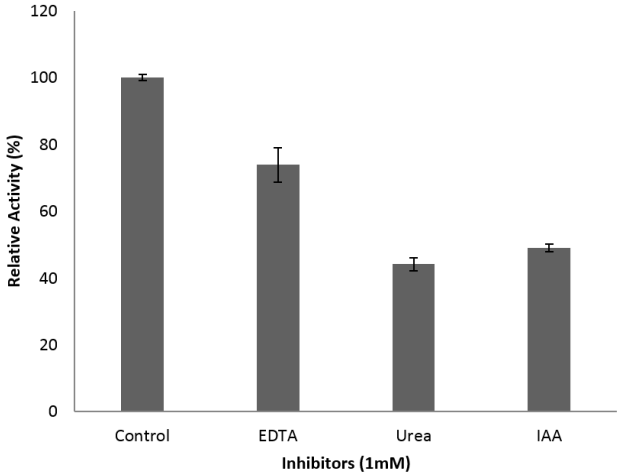

Fig. 7: Effect of inhibitors on enzyme activity

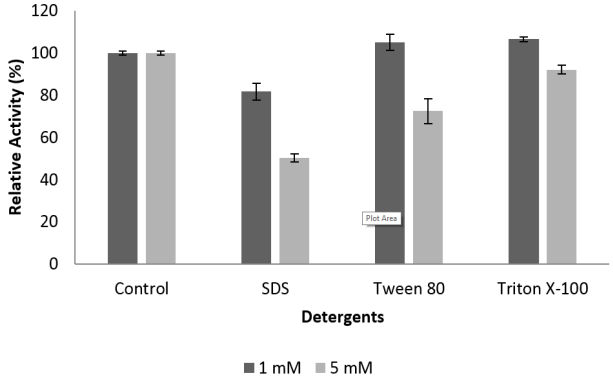

Fig. 8: Effect of detergents on enzyme activity

Impact of Metal Ions on enzyme activity

The impact of different metal ions on endoglucanase activity was investigated. The enzyme activity was enhanced in the presence of 1 mM concentrations of calcium, magnesium, potassium, and iron. Moreover, the enzyme's activity remained consistent with 1 mM sodium ions, although higher concentrations did not result in a notable increase (fig. 6). Conversely, mercury, chromium, and lead inhibited the enzyme's activity, with mercury causing more than 70% inhibition, while chromium and lead resulted in approximately 50% inhibition at 1 mM concentrations.

Impact of Inhibitors and detergents on Enzyme Activity

The influence of inhibitors on the enzyme was evaluated by incubating it with EDTA, urea, and indole-3-acetic acid (IAA) and measuring the remaining activity. At a concentration of 1 mM, all three inhibitors significantly reduced the activity of endoglucanase (fig. 7). To examine the effect of detergents, the enzyme was pre-incubated with commonly used detergents such as Tween 80, Triton X-100, and SDS. The enzyme maintained full activity in the presence of 1 mM Tween 80 and Triton X-100, while retaining approximately 80% of its activity with 1 mM SDS (fig. 8).

