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Research Article

Elucidating the cellulolytic potential of a locally Isolated common mould, *Cladosporium tenuissimum,* by optimizing its cellulase production

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Abstract
Cellulase is a complex of multiple enzymes that includes β-1,4 endoglucanase, β-1,4 exoglucanase, and β-glucosidase, all of which are recognized for breaking down cellulose into glucose monomers. Cellulolytic fungi play a crucial role in the biodegradation of cellulosic biomass by secreting extracellular cellulases. In the present study, soil samples were collected from various ecological niches of VNMKV Campus, Parbhani, Maharashtra (India). A total of 23 fungal isolates obtained after serial dilution were screened to assess their cellulolytic potentials. Qualitative screening using Carboxymethyl cellulose (CMC) and esculin plate assays demonstrated weak cellulolytic activity in other isolates and a strong positive reaction in a single fungal isolate, which was considered the most potent and selected for optimization studies. Optimization studies clearly indicated that there is a significant impact of temperature, pH and incubation period on cellulase production as maximum amount of cellulase enzyme was obtained at 30°C temperature (endoglucanase 147.60±0.1 IU/ml, exoglucanase 74.24±0.02 IU/ml and β glucosidase 80.65±0.2IU/ml**)** at pH 7 (endoglucanase 140.35±0.07 IU/ml, exoglucanase 66.64±0.1 IU/ml and β glucosidase 80.40±0.06 IU/ml)and day 5 incubation period (endoglucanase 143.90±0.1 IU/ml, exoglucanase 68.13±0.06 IU/ml and β glucosidase76.22±0.01 IU/ml) respectively. The isolate exhibited 99.43% homology with *the Cladosporium tenuissimum* strain*,* and the sequence was deposited in National Center for Biotechnology Information (NCBI)GenBank under accession number PV687687. The identified fungal strain holds potential for cellulase enzyme production and may be applied in diverse biotechnological processes, such as lignocellulosic biomass degradation, bioremediation and bioethanol production.

Keywords: Cellulase, *Cladosporium tenuissimum*, Internal transcribed spacer, optimization substrate,

INTRODUCTION

The most prevalent agricultural waste and source of biomass on the terrain is cellulose (Tomme *et al*., 1995). Cellulose, a naturally occurring linear organic polysaccharide, serves as the fundamental structural element of plant cell walls. Cellulose is composed of β-1,4-linked D-glucose units, forming a rigid, crystalline structure that is very difficult to dissolve or hydrolyze under natural conditions. It is the most abundant organic polymer on Earth and serves as the main structural component of plant cell walls. The main source of cellu-

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lose is plant tissue, where it is a major component of the cell wall, providing rigidity, strength, and resistance to mechanical stress (Hussain *et al*., 2017; Egwuatu and Appeh, 2018). Cellulose is resistant to physical decomposition, because of its strong glycosidic bonds. In particular, cellulose is environmentally friendly, multi-utility substrate. Plant cellulosic material is efficiently degraded by enzymes or multienzyme complexes involved in the hydrolysis of cellulose, known as cellulases (Lynd *et al.,* 2002; Jahangeer *et al.,* 2005). The efficient hydrolysis of cellulose requires the concerted action of at least three enzymes: (1) endoglucanases to randomly cleave inter monomer bonds; (2) exoglucanases to remove mono and dimers from the end of the glucose chain; and (3) β-glucosidase to hydrolyze glucose dimers (Deobald and Crawford, 1997). Cellulase enzymes play a critical role in breaking down lignocellulosic biomass, such as rice straw, wheat stubble and corn stalks. These multiple enzymes are secreted by soil-isolated fungi and bacteria that breakdown lignocellulosic biomass (Bruce *et al.,* 2010; Walters *et al.,* 2022). Habitats such as hypersaline lakes, riverine soils, and insects are home to a rich diversity of microbial communities, presenting numerous avenues for future investigation.

(Butinar *et al.,* 2005). Most fungi are capable cellulose degraders. However, their ability to facilitate rapid lignocellulose degradation has attracted attention from scientists and entrepreneurs alike (Malherbe and Cloete, 2002). Fungi outperform bacteria in breaking down lignocellulose because of their unique, more powerful enzyme systems, broader cellulase and hemicellulase diversity, synergistic enzyme secretion, and superior physical penetration and colonization, making them indispensable for biodegradation, nutrient recycling, and composting. The levels of enzymes produced by filamentous fungi are higher than those of yeast and bacteria, making them the preferred choice for commercial enzyme production (Bakri *et al.,* 2003). The degradation of cellulose via fungal cellulase has been well studied. Fungal species known to degrade cellulose encompass members of the *Acomycota* (Hernandez *et al.,* 2013, Schuberg *et al.,* 2016), *Basidiomycota* (Baldrian and Valášková, 2008). Cellulases have been produced and characterized from different aerobic fungi, such as *Aspergillus* (Bansal *et al*., 2012), *Trichoderma* (Ellilä *et al*., 2017), and *Penicillium* (Prasanna *et al.,* 2016). It is well known that *Aspergillus* and *Trichoderma* species effectively produce cellulases (Van Peij *et al.,* 1998; Bhat, 2000; Lynd *et al.,* 2002), and they are among the most prevalent fungi found in composting materials (Ashraf *et al.,* 2007). Several cellulose-degrading fungi, such as *Aspegillus niger, Cladosporium cladosporioides, C. sphaerospermum, Penicillium chrysegenum, scopulariopsis brevicualis, Stachybotrys schartarum, Verticillium cyclosporum, T. reesei* and *Chaetomium hamadae* have been identified for cellulase production based on their habitat (El-Morsy, 2000; Luo *et al.,* 2005, Maria *et al.,* 2005). Over the past 30 years, many research groups have dedicated their efforts to developing, analyzing, and utilizing cellulase enzymes across various industries. The annual sales of cellulase have reached 8% of the total enzyme market and are expected to exceed those of the protease market in the future (Horn *et al.,* 2012). The expense of acquiring commercial cellulase enzymes makes them less attractive, thereby increasing their value. Consequently, numerous research organizations are focusing on the production of cellulase for biomass conversion and biofuel generation, as the cost of cellulase poses a significant barrier to biomass hydrolysis and industrialization. The initial and vital step in enzyme technology is enzyme production, which demands attention because it influences the economic viability of the process.

Several fermentation conditions play fundamental roles in cellulase production, including fermentation method, carbon source, nitrogen source, pH, temperature, incubation time, aerations and fungal species (Norouzian, 2008; Okoye *et al.,* 2013, Saini *et al*., 2017). Optimization of cellulase production is critical for efficient, cost-effective production (Ahmed and Bibi, 2018). Microbial enzyme production is low-cost and economical, increasing the value of cellulase enzymes for commercial use (Gao *et al*., 2008). Fungal cellulases have significant applications in industries that deal with textiles, paper and pulp (Esteghlalian *et al*., 2001), food detergents (Fernandes, 2010), wine and breweries (Kuhad *et al*., 2011), and amino acid synthesis (Ahmed and Bibi, 2018). Fungal cellulase binds to lignocellulose biomass in the initial steps, followed by its degradation into components that are further absorbed by fungi for nutrition (Gerwick and Fenner, 2013). Standardization of process parameters significantly increases cellulase production and thus plays an important role in biomass conversion (Premalatha *et al.,* 2015), as fungal cellulase production is greatly affected by parameters such as temperature, pH, and incubation time. Several fungi produce cellulase and are gaining worldwide interest for the successful bioconversion of lignocellulosic biomass and crop residue waste management, food processing, and the paper and pulp industries, as they are more efficient in cellulolytic activities than others. Therefore, the present study focused on screening, identifying, and optimizing cellulose-degrading fungal strain *Cladosporium tenuissimum* for cellulase enzyme production.

