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# Exploring Fungal Cellulases: Determination Techniques, Properties, And Applications

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**ABSTRACT**:
Cellulases are enzymes that catalyze the hydrolysis of cellulose, a major component of plant cell walls. Fungal cellulases play a significant role in various biological processes, including the breakdown of plant biomass in ecosystems. In industrial applications, these enzymes are increasingly important in the bioconversion of lignocellulosic materials into fermentable sugars for biofuel production and other biotechnological processes. The determination of fungal cellulases is critical for optimizing their use in various industries. This paper reviews the different techniques used for determining fungal cellulases, the properties of these enzymes, and their applications in various sectors, including biofuels, food, textiles, and waste management. Techniques for enzyme determination include spectrophotometric methods, chromatographic analysis, and molecular biology approaches. The properties of fungal cellulases such as substrate specificity, pH, temperature stability, and kinetics are crucial for their industrial application. Additionally, fungal cellulases have shown great promise in diverse applications, including biomass degradation, bioremediation, and production of value-added products. This review aims to provide comprehensive insights into the methods of cellulase determination, their functional properties, and emerging industrial applications. The potential for further research to enhance cellulase activity and efficiency in industrial applications is also discussed.

**KEYWORDS**:
Fungal cellulases, enzyme determination techniques, cellulase properties, biofuel production, industrial applications.

## INTRODUCTION:

Cellulose is the primary structural component of plant cell walls and is the most abundant plant polysaccharide on Earth (Horwath, 2007; Henriksson & Lennholm, 2009). Approximately one-third of the organic matter produced by green plants is cellulose (Bhattacharjee et al., 2025). Cellulosic biomass has attracted considerable attention as a renewable feedstock for bioenergy and the production of bio-based products. Cellulolytic microorganisms, especially filamentous fungi, play a vital role in the enzymatic breakdown of cellulose (Rani et al, 2024).

Structurally, cellulose is a linear polymer composed of glucose units linked by β-1, 4-glycosidic bonds (Joseph et al., 2020; Zhang et al., 2024). A single cellulose polymer chain can consist of more than 10,000 D-glucose units (Zhu et al., 2025). The complexity of native cellulose arises from its close association with other plant cell wall components, such as hemicellulose, lignin, and pectin (Martínez-Sanz et al., 2015). The biological degradation of cellulose is one of the

most extensive mass hydrolytic processes occurring in nature (Hawksworth, 1991; Jayasekara & Ratnayake, 2019).

Fungi are considered the primary producers of cellulases, although certain bacteria and actinomycetes also exhibit cellulolytic activity (Bahatkar et al., 2023). These fungi are known for their ability to colonize a wide range of substrates and produce various bioactive metabolites, including cellulases. The enzyme has found diverse applications in industries such as pulp and paper, textiles, laundry detergents, biofuel production, food and feed processing, brewing, and agriculture (Kuhad et al., 2011). Cellulases exhibit high thermal stability compared to other plant cell wall-degrading enzymes, making them highly suitable for industrial use (Ejaz & Ghanemi, 2021). Globally, cellulases are the third most widely used industrial enzymes, with demand steadily increasing (Jayasekara & Ratnayake, 2019). Their commercial value lies in their efficiency in converting lignocellulosic biomass into glucose via enzymatic hydrolysis, which can be further processed into value-added products such as ethanol (Devi et al., 2022).

**Cellulases – Enzyme Complex:**

Organisms capable of efficiently degrading cellulose are known to produce a complex of enzymes collectively referred to as the *cellulase enzyme complex* (Bayer et al., 2006). Research into cellulases began during World War II, prompted by a biodeterioration issue encountered by the U.S. military in the South Pacific. Tents, sandbags, soldiers' uniforms, and other textiles were found to be deteriorating due to fungal activity. In response, several U.S. Army-associated organizations established research facilities to investigate and mitigate the problem. The fungus responsible was later identified as *Trichoderma viride* (Reese, 1976).

As investigations advanced, researchers discovered the potential industrial utility of cellulases—particularly in the conversion of cellulosic waste into glucose, soluble sugars, and other valuable chemicals. Since then, a wide variety of cellulolytic microorganisms capable of producing cellulases have been isolated (Ljungdahl, 2009; Béguin & Aubert, 1994; Liu et al, 2021).

Efforts to enhance cellulase production have involved various approaches, including mutation, protoplast fusion, and genetic engineering (Gosavi, 2024; Papzan et al, 2021). Much of this research has been motivated by the promising industrial applications of cellulases across numerous sectors.