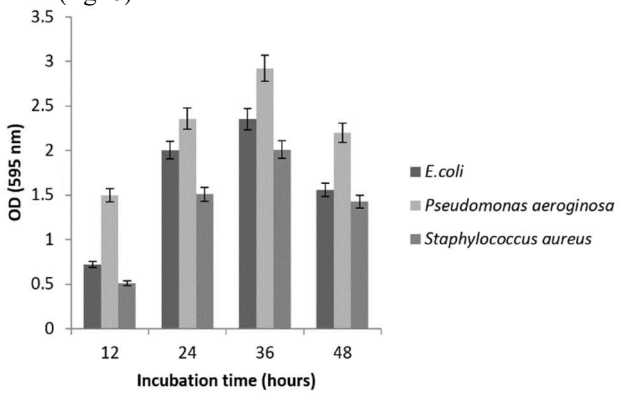

Fig. 9: Production of biofilm by pathogenic strains

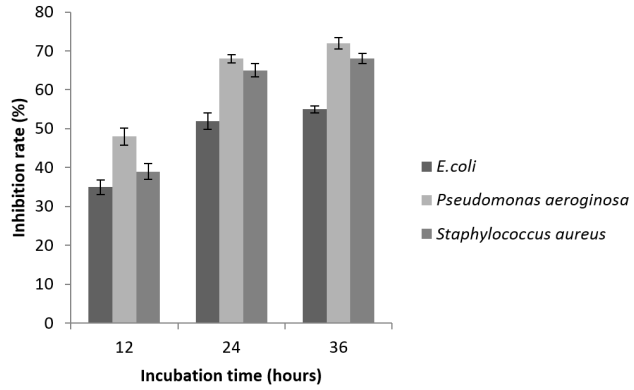

Fig. 10: Inhibition percentage of bacterial biofilm production by endoglucanase

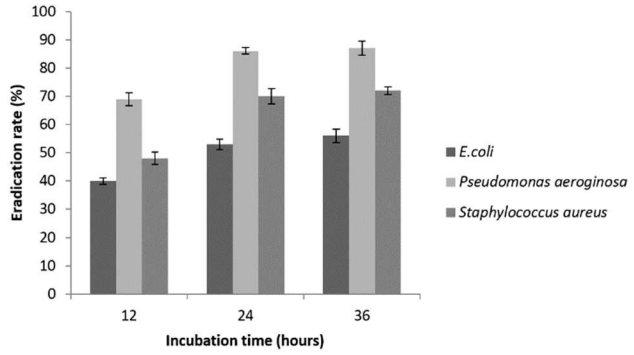

Fig. 11: Eradication of bacterial biofilm by endoglucanase

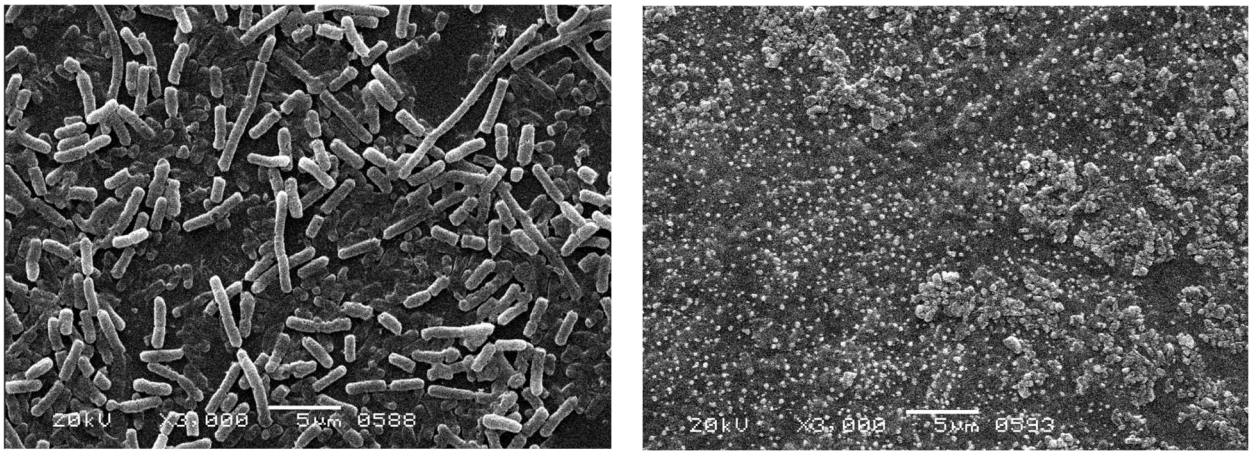

Fig. 12: Scanning electron microscopy (A) Biofilm formed by *Pseudomonas aeruginosa* (B) Eradication of biofilm by endoglucanase enzyme

Anti-biofilm potential of endoglucanase

The anti-biofilm potential of the partially purified endoglucanase was determined by the inhibition and eradication studies of biofilms produced by *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus.*

Biofilm formation by pathogenic strains

All three pathogenic strains of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* showed strong biofilm production under the given conditions. Maximum biofilm production was observed in the sample of 36 hours of incubation with *Pseudomonas aeruginosa* (fig. 9).