MATERIALS AND METHODS

Collection of soil samples

Soil samples used for fungal isolation were collected from 10 different locations on the Vasantrao Naik

Marathwada Krishi Vidyapeeth, Parbhani Campus, Maharashtra. The soil was dug up to a depth of 10-15 cm and transferred to sterile plastic petri plates (Tarsons). Approximately 5 g of the soil sample was brought to the Department of Plant Pathology Laboratory, College of Agriculture, Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani, Maharashtra, and stored at 4°C in a refrigerator for further use.

Isolation and maintenance of pure colonies

For isolation purposes, the serial dilution technique was employed (Cyrus and Juwon, 2015). 1 g of soil was added to 9 mL of sterile distilled water to achieve a 10^{-1} concentration. An aliquot of 0.1 ml was taken from the 10^{-1} concentration tube and transferred to the next tube containing 9 ml sterile distilled water to make it 10^{-2} concentration. In this manner, Serial dilutions ranging from 10^{-1} to 10^{-6} were prepared for the study. Following the dilutions, 200 µl aliquot from the 10^{-4} test tube was taken and spread onto the petri plates containing Sabouraud dextrose agar media (Sudha *et al*., 2018), (composition: dextrose 40 g/liter, peptone 10 g/liter, Agar 20 g/liter, distilled water 1000 ml) using a sterile L-shaped cell spreader (Tarsons). The Petri plates were incubated at 28 ± 2 °C for five days. After the incubation period, fungal colonies with diverse morphological characteristics were sub cultured on new agar plates to obtain pure cultures and maintained at 4°C in a refrigerator for qualitative screening assays.

Qualitative screening of fungi for cellulolytic activity

Following isolation and purification, a cellulolysis basal medium was used to determine the potential cellulolytic isolates (Pointing, 1999). All isolates were inoculated on cellulolysis basal medium (composition:C4HI2N206 5g/liter; KH2PO4 1g/liter; MgSO4.7H2O 0.5 g/liter; yeast extract 0.1 g/liter; CaCl2.2H2O 0.001 g/liter supplemented with 1% w/v CMC, 0.5% w/v Esculin and 1.6% w/v agar respectively). The final volume of the medium was adjusted to 1 L and the pH was maintained at 7. The medium was sterilized in an autoclave at 121°C and 15 psi for 15 to 20 min. Following autoclaving, the medium was transferred to sterile plastic Petri plates (Tarsons) and subsequently incubated at 28°C. Following a 5-day incubation period, the Petri dishes containing 1% CMC as the carbon source were immersed in a 2% aqueous Congo red solution for 15 minutes. Afterwards, the excess dye was removed, and the agar plates were rinsed with distilled water. After washing with distilled water, the plates were flooded with 1 M NaCl for 15 min. After 15 min, plates containing NaCl were discarded, and carboxymethyl cellulose degradation around the colonies was observed as a yellow, opaque area, indicating cellulase activity. The cellulolytic index was determined by zone of clearance, which is ratio of clearing zone diameter (cm) to Diameter of colony growth (cm) (Ashiqin and Azrimi, 2015Demissie *et al*., 2024). Esculine, also known as 6,7-dihydroxycoumarin β-d-glucose, is recognized as a substrate for β-glucosidase (Saqib and Whitney, 2006). Formation of black-coloured halos around the fungal colony was considered a positive indication of β-glucosidase activity (Pointing, 1999; Saroj *et al*., 2018).

Morphological identification of cellulolytic fungi

Fungal morphology was studied macroscopically for a single isolate that exhibited cellulolytic potential during qualitative screening. Morphological observations were recorded for colony colour, diameter, shape, and surface after sub-culturing on Sabouraud dextrose agar for 7 days at 28°C. Microscopic examinations were conducted to study the conidiophores and conidia. The morphological traits were identified using a Magnus Digital Microscope, with descriptions provided by Schubert and Braun (2007).

Initial assessment of enzyme activity

For the quantitative determination of cellulase, fungal isolate was inoculated into Erlenmeyer flask containing sterilized potato dextrose broth (250ml) supplemented with 1% CMC (for Endo 1, 4-β-D-glucanase activity), 1% avicel (for Exo 1, 4-β-D-glucanase activity), 1% pNPG (for β-glucosidase activity), and kept on a rotary shaker at 125 rpm for 7 days at 28±2°C. After 7 days, fungal broth was collected and centrifuged at 10,000 rpm using a cooling centrifuge (Eppendorf 5810R, Rotor model FA-48×2,2023-5) for 10 min (Montoya *et al*., 2020). After centrifugation, the cell-free supernatant obtained was stored at 4°C and used as a source of enzymes to determine the activities of Endo 1, 4-β-D-glucanase, Exo 1, 4-β-D-glucanase and β-glucosidase.

Quantitative estimation of cellulase

Crude cellulase was quantified using three different commercially available substrates: carboxymethyl cellulose salt (Qualigens, Thermo Fisher Scientific), Sigmacell 20 (Avicel) and p-nitrophenyl-β-D-glucopyranoside (Sigma Aldrich, USA). The substrates mentioned, carboxymethyl cellulose (CMC), Sigmacell 20 (Avicel) and p-nitrophenyl-β-D-glucopyranoside (pNPG) were used to estimate the activities of Endo (1, 4)-β-D-glucanase, Exo (1, 4)-β-D-glucanase and β-glucosidase, respectively. The reaction mixtures in the tube contained 0.5 ml of substrate at a 1% concentration suspended in 50 mM sodium citrate buffer (pH 5.0) and 0.5 ml of cell-free supernatant. Furthermore, the mixture was maintained at 50°C for 20 minutes, after which the reaction was terminated by the addition of 2 ml of 3,5-dinitrosalicylic acid (DNS) solution. Tubes were boiled for 5-10 minutes, then cooled to room temperature (Miller, 1959). The amount of reducing sugars

was calculated by measuring the absorbance at 540 nm using of Labman UV Vis. Double-Beam Spectrophotometer LMSP-UV1900 and D-glucose as the standard curve. Enzyme activity is measured as the quantity of enzyme that produces 1 µmol of reducing sugars per ml per minute. Enzyme assays were conducted with the inclusion of relevant controls. Furthermore, a heat-inactivated control was established by boiling the cell-free supernatant at 100 °C for 10 minutes before incubation. This step was crucial for confirming that any reducing sugars detected were solely attributable to fungal enzyme activity.

Optimization of different parameters for maximum cellulase production

Different parameters, such as temperature, pH, and incubation time, were optimized to study their effects on Cellulase (Endo1, 4-β-D-glucanase, Exo1, 4-β-D-glucanase, and β-glucosidase) production. To investigate the effect of temperature on enzyme activity, all reactions were carried out at the following temperature profiles: 25°C, 30°C, 35°C, 40°C, and 45°C (Shinde *et al.,* 2017). To examine the impact of pH on enzyme production, a range of buffers was utilized to stabilize pH levels at 4, 5, 6, 7, and 8, including50mM sodium acetate buffer (pH 4), 50mM sodium citrate buffer (pH 5), 50mM Tris base buffer (pH 6), 50mM potassium phosphate buffer (pH 7) and 50 mM Tris HCL (pH 8). To determine the optimum incubation period, crude enzyme from fungal culture was collected at 1-7 days, and absorbance was recorded at 24 h intervals (Kumar *et al*., 2024).