**Cellulolytic System:**

Reese and Levinson (1952) proposed a two-enzyme hypothesis, known as the C1–Cx concept, to explain the enzymatic breakdown of cellulose. Their model suggested the following sequence:

Cellulose → Reactive Cellulose → Cellobiose → Glucose

| **Stage** | **Enzyme Involved** | **Type of Reaction** |
|---|---|---|
| Cellulose → Reactive Cellulose | C1 | Unknown (possibly non-hydrolytic) |
| Reactive Cellulose → Cellobiose | Cx | Hydrolytic |
| Cellobiose → Glucose | β-glucosidase | Hydrolytic |

This conceptual model became foundational for subsequent research. It highlighted the synergistic action between C1 and Cx enzymes. Cx enzymes alone could not effectively

solubilize native cellulose (e.g., cotton fibers). However, Wood and McCrae (1979) demonstrated that supplementing Cx-rich fungal filtrates with C1 (extracted from *Trichoderma koningii* or *Fusarium solani*) led to significant solubilization of cotton.

Chromatographic studies further revealed that Cx is not a single enzyme but a complex of multiple enzymes with varying activities (Reese & Gilligan, 1953; Gilligan & Reese, 1954; Halliwell & Riaz, 1970).

With advances in purification and analytical techniques, a more refined understanding of the cellulolytic system emerged. C1 activity was later identified as Cellobiohydrolase (CBH), an enzyme that removes cellobiose units from the non-reducing ends of cellulose chains (Halliwell & Griffith, 1973; Wood & McCrae, 1979). Based on this, Reese (1976) proposed a revised mechanism of cellulose degradation:

**Updated Model of Cellulose Degradation**:

Crystalline Cellulose → Modified Cellulose → Cello-oligosaccharides → Cellobiose → Glucose

| **Enzyme** | **Action** |
|---|---|
| Endoglucanase (EC 3.2.1.4) | Randomly cleaves internal β-1,4 bonds in amorphous regions of cellulose |
| Cellobiohydrolase (CBH) (EC 3.2.1.91) | Removes cellobiose units from reducing and non-reducing ends |
| β-Glucosidase (EC 3.2.1.21) | Hydrolyzes cellobiose to glucose |
| Glucohydrolase (EC 3.2.1.74) | Degrades cello-oligosaccharides to glucose |

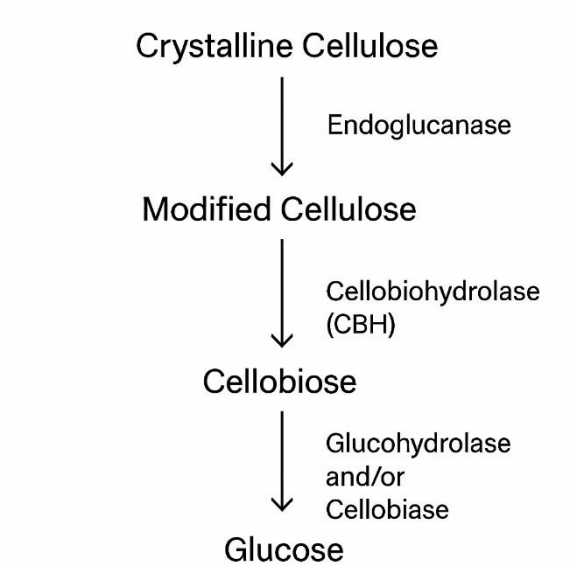
Eveleigh (1987) emphasized that β-glucosidase plays a critical role in completing the hydrolysis process. Systems with low β-glucosidase activity have reduced saccharification efficiency, as cellobiose accumulation can inhibit both CBH and endoglucanase activities (Wahal, 1998, Gosavi & Bagool, 2013).

**Oxidative Mechanisms in Cellulose Degradation:**

In addition to hydrolytic enzymes, oxidative enzymes also contribute to cellulose degradation. Eriksson (1978) proposed that oxidative processes may initiate or enhance the breakdown of cellulose. Key enzymes include:

- Cellobiose oxidase (EC 1.1.99.18)
- Cellobiohydrolase (EC 1.1.5.1) — oxidative variant
- Lactonases [D-glucano-1,5-lactonohydrolase (EC 3.1.1.17)]

These oxidative enzymes, isolated from fungi such as *Phanerochaete chrysosporium*, may induce hydrolytic enzyme expression or help remove inhibitory compounds (Zhang et al., 2015).