Inhibition of Biofilm by endoglucanase enzyme

The potential of the partially purified endoglucanase enzyme to inhibit biofilm was investigated. Results showed the decreased biofilm production by the addition of endoglucanase enzyme along with *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus.* Maximum anti-biofilm efficiency of endoglucanse enzyme was observed with *Pseudomonas aeruginosa* with 72% inhibition after 36 hours of incubation (fig. 10).

Eradication of Biofilm by endoglucanase enzyme

The biofilm detachment by endoglucanase enzyme was observed at various intervals of 12, 24 and 36 hours. Results showed that endoglucanase showed high detachment activity of 72 and 87% for *Pseudomonas aeruginosa* and *Staphylococcus aureus* at 36 hours respectively. The biofilm produced by *Escherichia coli* was also detached up to 56% after 36 hours of incubation by the partially purified endoglucanase enzyme (fig. 11). Scanning electron microscopy showed distinct difference in the eradication of biofilm produced by *Pseudomonas aeruginosa* by the action of endoglucanase (fig. 12). All experiments assessing biofilm eradication and inhibition were conducted in triplicate to ensure reproducibility and reliability of the data. The results are presented as mean values accompanied by their standard error (SE), providing a clear representation of the data's variability. The error bars displayed on the bar graphs indicate the standard error of the mean, illustrating the consistency among replicates and the precision of the measurements.

DISCUSSION

The potential of enzymes in addressing industrial and clinical challenges continues to expand, with endoglucanases gaining recognition for their multifaceted applications. This study highlights the partially purified endoglucanase from *Bacillus cereus* ARA-12 as a promising candidate for both industrial biocatalysis and biofilm inhibition.

The partial purification of endoglucanase is crucial for industrial applications, as crude enzymes often contain impurities that can negatively affect stability and specificity. In contrast, partially purified enzymes demonstrate increased efficiency, stability, and selectivity, making them more desirable for commercial use in various biotechnological applications such as bioconversion of lignocellulosic biomass, biofuel production and the inhibition of biofilms produced by pathogenic bacterial strains (Zahariev, 2024).

Partial purification is a cost-effective approach that retains sufficient enzyme activity while minimizing time, cost, and yield losses associated with complete purification. Studies have shown that partially purified enzymes often exhibit comparable stability and functionality to fully purified ones. For instance, *Pseudomonas aeruginosa* biofilms were effectively inhibited by partially purified endoglucanase (Ibrahim *et al.,* 2021), and similar findings were reported for cellulase applications in biofilm degradation (Kamali *et al.,* 2021). From an economic perspective, partial purification aligns with industrial biotechnology's emphasis on cost reduction and sustainability, maintaining efficiency while minimizing environmental and financial costs (Patel and Shah, 2021). In the present study, purification fold of 6.97, though lower than highly purified enzymes, remains sufficient for many

practical applications. Contaminants in partially purified preparations can influence enzyme performance either positively, by stabilizing the enzyme under harsh conditions, or negatively, by inhibiting activity through competitive binding (Salehi and Asoodeh, 2022). However, the observed activity of *Bacillus cereus* ARA-12 endoglucanase suggests minimal interference from contaminants. The effectiveness of enzyme in biofilm inhibition further supports its applicability without requiring extensive purification. Additional purification steps often increase costs without necessarily enhancing performance, the current purification level is a practical balance between functionality and economic feasibility for industrial and therapeutic applications (Grata, 2020).

The kinetics studies of the enzymes, including the Km and Vmax, are critical for assessing enzyme efficiency and substrate affinity. In this study, K_m of 3.25 mg/ml, indicating its high affinity for the substrate, and a V_{max} of 2500 µmol/min that reflects the maximum reaction rate when the substrate concentration reached to the maximum (Choi *et al.,* 2017). The kinetic parameters of the endoglucanase from *Bacillus cereus* ARA-12 demonstrated superior performance compared to several previously reported endoglucanases. The enzyme's lower *K*m value indicates a higher substrate affinity, surpassing those reported for other endoglucanases, such as *K*m values of 9.3 mg/mL (Alnoch *et al.,* 2023) and 12 mg/mL (Patel and Shah, 2021). In terms of *V*max, the endoglucanase from *Bacillus cereus* ARA-12 exhibited notably higher catalytic efficiency. For instance, the *V*max of endoglucanase from *Bacillus safensis* CF99 was reported as 53.88 µmol/min (Salehi and Asoodeh, 2022), while another study documented a *V*max of 556.5 µmol/min for endoglucanase produced by *Fomitopsismeliae* CFA2 (Patel and Shah, 2021). These findings underscore the exceptional catalytic potential of the *Bacillus cereus* ARA-12 endoglucanase. These findings differ from previous studies, where higher Km values of 7.2 mg/ml (Rawat *et al.,* 2012) was reported. However, the thermostable cellulase from *Bacillus licheniformis* PANG Lexhibited a Km of 1.8 mg/ml and a Vmax of 10.92 µg/ml/min (Shyaula *et al.,* 2023). Therefore the findings emphasize the importance of partially purified endoglucanase of *Bacillus cereus* ARA-12 for rapid carboxymethyl cellulose hydrolysis in industrial applications (Patel and Shah, 2021).