Molecular identification of cellulolytic fungi

Genomic DNA isolation and PCR amplification

For genomic DNA isolation, the isolate was grown in potato dextrose broth for 7 days at 25°C on a rotary shaker (120 rpm), and a small portion of fungal growth was collected into a 2-ml Eppendorf tube. Genomic DNA was extracted using the CTAB method (Moubasher *et al.,* 2019). The concentration of the isolated DNA was quantified using a Nano DropTM One Micro volume UV-Vis Spectrophotometer (Thermo Fisher Scientific). Further confirmation of the isolated DNA was performed by visualizing it on a 0.8% agarose gel. A single band of high-molecular weight DNA was observed and stored at -20°C for PCR amplification. The ITS region was amplified using the universal primer pair ITS1 and ITS4 (Kumar *et al.,* 2024; White *et al.,* 1990). The Genie PCR Master Mix kit (cat. no. 0602200011730) was used for PCR amplification. Primers were initially at a concentration of 10 µM, which were then diluted to achieve a final concentration of 0.2 µM for each primer in the reaction. In total, 30 PCR cycles were performed with the following conditions: an initial denaturation (95°C, 30 sec), annealing (55°C, 1 min), extension (72°C, 1 min), and a final extension (72°C, 7 min). Agarose gel 1.2% concentration was prepared to analyze the PCR amplicon. Gel images were captured using a Vilber Gel documentation system. The PCR amplicon was purified using Genei Pure^{TM} Quick PCR Purification kit. Following purification, a single discrete band was eluted and sent for sequencing.

Sequencing and phylogenetic analysis

The PCR product was sent for sequencing to a DNA sequencing facility at Barcode Biosciences Pvt. Ltd., Bangalore, Karnataka. DNA sequencing reactions in both forward and reverse directions were performed using ITS1 and ITS4 with the cycle sequencing kit (BDT v3.1) on an ABI Genetic Analyser. BLAST searches for ITS region sequences were performed online using NCBI GenBank (http://www.ncbi.nlm.nih.gov/blast/). Based on the maximum identity score, sequences were selected and aligned using the multiple alignment program ClustalW, with default gap-opening and gap-extension penalties of 10 and 0.1, respectively, and clearly specified alignment parameters. MEGA 12 software was used to construct the phylogenetic tree, incorporating bootstrap analysis with 1,000 replicates (Tamura *et al*., 2021) Bootstrap values were calculated to evaluate branch support, and the resulting tree was visualized and annotated using iTOL v7.2.

Statistical analysis

Enzyme assays were performed in biological triplicates, with each replicate derived from a separate culture grown under identical conditions. The statistical evaluation utilized the average of technical measurements. Enzyme activity results are expressed as mean ± standard deviation (SD) to reflect variability across independent biological replicates. The data were analyzed using the OPSTAT online web application (http://opstat.somee.com/opstat/), where all optimization experiments, including temperature, pH, and incubation period, underwent ANOVA followed by the Critical Difference (CD) test at $p < 0.05$ to identify pairwise differences among treatments.

RESULTS

Collection, isolation and maintenance of fungi

To isolate cellulolytic fungi, 10 soil samples were collected from different sampling sites of Vasantrao Naik Marathwada Krishi Vidyapeeth campus, Parbhani, India (Table 1). From these 10 soil samples, twenty-three fungal isolates were obtained by taking 200 µl aliquot from 10^{-4} serially diluted tube and spreading them on freshly prepared Sabouraud Dextrose Agar media plates (Fig. 1). All the fungal isolates, denoted as F1 to F23 were maintained at 4°C in their pure form for fur-

Table 1. Soil samples collected from different locations in Vasantrao Naik Marathwada Krishi Vidyapeeth campus, Parbhani

No.	Sample source	Date of sample collection	Sample location
1	Fodder cafeteria soil	August 3, 2024	Latitude N19^{0}15'40.62384 Longitude E 76^{0}46.16.83408
2	Cattle shed soil	August 8, 2024	Latitude N19^{0}15'10.99656 Longitude E 76^{0}46.16.83408
3	Cow dung soil	August 12, 2024	Latitude N19^{0}15'10.84577 Longitude E 76^{0}46.16.83408
4	Biomix plot soil	August 19, 2024	Latitude N19^{0}14'41.74152 Longitude E 76^{0}40.25.8882
5	Rhizospheric soil 1	September 3, 2024	Lattitude N19^{0}15'10.02384 Longitude E 76^{0}46.58.37592
6	Rhizospheric soil 2	September 7, 2024	Lattitude N19^{0}14'14.81136 Longitude E 76^{0}47.30.51528
7	Rhizospheric soil 3	September 22, 2024	Lattitude N19^{0}13'11.88372 Longitude E 76^{0}47.27.79548
8	Rhizospheric soil 4	November 5, 2024	Lattitude N19^{0}13'12.746826 Longitude E 76^{0}46.44.152528
9	Rhizospheric soil 5	November 17, 2024	Lattitude N19^{0}15'40.12140 Longitude E 76^{0}47.34.72188
10	Rhizospheric soil 6	December 1, 2024	Lattitude N19^{0}14'14.8056 Longitude E 76^{0}47.30.49044

ther studies.

Qualitative screening of fungi for cellulolytic activity

In the present study, all twenty-three fungal isolates were screened for their cellulase activity on cellulolysis basal media supplemented with CMC and aesculin as the sole carbon source. Carboxymethyl cellulose is a chemically modified cellulose derivative. Breakdown of cellulose can occur through chemical, enzymatic, or microbial degradation. During microbial degradation, fungi metabolize CMC and aesculin as carbon sources by hydrolyzing them into smaller sugars. In the case of Petri plates containing CMC as the only carbon source, the development of a yellow opaque area around the fungal colony (isolate F18) against red color was observed and considered as an indication of cellulose degradation (Fig. 2a). The cellulose solubilization index for isolate F18 was found to be 2.66 cm, which is the ratio of halo zone diameter to diameter of colony growth. However, on plates containing aesculin, the development of black colour around the fungal colony (isolate F18) was clearly observed (Fig. 2b). Based on the CMC and aesculin plate assays, out of twenty-three isolates, a single isolate, denoted as F18, exhibited both extracellular CMCase and β-glucosidase activities. Isolate F18 was subjected to further investigation.

Morphological identification of cellulolytic fungi

Fungal isolate F18 exhibited moderate to rapid growth on Sabouraud dextrose agar (SDA) media at 28°C for 7 days with colonies initially appearing olive-grey to greenish-grey. As the culture matured, the colony gradually turned dark olivaceous or greyish-black. The colony surface was velvety to powdery owing dense sporulation (Fig. 3a). Microscopic examination revealed a filamentous fungal structure composed of septate hyaline hyphae that supported clusters of conidia. The hyphae are smooth-walled and branched, leading to short conidiophores that end in chains or small groups of globose or subglobose conidia. The conidia have thin walls and appear pale to light greenish-blue owing to staining, with a consistent size observed across the field (Fig.3b) Several conidiogenous cells displayed blastic conidiation, with conidia forming singly or in short, acropetal chains. The overall configuration of septate hyphae, blastic conidiogenesis, and ellipsoidal to globose conidia in loosely branched clusters is typical of dematiaceous hyphomycetes, such as *Cladosporium*-like species. These morphological features support the initial identification based on the colony characteristics.