**Techniques for Determining Fungal Cellulases:**

The determination of fungal cellulases involves measuring their activity, stability, and efficiency in various conditions. Several techniques are available for this purpose, each with its strengths and limitations.

1. **Enzyme Assay:**

The cellulolytic activity of the filtrate was determined using the method described by Ghose (1987). The total endoglucanase activity was assayed by measuring the amount of reducing sugars released from carboxymethyl cellulose (CMC), while exoglucanase activity was assessed using cotton as the substrate. The production of cellulase enzymes by the fungal organism is induced only in the presence of a cellulosic substrate (Gosavi & Bagool, 2013).

Previous studies have shown that cotton is the most suitable substrate for exoglucanase activity, whereas CMC is optimal for analyzing endoglucanase activity (Teeri, 1997; Enari, 1983; Soni & Soni, 2010; Sulyman et al., 2020).

The protein concentration was determined by the method of Lowry et al. (1951), using Bovine Serum Albumin (BSA) as the standard. A standard curve was used to estimate the protein concentration. The enzyme activities were calculated based on the amount of reducing sugars released, measured using 3,5-dinitrosalicylic acid (DNSA) reagent (Mandels et al., 1976).

**Exoglucanase Activity**

Dewaxed cotton served as the substrate for assessing the exoglucanase activity. The procedure followed the methods of Bucheli et al (1985) and Wood & Bhat (1988). The assay mixture consisted of:

- 50 mg of absorbent cotton
- 0.5 mL of 0.05 M citrate buffer (pH 4.8)
- 1 mL of enzyme extract

The reaction mixture was incubated at 50°C for 24 hours. A blank control was maintained under identical conditions. The total cellulase activity was determined by measuring the amount of reducing sugars released from cotton.

**Endoglucanase Activity**

Carboxymethyl cellulose (CMC) was used as the substrate to determine endoglucanase activity, following the protocols of Bucheli et al (1985) and Wood & Bhat (1988). The assay mixture included:

- 0.5 mL of 1% CMC (prepared in 0.05 M citrate buffer, pH 4.8)
- 1 mL of enzyme extract (appropriately diluted, if needed)

The mixture was incubated at 50°C for 30 minutes. To each tube, 3 mL of DNSA reagent was added, and the tubes were placed in a boiling water bath for 10–15 minutes, then cooled to room temperature. The final volume was adjusted to 20 mL using distilled water.

Triplicate blanks were prepared and treated similarly. Absorbance was measured at 540 nm using a colorimeter. Enzyme activity was expressed as mg of reducing sugars per mg of protein.

**Definition of Enzyme Activity**

The activity of exoglucanase and endoglucanase was defined in International Units (IU), where one unit is the amount of enzyme that releases 1 µmol of glucose per minute under assay conditions (Patil & Talhande, 2023).

## 2. Chromatographic Techniques for Determining Fungal Cellulases:

Several chromatographic techniques were employed to analyze, isolate, and characterize the fungal cellulase enzymes. These methods help in determining enzyme purity, molecular weight, isoforms, and activity. The most commonly used methods include ion exchange chromatography, gel filtration chromatography, affinity chromatography, and high-performance liquid chromatography (HPLC).

**Ion Exchange Chromatography (IEC):** Ion exchange chromatography was utilized to fractionate cellulase components based on their net surface charge. Crude fungal enzyme extract was passed through a DEAE-cellulose column equilibrated with 50 mM sodium acetate buffer (pH 5.0), following the methods of Wood and Bhat (1988). Proteins were eluted using a linear NaCl gradient (0–1.0 M), and each fraction was monitored for cellulase activity using the DNSA method (Ghose, 1987). This technique effectively separated endoglucanase and exoglucanase isoforms.

**Gel Filtration Chromatography:** The molecular weights of cellulase enzymes were estimated using gel filtration chromatography on a Sephadex G-100 column, pre-equilibrated with citrate buffer (pH 4.8), as described by Mandels and Weber (1969). The eluted fractions were analyzed for enzyme activity and protein content. This technique helped confirm the oligomeric nature of the enzymes and monitor their purification progress.

**Affinity Chromatography :** For high-purity isolation of cellulases, affinity chromatography was conducted using a cellulose-agarose column, where the enzyme binds specifically to the substrate analog. The bound cellulase was eluted using a 1.0 M NaCl or cellobiose gradient, following the methods adapted from Tuohy et al. (1990). This technique enabled selective recovery of active cellulase components with minimal contaminants.