The optimum temperature is crucial in enzyme-catalyzed reactions, influencing reaction rates and enzyme stability. Our results demonstrated that the partially purified endoglucanase is thermostable, retaining over 80% activity at 50°C for 120 minutes, making it suitable for industrial applications (Kabir *et al.,* 2023). Thermostable enzymes enhance substrate solubility and minimize denaturation risks, benefiting industries like textiles, food processing, and paper manufacturing (Liu *et al.,* 2021). Notably, the thermostability of *Bacillus cereus* ARA-12 endoglucanase compares well with other reported enzymes, such as *Talaromycesemersonii* Egl5A, which retains full activity at 70°C for 1 hour, and *Stegonsporiumopalus* Cel5, which loses over 80% activity within 10 minutes at the same temperature (Zheng *et al.,* 2019). Similarly, an *Aspergillus niger* endoglucanase exhibited enhanced thermostability in high salt conditions (Cai *et al.,* 2022). While some endoglucanases show superior stability at higher temperatures, *Bacillus cereus* ARA-12 endoglucanase maintains substantial activity at moderate temperatures, making it ideal for applications requiring prolonged enzymatic function.

The enzyme also exhibited high activity at neutral and alkaline pH, which is advantageous for industrial processes such as detergent formulation, textile treatment, biofuel production, and paper deinking (Yakubu and Vyas, 2023). The combined thermostability and alkaline stability of *Bacillus cereus* ARA-12 endoglucanase underscore its potential for diverse industrial applications, eliminating the need for extensive protein modifications (Hussain and Leong, 2023).

Metal ions play a significant role in enzyme catalysis by either enhancing or inhibiting enzyme activity, and by maintaining enzyme structure. In contrast to the results of the present study, where Mg^{+2} found as an activator, Sulyman *et al.* (2022) reported Mg2+ as competitive inhibitor of cellulase while Zn^{2+}, Cu^{2+}, Ca^{2+} and Fe^{2+} were found as noncompetitive inhibitors of cellulase. In addition to ionic charge, the ion radius significantly affects enzyme activity and stability. Studies have shown that ions with larger radii exert less influence on catalytic amino acids, whereas smaller radii can more strongly attract charged amino acids. This intense interaction may alter the enzyme's overall conformation, potentially causing damage to the catalytic site and impairing its functionality. These ions can influence enzymatic activity by either activating or inhibiting it through interactions with the amino acid residues; specifically the amine or carboxylic acid groups (De Cassia *et al.,* 2017).Studies have also suggested that conserved glutamate residues at the catalytic site may influence the enzyme's activity upon metal ion binding (Adab *et al.,* 2024).

Endoglucanase from *Bacillus cereus* ARA-12 was inhibited by various inhibitors, including EDTA, which likely interfered with enzyme activity by chelating essential metal ions. As the enzyme is activated by divalent cations, this finding aligns with previous studies on metal-dependent enzymes (Sreedharan, 2023). The enzyme remained stable in the presence of detergents such as SDS, Tween 80, and Triton X-100, indicating its potential for the detergent and textile industries (Jefferson and Copeland, 2024). The reduction in enzyme activity upon exposure to EDTA, urea, and indole acetic acid (IAA) resulted from their interactions with the enzyme's structure and function. The inhibition of endoglucanase by these inhibitors has

been reported, as they disrupt the enzyme's secondary and tertiary structures, leading to the loss of activity (Almarza *et al.,* 2013; Lopata *et al.,* 2019).