Preliminary cellulase enzyme activity

Fungal isolate F18 was explored for its initial cellulase activity to support further studies. According to the studies, preliminary enzyme production for all three enzymes, viz., Endo 1, 4-β-D-glucanase, Exo 1, 4-β-D-glucanase, and β-glucosidase, was 45.57 IU/ml, 27.38 IU/ml, and 29.12 IU/ml, respectively.

Optimization of cellulase production

Three important parameters, such as temperature, pH, and incubation time, were investigated to optimize maximum cellulase production by the potential isolate F18 (Table 2). The temperature significantly impacts the activity, stability and efficiency of cellulase production, which are crucial for hydrolysis of cellulose to glucose. In this study, cellulase production was observed to be maximum at 30°C. Later, cellulase enzyme production decreased with increase in temperature. The optimum cellulase enzyme activity with respect to Endo 1, 4-β-D-glucanase, Exo 1, 4-β-D-glucanase and β-glucosidase was 147.60 IU/ml, 74.24 IU/ml and 80.65 IU/ml respectively. One-way ANOVA indicated that temperature had a significant effect on cellulase production ($p < 0.05$), and CD analysis showed that the activity at 30 °C was significantly higher than that at other temperatures.

Table 2. Optimization of different parameters investigated in the present study

Sr. No.	Parameter investigated	Range	Unit	Optimum condition
1	Temperature	25 to 45	°C	
2	pH	4 to 8	-	
3	Incubation time	1 to 7	days	

Table 3. Effect of temperature on cellulase activity

Sr. No.	Cellulase enzyme	Incubation temperature range				
		25 °C	30 °C	35°C	40°C	45°C
1	Endo glucanase activity (IU/ml)	118.84±0.3	147.60±0.1	109.94±0.1	51.01±0.03	34.49±0.06
2	Exo glucanase activity (IU/ml)	39.41±0.02	74.24±0.02	45.94±0.1	17.47±0.07	10.67±0.04
3	β glucosidase activity (IU/ml)	45.12±0.1	80.65±0.2	46.89±0.06	24.70±0.04	12.78±0.08

Values represent mean ± SD of three independent biological replicates

Table 4. Effect of pH on cellulase activity

Sr. No.	Cellulase enzyme	pH range				
		4	5	6	7	8
1	Endo glucanase activity (IU/ml)	27.38±0.1	38.50±0.2	79.41±0.2	140.35±0.07	110.42±0.2
2	Exo glucanase activity (IU/ml)	11.64±0.1	13.82±0.1	19.20±0.2	66.64±0.1	37.35±0.1
3	β glucosidase activity (IU/ml)	9.54±0.1	12.96±0.1	25.27±0.2	80.40±0.06	58.34±0.1

Values represent mean ± SD of three independent biological replicate

(Table 3,). Similarly, pH exerted a significant influence ($p < 0.05$), with pH 7 showing the highest enzymatic activity, which was statistically different from the adjacent pH treatments. Maximum cellulase activity exhibited at pH 7 was 140.35 IU/ml, 66.64 IU/ml and 80.40 IU/ml for Endo 1, 4-β-D-glucanase, Exo 1, 4-β-D-glucanase and β-glucosidase respectively. (Table 4,). The incubation time significantly affects cellulase enzyme production by influencing microbial growth, enzyme synthesis and substrate degradation. In the current investigation, supernatant from potential isolate was collected at regular intervals and cellulase activity was measured. The peak cellulase enzyme production was observed on the 5th day and gradually decreased thereafter. Maximum yield of Endo 1,4-β-D-glucanase, Exo 1, 4-β-D-glucanase and β-glucosidase obtained at 5^{th} day were 143.9 IU/ml, 68.13 IU/ml and 76.22 IU/ml respectively. The incubation period also differed significantly among the treatments ($p < 0.05$), with maximum activity recorded on the 5th day, which was significantly higher than that on earlier or later incubation periods (Table 5).

Molecular identification of cellulolytic fungi

Based on qualitative and quantitative enzyme assays, a single potential isolate, denoted F18, isolated from a soil sample, was subjected to molecular identification by extracting genomic DNA and performing PCR amplification. A single discrete PCR amplicon of ~600 bp was obtained using a pair of primers specific to the internal transcribed spacer region (ITS1 and ITS4). The PCR amplicon was purified and sequenced. After sequencing, the data were analyzed using BLASTn against the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/blast/). A BLAST search at NCBI revealed that the cellulolytic fungal isolate denoted as F18 shares 99.43% homology with *C. tenuissimum* strains (Table 6). The ITS sequence data obtained from this study were deposited in NCBI GenBank under the accession number PV687687.

Phylogenetic analysis

A neighbour-joining tree was constructed from ITS sequences of 40 different *Cladosporium* strains available in the NCBI GenBank database and was finally visualized in iTOL, clearly illustrating the highest similarity with their Ex-type strains retrieved from NCBI GenBank. The ITS region sequence analyses placed potential isolate F18 with its closely related strains (Fig. 4).

DISCUSSION

A systematic isolation and screening approach led to the identification of a potent cellulolytic fungal isolate from soil samples collected from the VNMKV campus. A total of 23 fungal isolates (F1–F23) were obtained