**High-Performance Liquid Chromatography (HPLC) :** HPLC was used to analyze the sugar products released by cellulase action. Reverse-phase HPLC and ion-exchange HPLC were both employed to separate and quantify glucose, cellobiose, and other oligomers. The analysis was carried out using a Shimadzu HPLC system with a refractive index detector, as per the protocols of Soni and Soni (2010) and Sulyman et al. (2020). The retention times and peak areas were compared with standards to confirm the enzymatic breakdown products.

**Thin Layer Chromatography (TLC):** Thin layer chromatography was used for the qualitative analysis of cellulolytic hydrolysates. Hydrolyzed samples were spotted on silica gel plates and developed in a solvent system of butanol:acetic acid:water (2:1:1). After development, the sugars were visualized using aniline-phthalate reagent and heating, following methods described by Enari (1983).

### 3. Molecular Biology Techniques for Determining Fungal Cellulases:

Molecular biology techniques are essential for identifying, cloning, and expressing fungal cellulase genes, as well as for analyzing gene expression patterns under different environmental conditions. These tools complement biochemical and chromatographic methods by enabling a deeper understanding of the genetic regulation and sequence diversity of cellulase-producing fungi.

**Genomic DNA Isolation:** Genomic DNA was extracted from fungal mycelium using the CTAB method, as described by Doyle and Doyle in 1990 (Tripathy et al., 2017). Mycelia were harvested, frozen in liquid nitrogen, and ground to a fine powder. The powder was suspended in CTAB extraction buffer, and DNA was purified through chloroform: iso-amyl alcohol extraction followed by ethanol precipitation (Rygiewicz & Armstrong, 1991). DNA quality and concentration were assessed by spectrophotometry (A260/A280 ratio) and agarose gel electrophoresis.

**PCR Amplification of Cellulase Genes:** Polymerase Chain Reaction (PCR) was employed to amplify cellulase genes using gene-specific primers targeting conserved regions of endoglucanase (eg) and exoglucanase (cbh) genes (Budathoki, 2018). The PCR reaction conditions followed the protocol of Sambrook and Russell (2006), with the following thermal profile: initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, annealing at 55–60°C (depending on primer Tm) for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. Amplified products were visualized using 1.5% agarose gel stained with ethidium bromide.

**Reverse Transcription PCR (RT-PCR):** To evaluate cellulase gene expression under different substrates (e.g., CMC and cotton), total RNA was isolated from fungal cultures using TRIzol reagent, and complementary DNA (cDNA) was synthesized using oligo (dT) primers and reverse transcriptase (Invitrogen kit), as described by Chomczynski and Sacchi (1987). RT-PCR was conducted to compare transcript levels of cellulase genes under inducing and non-inducing conditions.

**Quantitative Real-Time PCR (qRT-PCR):** Quantitative real-time PCR was used to quantify the relative expression of cellulase genes. SYBR Green Master Mix was used in a 96-well plate format (Applied Biosystems StepOnePlus system), and reactions were performed in triplicates. The ΔΔCt method was used for data analysis, with β-actin or GAPDH serving as the internal reference gene (Livak & Schmittgen, 2001).

**Cloning and Sequencing of Cellulase Genes:** PCR products were purified and cloned into a pGEM-T Easy vector (Promega), followed by transformation into *Escherichia coli* DH5α competent cells. Recombinant colonies were screened using blue-white selection and confirmed by colony PCR. Plasmids were extracted using a mini-prep kit (Qiagen), and sequencing was carried out using T7/SP6 primers. The obtained sequences were analyzed using

BLAST and multiple sequence alignment tools (ClustalW) to identify homologous cellulase genes (Hou et al., 2007).

**In Silico Analysis and Phylogenetics :** The deduced amino acid sequences were analyzed for conserved domains using the NCBI Conserved Domain Database (CDD) and Pfam. Phylogenetic trees were constructed using MEGA X software (Kumar et al., 2016) employing the neighbor-joining method with 1,000 bootstrap replicates, revealing evolutionary relationships between fungal cellulases and known glycoside hydrolase families (Tamboli et al., 2017).

**Properties of Fungal Cellulases**

The properties of fungal cellulases are crucial in determining their effectiveness in industrial applications. Some of the key properties include:

**1. Substrate Specificity:** Fungal cellulases exhibit varying degrees of specificity toward different forms of cellulose, such as amorphous or crystalline cellulose. The effectiveness of fungal cellulases is influenced by the structure of the cellulose substrate, with amorphous cellulose being more easily degraded than crystalline cellulose (Vlasenko et al., 2010 & Payne et al., 2015).