Beyond its industrial potential, partially purified endoglucanase exhibited significant anti-biofilm properties. It has been observed that *Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus* formed biofilms that matured between 24-36 hours and started dispersing by 36-48 hours. When endoglucanase was introduced along with bacterial suspensions, biofilm formation significantly decreased. Additionally, biofilm eradication was assessed using a colorimetric microtiter plate assay, with untreated samples serving as controls. The test samples showed notable inhibition, as indicated by lower absorbance values. The enzyme effectively eradicated biofilms after 24 and 36 hours, beyond which eradication rates showed no significant difference. Since biofilm biomass naturally declined after 36 hours, results from both inhibition and eradication assays emphasized endoglucanase's potential as a biofilm inhibitor (Li *et al*., 2022).

Enzyme-substrate specificity is a critical aspect of endoglucanase activity, particularly in its ability to target and degrade the polysaccharide components of the extracellular polymeric substance (EPS) matrix in biofilms. The endoglucanase from *Bacillus cereus* ARA-12 demonstrated specificity towards β-1,4-glycosidic bonds present in polysaccharides such as cellulose and its derivatives, which are major constituents of the EPS matrix in bacterial biofilms. The EPS matrix is a heterogeneous structure composed of polysaccharides, proteins, nucleic acids, and lipids, with polysaccharides often forming the dominant structural scaffold. The observed variation in endoglucanase efficacy against biofilms of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* can be attributed to differences in their extracellular polymeric substance (EPS) compositions. *S. aureus* biofilms are primarily composed of polysaccharides, proteins, and extracellular DNA (eDNA). Notably, the exopolysaccharide poly-N-acetylglucosamine (PNAG) is a significant component, providing structural integrity to the biofilm. Endoglucanases, which hydrolyze β-1,4-glycosidic bonds in polysaccharides, can effectively degrade PNAG, leading to substantial biofilm detachment. This aligns with findings where enzymatic treatments targeting polysaccharides demonstrated significant disruption of *S. aureus* biofilms (Quan *et al.,* 2024). In *P. aeruginosa*, the biofilm matrix is rich in exopolysaccharides such as alginate, Pel, and Psl. These components are crucial for biofilm formation and architecture. The Pel polysaccharide, in particular, is a cationic exopolysaccharide that cross-links with eDNA, contributing to the biofilm's structural stability. Endoglucanases can target these polysaccharides, resulting in effective biofilm disruption (Hou *et al.,* 2018). Conversely, *E. coli* biofilms exhibit a more complex EPS composition, including proteins, lipids, and nucleic acids. The reduced polysaccharide content limits the substrates available for endoglucanase action, resulting in lower detachment efficacy compared to *S. aureus* and *P. aeruginosa*. This complexity contributes to the resilience of *E. coli* biofilms against enzymatic degradation (Martino, 2018).

In this study, the enzyme was particularly effective against biofilms produced by *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli*. The results suggest that the enzyme hydrolyzes the cellulose-like polysaccharides in the EPS matrix, disrupting the integrity of the biofilm. The high catalytic efficiency, as indicated by its low *K*m and high *V*max, further supports its strong substrate affinity and robust activity in targeting these components. Comparatively, other endoglucanases, such as those reported by Salehi and Asoodeh (2022), also exhibit similar specificity for cellulose and related polysaccharides. However, their activity often depends on the structural complexity of the EPS matrix. For instance, endoglucanases from *Fomitopsismeliae* CFA2 and *Bacillus safensis* CF99 have been shown to degrade cellulose effectively but may have limited activity against more complex or highly substituted polysaccharides within the EPS (Patel and Shah, 2021). The enzyme from *Bacillus cereus* ARA-12 appears to compare favorably, as its activity results in significant biofilm inhibition and eradication, suggesting it can efficiently target the critical polysaccharide backbones within the EPS matrix. This broad substrate specificity is consistent with its demonstrated effectiveness in degrading biofilms from diverse bacterial strains. Additionally, synergistic interactions with other enzymes present in the partially purified preparation may enhance substrate degradation, particularly for biofilms containing mixed polysaccharide types. Previous studies have highlighted how such synergistic effects can increase the effectiveness of enzymes in breaking down EPS (Ibrahim *et al.,* 2021). Overall, the enzyme-substrate specificity of *Bacillus cereus* ARA-12 endoglucanase aligns well with its targeted application in biofilm control and compares favorably with other reported endoglucanases, particularly in its ability to act on the polysaccharides critical for biofilm structure. These findings highlight its potential for both therapeutic and industrial applications where robust biofilm degradation is required.