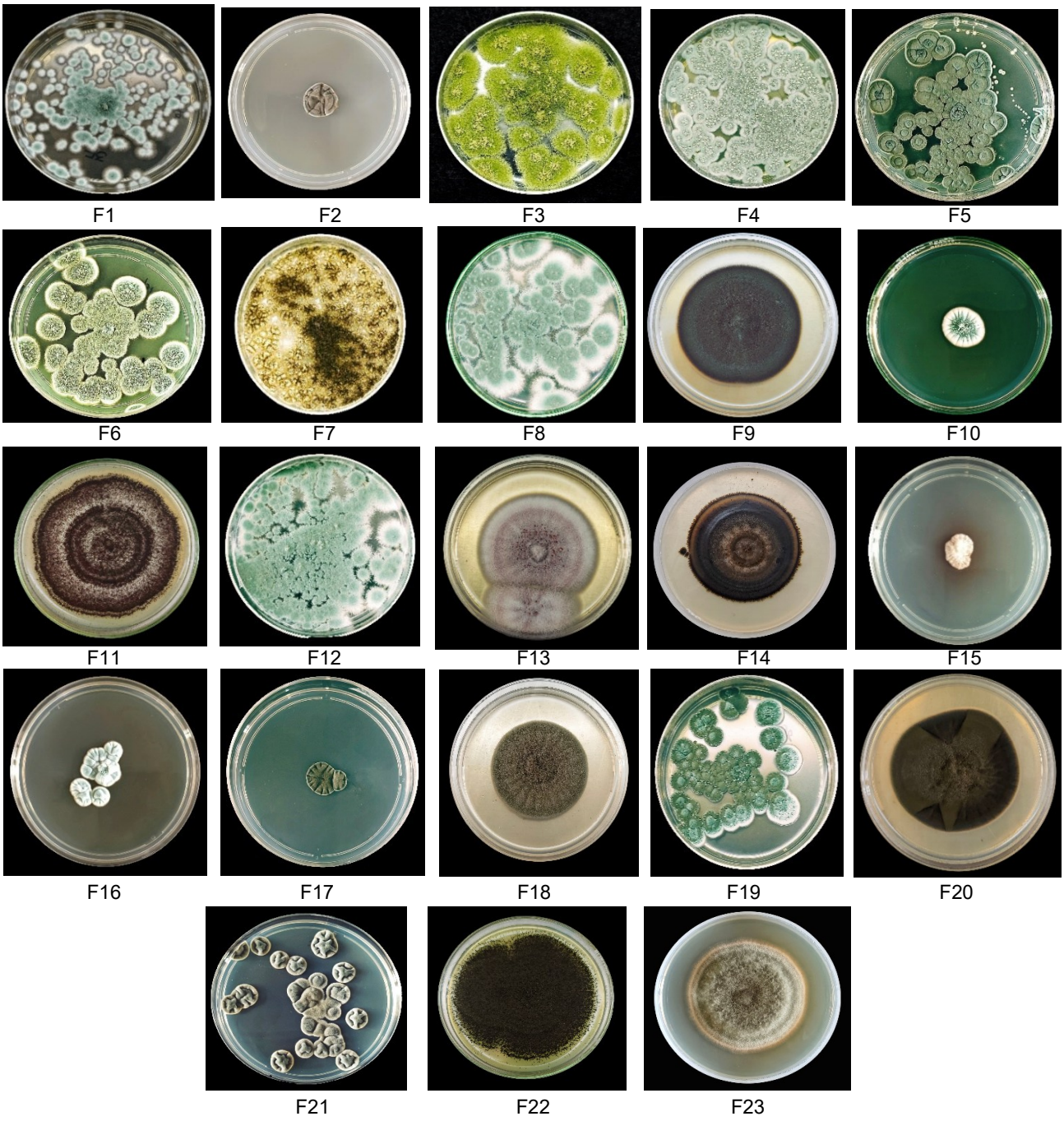


Fig. 1. *Colonies of twenty-three fungal isolates (F1 to F23) on Sabouraud dextrose agar (SDA) media*

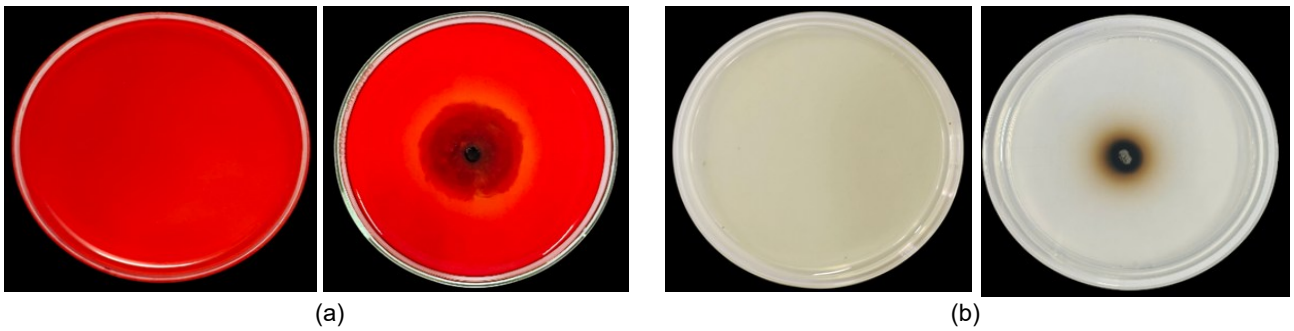
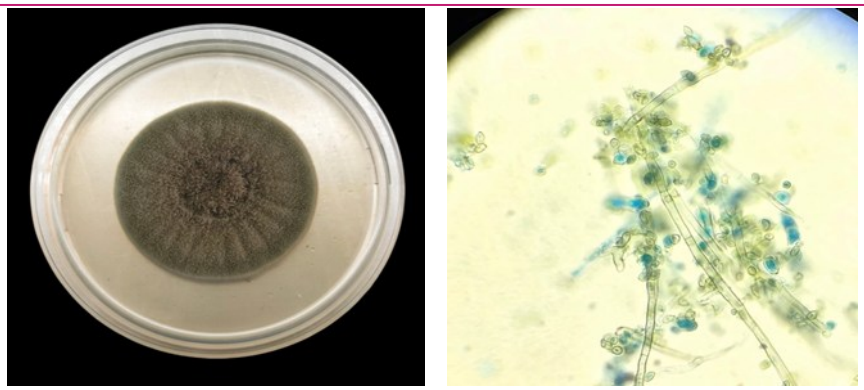


Fig. 2. *Qualitative screening results (a): First plate as negative control with no CMCase activity, second plate indicating CMCse activity (b) First plate as negative control with no β-glucosidase activity, second plate indicating β-glucosidase activity*



(a) (b)

Fig. 3. *Morphological features of dematiaceous fungal isolate F18. (a): Front side (b) Microscopic view showing conidia morphology*

Table 5. Effect of incubation period on cellulase activity

Sr. No.	Cellulase enzyme	Incubation period (days)						
		1	2	3	4	5	6	7
1	Endo glucanase activity (IU/ml)	20.64±0.1	37.44±0.07	61.82±0.1	100.58±0.1	143.90±0.1	128.53±0.09	106.04±0.2
2	Exo glucanase activity (IU/ml)	9.48±0.2	15.37±0.2	20.85±0.1	49.57±0.06	68.13±0.06	50.62±0.15	33.98±0.1
3	β glucosidase activity (IU/ml)	8.85±0.2	12.99±0.1	24.03±0.05	54.00±0.18	76.22±0.1	58.29±0.16	36.10±0.2

Values represent mean ± SD of three independent biological replicates

from 10 soil samples using serial dilution and spread plate methods on SDA medium, indicating considerable fungal diversity in campus soils. Primary qualitative screening on cellulolysis basal medium supplemented with CMC and aesculin identified a single isolate, F18, with strong extracellular cellulase activity. The formation of a yellow opaque halo on CMC plates confirmed endoglucanase activity, whereas a black precipitate on aesculin plates indicated β-glucosidase production. Isolate F18 showed a cellulose solubilization index of 2.66 cm, which was significantly higher than that of the other isolates. Microscopic features, including septate hyaline hyphae, and globose to subglobose conidia, were consistent with those of *Cladosporium*-like dematiaceous hyphomycetes. Quantitative assays confirmed that isolate F18 produced all major cellulase components, with preliminary activities of 45.57 IU mL^{-1} (endoglucanase), 27.38 IU mL^{-1} (exoglucanase), and 29.12 IU mL^{-1} (β-glucosidase), justifying further optimization. Optimization experiments demonstrated that temperature, pH, and incubation time significantly affected cellulase production ($p < 0.05$). Maximum enzyme activity was recorded at 30 °C and pH 7. The highest enzyme yields were observed on the 5th day of incubation. Molecular identification based on ITS rDNA sequencing revealed 99.43% similarity to *C. tenuissimum*. The ITS sequence was deposited in GenBank under accession number PV687687, and phylogenetic analysis using the neighbour-joining method placed isolate F18 within the C. tenuissimum clade, confirming its taxonomic identity. Similar to these findings, Carboxymethyl cellulose (CMC) was used as substrate for cellulase screening (Andriani *et al*., 2020). A total of 151 fungal colonies were isolated and screened on selective media (i.e., CMC) to determine the potency of microbes in producing cellulase, as indicated by clear