**2. pH and Temperature Stability:** Fungal cellulases typically show a broad range of optimal pH and temperature conditions. For instance, cellulases from *Trichoderma reesei* exhibit optimal activity at slightly acidic pH and moderate temperatures (50–60°C). Understanding these stability profiles is vital for optimizing cellulase activity in industrial processes, such as biofuel production (Barapatre et al., 2020 & Bhardwaj et al., 2021).

**3. Kinetic Parameters:** The catalytic efficiency of fungal cellulases is characterized by kinetic parameters, including the Michaelis constant (Km) and the maximum velocity (Vmax). These parameters are crucial for assessing the efficiency of cellulases in various applications, including enzymatic hydrolysis of cellulose for biofuel production (Babalola et al., 2017 & Hashem et al., 2022).

**Applications of Fungal Cellulases:**

Cellulases constitute a collection of enzyme whose primary function is to hydrolase cellulose into glucoses units. The process of cellulolysis, apart from being looked upon as a 'necessary evil' for the maintenance of the carbon cycle in nature, has found a wide range of applications in wooden technology. The past few years have seen a growing invest in all aspects of cellulases. Today, we are trying to exploit these enzymes for the benefit of mankind.

Commercial applications of cellulases include food processing, animal feed (Chahal & Young, 1982; Hecht et al., 1985; Young, 1985 and Grujic *et al*., 1992*)*, enrichment of proteins (Prendergast & Booth, 1984; Chahal *et al*., 1987 and Carrizales & Saenz, 1988), production of metabolites, fermentation technology and SCP production (Ulmer *et al*., 1980; & Abdulla *et al*., 1985), detergent, paper and pulp, textile industries and environmentally friendly processes (Janshekar *etal*., 1982).

The major applications of cellulases are discussed below:

**1. Biofuel Production:** One of the most significant applications of fungal cellulases is in the production of biofuels, particularly bioethanol (Areeshi, 2022). Fungal cellulases are used to hydrolyze lignocellulosic biomass (e.g., agricultural residues, wood chips, and switchgrass) into fermentable sugars, which are then converted into ethanol by fermentation. The use of

fungal cellulases in biofuel production offers a sustainable and cost-effective method for converting waste biomass into renewable energy (Verma et al., 2021).

**2. Textile Industry:** Cellulases are used in the textile industry for the biopolishing of cotton fabrics (Korsa et al., 2023). Fungal cellulases remove microfibrils from cotton fibers, enhancing fabric softness and improving the appearance by creating a smoother surface. This biotechnological process is environmentally friendly and offers a greener alternative to conventional chemical treatments (Choudhury, 2020).

**3. Food and Beverage Industry:** In the food and beverage industry, fungal cellulases are used in the processing of fruit juices, wine, and other beverages (de Souza & Kawaguti, 2021). The enzymes are used to break down cell walls and release juices, enhancing extraction yields. In addition, cellulases are used to improve the texture and quality of baked goods, such as bread and cakes, by affecting the dough's consistency (Liu et al., 2024).

**4. Bioremediation:** Fungal cellulases are also being explored for use in bioremediation. These enzymes help in the degradation of lignocellulosic waste, such as paper and wood waste, contributing to waste management and environmental sustainability (Vermelho et al., 2012). By breaking down complex pollutants, cellulases can aid in the detoxification of contaminated sites (Sharada et al., 2014).

**5. Paper and Pulp Industry:** In the paper and pulp industry, cellulases are used in the bleaching and softening of paper products (Singh et al., 2016). The use of fungal cellulases reduces the need for harsh chemicals, making the process more environmentally sustainable. Moreover, cellulases help in improving the quality and durability of paper products (Bhati et al., 2021).

## FUTURE PROSPECTS AND CHALLENGES:

Despite the numerous advantages of fungal cellulases, several challenges remain, such as their high production costs and low activity against crystalline cellulose. Research into genetic engineering, enzyme optimization, and the use of mixed enzyme systems may help overcome these challenges. Future developments in enzyme stabilization, cost reduction, and process integration will enhance the industrial applicability of fungal cellulases.

## CONCLUSION:

Fungal cellulases are indispensable enzymes in both natural and industrial processes due to their ability to degrade cellulose. The development of efficient techniques for determining cellulase activity, coupled with an understanding of their properties, is essential for maximizing their industrial potential. Applications in biofuels, textiles, food, and waste management highlight the diverse utility of these enzymes. Ongoing research and innovation will undoubtedly expand the scope of fungal cellulases in various biotechnological applications, making them a key component of sustainable industrial processes.

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