While the partially purified endoglucanase from *Bacillus cereus* ARA-12 demonstrated exceptional thermostability, pH stability, and biofilm inhibition potential, addressing practical considerations enhances its industrial relevance. The cost-efficient partial purification process aligns with industrial demands, offering a balance between economic feasibility and functional activity. Although large-scale production may require stabilizers to maintain activity during storage and transport, these adjustments are standard in enzyme formulations and do not diminish the

enzyme's inherent efficiency (Jefferson and Copeland, 2024). Enzymatic processes are generally considered environmentally friendly; however, ensuring sustainable practices, such as recycling enzyme formulations and using biodegradable buffers, can further reduce any potential environmental footprint (Sulyman *et al.,* 2022). Long-term storage stability is another important consideration. The inclusion of cryoprotectants or stabilizers, such as glycerol or sugars, could extend the enzyme's shelf life, ensuring consistent performance over time (Sreedharan, 2023). Similarly, testing the enzyme's activity across repeated cycles or immobilization strategies could unlock its potential for cost-effective and continuous industrial applications (Adab *et al.,* 2024).

CONCLUSION

This study establishes a strong foundation for the industrial and therapeutic applications of the partially purified endoglucanase from *Bacillus cereus* ARA-12. Its remarkable thermostability, alkaline activity, and significant anti-biofilm potential highlight its suitability for diverse industrial processes, including biofuel production, textile processing, and biofilm inhibition. Future research should focus on engineering the enzyme to enhance its catalytic efficiency and broaden its substrate specificity, potentially expanding its applicability to more complex polysaccharides found in industrial and clinical settings. Additionally, strategies such as immobilization could be explored to improve the enzyme's reusability and stability, particularly under extreme operational conditions. Long-term storage stability, including the development of formulations with cryoprotectants or stabilizers, should be assessed to ensure the enzyme's performance in commercial applications. Pilot-scale studies to evaluate the enzyme's performance under real-world conditions will be critical for its commercialization. Moreover, integrating this enzyme into existing industrial workflows and exploring its synergistic effects with other enzymes or antimicrobial agents could unlock its full potential for therapeutic applications, such as combating biofilm-associated infections. These forward-looking steps will bridge the gap between laboratory findings and practical implementation, paving the way for the enzyme's widespread adoption in sustainable industrial processes and innovative therapeutic strategies.

Conflict of interest

Authors declare no conflict of interest.

REFERENCES

Adab FK, Yaghoobi MM and Gharechahi J (2024). Enhanced crystalline cellulose degradation by a novel metagenome-derived cellulase enzyme. *Sci. Rep.*, **14**: 8560-8569.

Almarza J, Rincón L, Bahsas A and Bahsas F (2013). Urea's effect on the ribonuclease a catalytic efficiency: A kinetic, 1H NMR and molecular orbital study. *Protein J.*, **32**: 118-125.

Alnoch RC, Salgado JCS, Alves GS, de Andrades D, Meleiro LP, Segato F, Berto GL, Ward RJ, Buckeridge MS and Polizeli MM (2023). Biochemical characterization of an endoglucanase GH7 from thermophile *Thermothielavioidesterrestris* expressed on *Aspergillus nidulans*. *Catalysts*, **13**: 582.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**: 248-254.

Cai LN, Xu SN, Lu T, Liu ZJ, Wu BL, Chen YC and Zhang HZ (2022). Salt-tolerant and thermostable mechanisms of an endoglucanase from marine *Aspergillus niger*. *Bioresour.Bioprocess.*,**9**: 44.

Choi B, Rempala GA and Kim JK (2017). Beyond the Michaelis-Menten equation: Accurate and efficient estimation of enzyme kinetic parameters. *Sci. Rep.*, **7**: 1-11.