Table 6. Molecular identification of potential cellulolytic strain

Sr. No.	Name of isolate	Maximum homology	Sequence similarity (%)	Genbank accession number
1	Fungal isolate F18	*Cladosporium Tenuissimum*	99.43%	LC847972 MF473304 MF473303 MF473302 MF473299

zones of growth around the cultures (Bairagi, 2016). The study conducted by Bhavsar *et al*. (2015) showed the isolation and screening of cellulolytic fungi directly from local compost pits. Cellulase-producing fungi were primarily screened on plates containing Basal Salt Media supplemented with 1% carboxymethyl cellulose as the sole carbon source, which were flooded with 1% Congo red dye, followed by destaining with 1 M NaCl. The fungal colony showing the largest zone of decolourization was selected for cellulase production.The potent cellulolytic fungal species, *Aspergillus flavus, Aspergillus terreus, Ramichloridium apiculatum, Fusarium pallidoroseum* were isolated from soil and tree bark samples,later subjected to primary screening on the media plates containing carboxymethyl cellulose and considered as cellulose degrading based on clear zone formation after staining with congo red indicator dye (Devi *et al*., 2024). In a prior investigation, 10 fungal isolates from organic-rich soils exhibited zones of clearance on 1% carboxymethyl cellulose (CMC) plates after staining with 1% Congo red dye (Kanakraju *et al*., 2022). Kumar *et al*. (2024) reported similar findings, isolating fungal consortia *of Cladosporium oxysporum, Aspergillus sigurros, and Cladosporium cladosporoides* from humus-rich soil and assessing their cellulolytic activity by zone of clearance. Thirteen different fungi were isolated from soil samples and screened for cellulolytic activity; the fungi with the largest clear zones were selected (Nhan *et al*., 2021). Fungi isolates, *A. niger* and *T. reesei,* were cultured on CMC agar plates; a clear zone was observed by staining the plates with Congo red, followed by the formation of *a* clear zone (Septiani *et al*., 2019). In this context, a study was carried out for isolation of cellulolytic fungi from the soil samples among which nine isolates, *Aspergillus niger, Aspergillus flavus, Nigrospora sphaerica, Chaetomium globosum, Cladosporium cladosporides, Trichoderma sp., Penicillium sp., Fusarium oxysporium, Acremonium sp.,*found to have cellulolytic activity on the basisof their ability to hydrolyze cellulose byforming diameter zone of clearance (Reddy *et al*., 2014). In another study, *Aspergillus niger* and *Trichoderma viride* were isolated

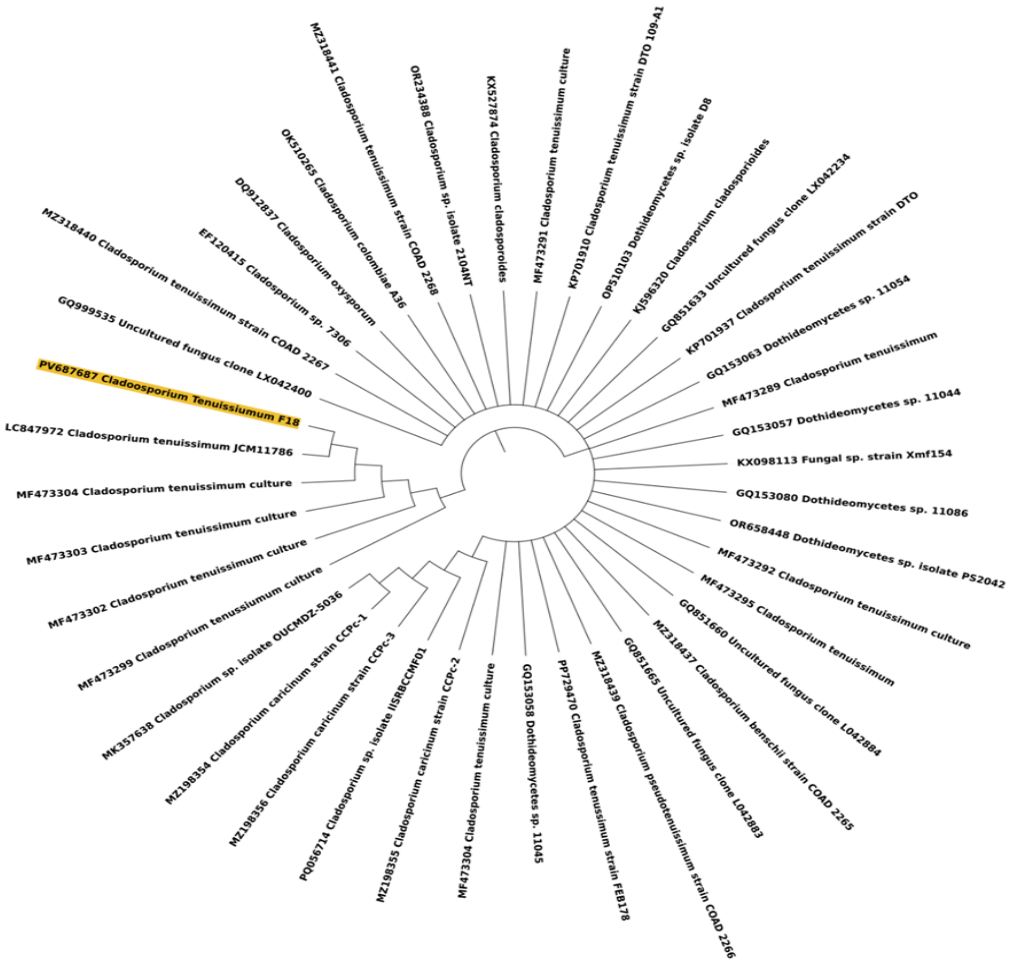


Fig. 4. *Phylogenetic Tree of Cladosporium tenuissimum based on ITS sequences of 40 different Cladosporium strains shows genetic relationships among closely related strains, illustrating evolutionary relationships and genetic diversity among strains*

from soil and a decaying wood sample and exhibited cellulose-degrading ability based on the Congo Red assay (Shareef *et al*., 2015).

In the present investigation, the *C. tenuissimum* strain exhibited both Endoglucanase and β-glucosidase activities. β-Glucosidase is an important component of the cellulose-degrading enzyme system, and esculin (6,7-dihydroxycoumarin-β-d-glucose) has been shown to be a substrate for β–glucosidase (Saqib and Whitney, 2006). β-glucosidase activity on an agar plate containing Esculin as the sole carbon source was easily assessed by the production of a black colour in the growth medium. Similar to these findings, Saroj *et al*. (2018) reported screening 15 isolates, and β-glucosidase activity was assessed by black zone formation. Selection of β-glucosidase producers used the esculin gel diffusion assay to detect β-glucosidase in fungal culture extracts, with positive extracts forming dark-coloured halos (Robl *et al*., 2013). Four of the bacteria from the dairy effluent and five from the barley source exhibited β-glucosidase activity by blackening of the medium around the colony (Veena and Paramsivan, 2011). Twenty bacterial isolates were isolated from termite gut, and their β-glucosidase activity was assessed by observing the appearance of a black colour around the colony (Shinde *et al*., 2017). Thus, on the basis of qualitative screening plate assays, a single isolate, denoted F18, exhibiting cellulolytic abilities was selected for further investigation.