De Cassia Pereira J, Giese EC, De Souza Moretti MM, dos Santos Gomes AC, Perrone OM, Boscolo M and Martins DAB (2017). Effect of metal ions, chemical agents and organic compounds on lignocellulolytic enzymes activities. *Enzyme Inhib. Activ.*, **29**: 139-164.

Grata K (2020). Determining cellulolytic activity of microorganisms. *Chem. Didact. Ecol. Metrol.*, **25**: 133-143.

Hou J, Veeregowda DH, van de Belt-Gritter B, Busscher HJ and van der Mei HC (2018). Extracellular polymeric matrix production and relaxation under fluid shear and mechanical pressure in *Staphylococcus aureus* bio films. *Appl. Environ. Microbiol.*, **84**: e01516-17.

Hussian CHAC and Leong WY (2023). Thermostable enzyme research advances: A bibliometric analysis. *J. Genet. Eng. Biotechnol.*,**21**: 37-49.

Ibrahim AM, Hamouda RA, El-Naggar NE and Al-Shakankery FM (2021). 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**(1): 9754.

Jefferson CJ and Copeland DA (2024). Enzyme Purification Techniques. *Enzyme Engineering*, Springer, pp.249-267.

Kabir MF and Ju LK (2023). On optimization of enzymatic processes: Temperature effects on activity and long-term deactivation kinetics. *Process Biochemistry*, **130**: 734-746.

Kalia VC, Patel SKS and Lee JK (2023). Bacterial biofilm inhibitors: An overview. *Ecotoxicol. Environ. Saf.*, **264**: 115389.

Kamali E, Jamali A, Izanloo A and Ardebili A (2021). *In vitro* activities of cellulase and ceftazidime, alone and in combination against *Pseudomonas aeruginosa* biofilms. *BMC Microbiol.*, **21**: 347.

Ladeira SA, Cruz E, Delatorre AB, Barbosa JB and Martins MLL (2015). Cellulase production by thermophilic *Bacillus* sp. SMIA-2 and its detergent compatibility. *Electron. J. Biotechnol.*, **18**(2): 110-115.

Li Y, Dong R, Ma L, Qian Y and Liu Z (2022). Combined anti-biofilm enzymes strengthen the eradicate effect of *Vibrio parahaemolyticus* biofilm: Mechanism on cpsA-J expression and application on different carriers. *Foods*, **11**(9): 1305.

Lineweaver H and Burk D (1934). The determination of enzyme dissociation constants. *J. Am. Chem. Soc.*, **56**: 658-666.

Liu M, Mandeep H and Shukla P (2021). Synthetic biology and biocomputational approaches for improving microbial endoglucanases toward their innovative applications. *ACS Omega*, **6**: 6055-6063.

Lopata A, Jójárt B, Surányi ÉV, Takács E, Bezúr L, Leveles I, Bendes ÁÁ, Viskolcz B, Vértessy BG and Tóth J (2019). Beyond chelation: EDTA tightly binds Taq DNA polymerase, MutT and dUTPase and directly inhibits dNTPase activity. *Biomolecules*, **9**(10): 621.

Martino P (2018). Extracellular polymeric substances, a key element in understanding biofilm phenotype. *AIMS Microbiology*, **4**(2): 274-288.

Nagl M, Haske-Cornelius O, Skopek L, Pellis A, Bauer W, Nyanhongo GS and Guebitz G (2021). Biorefining: The role of endoglucanases in refining of cellulose fibers. *Cellulose*, **28**(12): 7633-7650.

Nema N, Alamir L and Mohammad M (2015). Production of cellulase from *Bacillus cereus* by submerged fermentation using corn husks as substrates. *Int. Food Res. J.*, **22**(5): 1831-1836.

Pal N and Chakraborty M (2021). Bioprocess developments for improved cellulase production. *Current Status and Future Scope of Microbial Cellulases*, Elsevier, pp.85-93.

Patel A and Shah A (2021). Purification and characterization of novel, thermostable and non-processive GH5 family endoglucanase from *Fomitopsismeliae* CFA 2. *Int. J. Biol. Macromol.*, **182**: 1161-1169.

Quan L, Maokun S, Yanjun T, Bing L, Zhaodi K, Yingying Y, Zhenbo X, Thanapop S, Liang G, Huzhi S, Yan G, Lingyun Y, Qiang P and Yulong T (2024). Antibiofilm activity and synergistic effects of DNase I and lysostaphin against *Staphylococcus aureus* bio films. *Food Qual. Saf.*, **8**: fyae024.

Rahman MS, Fernando S, Ross B, Wu J and Qin W (2018). Endoglucanase (EG) activity assays. *Methods Mol. Biol.*, **1796**: 169-183.

Rawat R and Tewari L (2012). Purification and characterization of an acid other mophilic cellulose enzyme produced by *Bacillus subtilis* strain LFS3. *Extremophiles*, **16**(4): 637-644.

Riaz A, Idrees I, Ahmad S, Siddiqui A and Qader SAU (2023). Enhanced biosynthesis and purification of proteases from *Bacillus* sp. AI-5 by SmF: A green approach for degradation of peptide bonds in complex proteins. *J. Chem. Soc. Pak.*, **45**(2): 128-135.

Riaz H, Jabbar A, Rashid MH, Riaz S and Latif F (2014). Endoglucanase production by *Humicolainsolens*: Effect of physiochemical factors on growth kinetics and thermodynamics. *Int. J. Agric. Biol.*, **16**: 1141-1146.

Salehi ME and Asoodeh A (2022). Extraction, purification, and biochemical characterization of an alkalothermophilic endoglucanase from bacterial flora in the gastrointestinal tract of *Osphranteria coerulescens* larvae. *Waste Biomass Valorization*, **14**: 1-15.

Sherief AA, El-Naggar NE and Hamza SS (2010). Bioprocessing of lignocellulosic biomass for production of bioethanol using thermotolerant *Aspergillus fumigatus* under solid-state fermentation conditions. *Biotechnology*, **9**(4): 513-522.

Shyaula M, Regmi S, Khadka D, Poudel RC, Dhakal A, K oirala D, Sijapati J, Singh A and Maharjan J. (2023). Characterization of thermostable cellulase from *Bacillus licheniformis* PANG L isolated from the himalayan soil. *Int. J. Microbiol.*, pp.3615757.

Siddiqui A, Zohra RR, Riaz A, Fatima A, Imtiaz A, Ahmad M and Zohra RR (2024). Enzymatic hydrolysis of complex agro wastes by *Bacillus cereus* ARA-12: A sustainable approach for biofuel production. *NUST J. Nat. Sci.*, **9**(1): 23-32.

Sreedharan B (2023). Cellulase characterization and structure-function relationships. *J. Mol. Biol.*, **29**: 321-333.

Sulyman AO, Igunnu A and Malomo SO (2022). Effect of metal ions on the activity of cellulase produced by *Aspergillus niger* using *Arachishypogaea* shells. *Niger. J. Biochem. Mol. Biol.*, **37**(1): 58-63.

Ugras S, Isik Bicen HE and Emire Z (2024). Determination of cellulase enzyme produced by *Bacillus cereus* DU-1 isolated from soil and its effects on cotton fiber. *Braz. Arch. Biol. Technol.*, **67**: e24230391.

Yakubu A and Vyas A (2023). Industrial application of alkaline cellulase enzymes in pulp and paper recycling: A review. *Cellul. Chem. Technol.*, **57**: 17-28.

Yennamalli RM, Thangavel S, Anishetty S, Pennathur G and Singaravelu S (2013). Endoglucanases: Insights into thermostability for biofuel applications. *Biotechnol. Biofuels*, **6**: 136-144.

Zahariev LJ (2024). Revisiting cellulase properties in microbial enzymatic systems. *Curr. Opin. Biotechnol.*, **45**: 55-64.

Zheng F, Vermaas JV, Zheng J, Wang Y, Tu T, Wang X, Xie X, Yao B, Beckham GT and Luo H (2019). Activity and thermostability of GH5 endoglucanase chimeras from mesophilic and thermophilic parents. *Appl. Environ. Microbiol.*, **85**: e02079-18.

Zumstein L (1994). Dialysis and ultrafiltration. *Curr. Protoc. Mol. Biol.*, Wiley, New York.