The genus *Cladosporium* is notoriously difficult to identify solely on morphological grounds due to several key challenges. Many *cladosporium* species look almost identical under the microscope differing only in subtle features and are frequently confused due to overlapping characteristics. Although conidiophore and conidia size and shape are important characteristics of different *cladosporium* species, dimensions usually overlap among species in the genus (Yang *et al*., 2023). The Phenotypic appearance (colony colour) of *Cladosporium* varies widely with temperature and culture media, potentially leading to misidentification. Unlike *Aspergillus* and *Penicillium*, *Cladosporium* does not produce unique fruiting bodies or specialized spores that aid in identification. *Cladosporium* is a common soil and airborne fungi, if multiple species grow together in the laboratory, morphological separation become nearly impossible. Considering the above facts, reliance on morphological features alone is insufficient for species level identification. Due to high morphological ambiguity, a molecular-level approach was used to obtain accurate species-level identification of *Cladosporium*, as discussed later.

All strains of fungi (*Aspergillus sp. and Trichoderma sp*.) and bacteria (*Bacillus sp.* and *Micrococcus sp*.) isolated from different partially decomposed cellulose-rich substrates showed a detectable range of cellulase production potential, which was effectively correlated with the reducing sugar content (Roy *et al*., 2024). Arnthong *et al* (2024) isolated *Aspergillus terreus, Penicillium oxalicum, Talaromyces siamensis* and *Trichoderma afroharzianum* and determined their total FPase (CMCase, xylanase and β-glucosidase) activities by quantitative screening strategy and reported maximum CMCase production 20.16 U/ml and β-glucosidase enzyme production 7.93 U/ml by *Penicillium oxalicum* strains AG496 and AG498, respectively. Jagadeesh and Muthuraju, (2022) isolated lignocellulolytic microbes from cow dung, forest soil, etc., and estimated cellulase (endoglucanase, exoglucanase, and β-glucosidase) activity. Among 19 fungal isolates, the maximum enzyme activity was exhibited by isolate UASFW, viz., exoglucanase 19.826 U/ml, endoglucanase 232.42 U/ml, and β-glucosidase 357.66 U/ml. Gautam *et al*. (2011) reported cellulase enzyme production from *Aspergillus niger* isolated from municipal solid waste samples, and the isolate exhibited endoglucanase activity of 1.67 U/ml, exoglucanase activity of 1.97 U/ml, and β-glucosidase activity of 2.31 U/ml, respectively. Before optimizing the cellulase enzyme, it was crucial to determine its baseline activity under controlled conditions. This preliminary assessment helped identify the roles of key parameters in further optimizing the cellulase produced by the *C. tenuissimum* strain.

The effect of incubation temperature on cellulase enzyme production by the *C. tenuissimum* strain is discussed herein, based on earlier results. Prasanna *et al*. (2016) investigated the effect of temperature on the maximum cellulase activity of *Penicillium sp*. by inoculating into Czapek Dox medium and found optimal enzyme production at 30°C for all three enzymes (endoglucanase, exoglucanase, and β-glucosidase). Saini *et al* (2017) studied cellulase production using *Trichoderma reesei* (NCIM 992) on *Parthenium* biomass. The effect of temperature was studied by producing cellulase at 20, 25, 30, 35, and 40°C; maximum enzyme activity was observed at 30°C, while minimum activity was observed at 40°C.The optimum temperature of the enzyme was determined by incubating at different temperature profiles (20 to 45 °C), and the highest CMCase activity of *A. terreus* was recorded at 35-40 °C, followed by a decrease in activity beyond 45 °C (El-Baroty *et al*., 2019). Many researchers concluded that the optimum incubation temperature for the cellulase enzyme is between 25 and 40°C and suggested that the optimal temperature for cellulase production also depends on the strain of microorganism (Lu *et al*., 2003). In the current investigation, *the C. tenuissimum* strain exhibited maximum enzyme activity at 30°C, with activity decreasing above 35°C. Like temperature, pH significantly influences enzyme activity. Similar to the findings, Kumar *et al*. (2024) optimized pH conditions for the assessment of cellulase activity in *Cladosporium*

oxysporum N5 isolated from humus-rich soils, and peak enzyme production was observed at pH 7. Cellulolytic bacteria *Pseudomonas, Klebsiella, Salmonella, Serratia*, and *Enterobacte*r were isolated from termite guts, and efforts were made to optimise pH for cellulase production; maximum CMCase and FPase activities were observed at pH 7 (Shinde *et al*., 2017). Fungal isolates *Aspergillus sp., Penicillium sp. and Fusarium sp.* were obtained from cellulosic waste material and sea sands and investigated for maximum cellulase production at different pH range. Cellulase activity was significantly high over a broad pH range from 5.0 to 8.0, with maximum activity at pH 6.0 (Hasan *et al*., 2016). Nine fungal isolates *Aspergillus niger, Aspergillus flavus, Nigrospora sphaerica, Chaetomium globosum, Cladosporium cladosporides, Trichoderma sp, Penicillium sp. Fusarium oxysporium, Acremonium sp.* exhibited cellulolytic activity (β-glucosidase, FPase and CMCase) and later investigated for optimization of pH for all the three-enzyme production. It was found that all the three enzymes possessed maximum activity between pH 5 to 6. Most of the discussed research articles showed maximum enzyme activity between 5 and 7pH. The present studies showed that *the C. tenuissimum* strain exhibits maximum enzyme activity at neutral pH (pH 7), and activity decreases as pH increases towards alkalinity or acidity.

Goukanapalle *et al.* (2020) studied cellulase production by the novel endophytic fungus *Pestalotiopsis microspora* TKBRR in solid-state fermentation, optimizing the incubation period to 3-7 days, and enzyme activities were examined at 24-h intervals. The findings indicated that a 5–6-day incubation period was ideal for maximizing cellulase enzyme activity recovery. *Cladosporium oxysporum* N5 obtained from humus rich soils exhibited maximum amount of cellulase enzyme at 5th day of incubation time (Kumar *et al.,* 2024). El Said *et al.* (2014) studied the cellulase activity of some fungal species by evaluating their capacity to produce both exo- and endo-1,4-glucanase enzymes (C1 and Cx). The highest levels of exo- and endo-1,4-glucanases produced by these fungi were observed after incubation periods of 6 and 8 days, respectively.

Nagah *et al*. (2016) reported maximum FPase production at the 6th day, CMCase production at the 9th day, and β-glucosidase production at the 9th day by *Penicillium sp*. and *Aspergillus sp*., respectively. These findings clearly indicate that the optimum incubation period for cellulase enzyme production relay between days 4 to 9. *Cladosporium tenuissimum* strain investigated in the present study exhibited maximum enzyme activity on day 5. This study identified the optimal conditions for cellulase production by *C. tenuissimum* F18 as 30 °C, pH 7, and 5 days. However, it is crucial to recognize that these conditions may not apply to all *C. tenuissimum* isolates or to other cellulolytic fungi. Fungal cellulase expression is highly strain-specific and is influenced by genetic makeup, ecological environment, substrate history, and differences in enzyme-induction pathways. Even within a single species, isolates can show significant variability in terms of enzyme production, temperature adaptability, pH preference, and secretion characteristics. Studies conducted between 2022 and 2024 have shown that *Cladosporium cladosporioides* and *Cladosporium herbarum* exhibit lower cellulase activity when subjected to similar submerged fermentation conditions. This suggests that strain F18 has a more advanced enzymatic profile than other members of its genus. Moreover, compared with well-known industrial producers such as *Trichoderma reesei* and *Aspergillus niger*, which typically achieve CMCase yields of 1.8–3.5 U/mL under optimal conditions, F18's enzyme production is competitive. This positions it as a promising alternative cellulase source for industrial applications.

Although this study used a controlled in vitro system to assess enzyme production, such conditions capture only a portion of the complexity of natural lignocellulosic breakdown. In actual environments, a variety of microbial communities, diverse substrate compositions, and changing physicochemical factors affect enzyme expression and activity. These interactions and variations cannot be replicated completely in laboratory settings. Thus, although the in vitro model provides a useful baseline for evaluating the strain's potential, its performance in natural or industrial settings may vary. Conducting studies with mixed microbial communities, experimenting with different lignocellulosic substrates, and validating the strain in pilot-scale bioreactors would offer a more thorough understanding of its practical applications.

The internal transcribes spacer (ITS) region is the most widely used DNA barcode for fungal identification due to its high variability and universal presence across fungal species. Therefore, molecular identification of cellulolytic fungi was performed by genomic DNA isolation, PCR amplification of the ITS region, amplicon sequencing, and *in silico* analysis, which helped characterize the potential isolate as *C. tenuissimum* strain. Similar findings have been reported by many researchers, and some of them are discussed here. Jin *et al*. (2012) reported the isolation of a lignin- and cellulose-degrading fungal strain, Bio-1, from soil. Due to its extracellular laccase and cellulase activities, Bio-1 was subjected to DNA extraction, PCR amplification of the ITS region, and sequencing. ITS sequence analysis revealed Bio-1 belonged to the genus *Cladosporium*. Coronado *et al*. (2018) isolated cellulolytic fungi from a nineteenth-century French collection of drawings and lithographs, as they were able to degrade cellulose, and identified them through DNA sequencing of ITS regions. BLAST searches in the GenBank database

showed that 19 isolates had at least 98% similarity with known species, mainly *Cladosporium sphaerospermum, Cladosporium tenuissimum, Cladosporium angustisporum*, and *Cladosporium cladosporioides.* Molecular characterizations, particularly DNA sequence analysis of the two internal transcribed spacer (ITS) regions of ribosomal DNA, ITS1 and ITS2, were used for species identification of cellulolytic fungi isolated from three sites of an old house and identified as *Thielavia hyalocarpa*, *Penicillium crustosum*, *Penicillium granulatum*, *Penicillium commune*, *Penicillium chrysogenum, Penicillium expansum* and *Cladosporium cladosporioides* (Zyani *et al*., 2009). Potential cellulolytic fungi were isolated from a humus soil sample; their cellulase activity was investigated, and they were molecularly identified using the universal primers ITS-1 and ITS-4, and further sequenced. The sequenced amplicons were analyzed using BLAST and identified as *Cladosporium oxysporum*, *Aspergillus sigurros*, and *Cladosporium cladosporioides* (Kumar *et al*., 2024). Arnthong *et al*. (2024) isolated 297 fungal strains from several cellulose-containing samples, selected 9 based on their potential for cellulase production, and subjected them to genomic DNA isolation, PCR amplification of the ITS region, and finally sequencing. *Insilico* analysis revealed the identities of nine isolates as *Aspergillus terreus*, *Penicillium oxalicum, Trichoderma afroharzianum*, and *Talaromyces siamensis.* Montoya *et al*. (2020) isolated and purified 42 microorganisms from *M. oleifera* biomass, and the fungi with the largest hydrolytic halos in carboxymethylcellulose as a substrate were molecularly identified as *Penicillium funiculosum* (FG1), *Fusarium verticillioides* (FG3), and *Cladosporium cladosporioides* (FC2) by amplifying the ITS rDNA region. The fungi that exhibited the largest hydrolytic zones on carboxymethyl cellulose as a substrate were identified at the molecular level as *Penicillium funiculosum* (FG1), *Fusarium verticillioides* (FG3), and *Cladosporium cladosporioides* (FC2).

ITS (internal transcribed spacer) sequencing of four potential cellulolytic strains revealed their identity as *Penicillium oxalicum, Talaromyces pinophilus*, *Penicillium griseofulvum and Trichoderma reesei* (Singh *et al*., 2025). Roy *et al*. (2024) isolated cellulose-degrading fungi from different partially decomposed cellulose-rich substrates, and genus-level identification of potential fungi was performed using the universal primers ITS1 and ITS4.

Analysis of partial ribosomal gene sequences indicated that DAJ2 (PP086700) is completely identical to *Aspergillus foetidus*, while DTJ4 (PP086699) exhibits a 99.74% similarity to *Trichoderma atrobrunnium*. The genus *Cladosporium* poses a complex taxonomic challenge due to significant morphological overlap, which often hinders accurate species identification. While ITS sequencing is widely recognized as the primary DNA barcode for fungi, it sometimes fails to resolve closely related *Cladosporium* species. In this study, the isolate exhibited a 99.43% ITS sequence similarity with existing strains, offering strong initial evidence for its identification at the genus level. However, achieving precise species-level classification within *Cladosporium* generally necessitates multi-locus sequencing (e.g., TEF1-α, ACT, and CAL) or whole-genome phylogenomic analysis. Future research employing these additional genetic markers could improve taxonomic resolution and provide a more definitive classification of the isolates.

Conclusion

A total of 23 fungal isolates were obtained from the Vasantrao Naik Marathwada Krishi Vidyapeeth campus in Parbhani and maintained in pure culture to assess cellulase (endoglucanase and β-glucosidase) activity. Qualitative screening results demonstrated a single potential cellulolytic fungus capable of utilizing both CMC and esculin as sole carbon sources for growth, as evidenced by a zone of clearance (endoglucanase activity) and black colour development (β-glucosidase activity). The fungus was subjected to optimization of different parameters for maximum enzyme production. Peak cellulase enzyme production was observed on day 5 of incubation at 30°C and pH 7. Molecular identification using an ITS-based molecular method confirmed that the prominent fungal isolate belongs to the Genus *C. tenuissimum*. The fungal isolate *C. tenuissimum* exhibited considerable potential for the production of cellulolytic enzymes, viz., endoglucanase, exoglucanase, and β-glucosidase. To convert the optimized cellulase production at the laboratory scale into viable biotechnological applications, a series of targeted experimental steps is necessary. The strain should be tested in simulated environmental settings, such as cellulose-rich solid waste or soil contaminated with plant debris. This approach would enable examination of the strain's stability, persistence, and enzyme activity under varying pH, moisture, and nutrient conditions characteristic of natural environments. To summarize, although the fungal strain demonstrated significant cellulolytic activity under controlled laboratory conditions, the next essential step is to assess its effectiveness on actual lignocellulosic materials, examine its potential for environmental applications, and incorporate its enzyme system into pilot-scale bioethanol production.

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Data availability

For the transparency and reproducibility of the computational analyses, all raw sequence data produced in this study, along with the associated FASTA files, multiple sequence alignments, files supplementary materials, along with the GenBank accession number can be provided by the corresponding author on request.

Conflict of interest

The authors declare that they have no conflict of interest.

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