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Optimization of Cellulases under Solid State Fermentation by Newly Isolated Fungus: An Environmentally Sustainable Approach

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Abstract

There exists a substantial interest in advancing the commercial production of cellulolytic enzymes. This drive is fueled by the pursuit of cost-effective substrates and energy-efficient fermentation processes, all aimed at enhancing the economic viability of enzymatic conversion of lignocellulosic biomass (LB) into bioethanol. In the present study, emphasis was given to the isolation of superior cellulase-producing fungal isolate. A comprehensive collection of 199 fungal isolates was derived from diverse soil samples, and the fungal isolate with the largest hydrolytic halos was identified as *Trichoderma atroviride* AD-130 through molecular analysis. The potential of cellulase production was explored and optimized during solid state fermentation (SSF) using inexpensive substrates such as *Eichhornia crassipes* and Municipal Solid Waste (MSW). *Trichoderma atroviride* AD-130 displayed the maximum cellulase production on the fifth day at pH 7.0 at a substrate-moisture ratio of 1:6 for *E. crassipes* and 1:2 for MSW under solid-state fermentation. The cellulases obtained were partially purified and characterized for their optimal pH (6.0, 4.5, and 5.0 for FPase, CMCase, and BGL respectively) and temperature conditions (60°C, 50°C, and 70°C for FPase, CMCase, and BGL respectively). Zymogram analysis revealed that the cellulolytic fungus *T. atroviride* AD-130 possessed multiple alleles for the synthesis of CMCase, which is crucial for the effective degradation of various types of lignocellulosic substrates.

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Introduction

Bioethanol, derived from lignocellulosic biomass (LB), possesses the potential to substitute our reliance on conventional petroleum fuel. LB, being a renewable and carbon-neutral resource, serves as an excellent raw material with vast potential for sustainable bioethanol production. This, in turn, can contribute to mitigating climate change and ensuring long-term energy security. The bioconversion process of lignocellulosic biomass (LB) into ethanol involves three main steps: pretreatment, saccharification/ hydrolysis, and fermentation. Cellulases play a crucial role in generating fermentable sugars for ethanol production. Consequently, a significant quantity of cellulase enzymes is required to scale up the conversion process to an industrial level. However, the high production cost of commercially available cellulases makes the entire process expensive. In recent years, numerous studies have focused on exploring new approaches to develop a cost-effective enzyme production process.[1] In this particular context, extensive research has been executed on various microorganisms, particularly filamentous fungi such as *Aspergillus niger*,[2-3] *Trichoderma* sp.,[4-5] and *Penicillium* sp.,[6] to explore their capacity for producing cellulases in high quantities. Additionally, strain improvement using genetic engineering and recombinant DNA technologies has also been investigated to enhance cellulases production. These efforts hold significant potential for creating cost-effective and efficient enzyme production processes.

In recent years, remarkable inclination towards adopting solid-state fermentation instead of submerged fermentation for producing microbial enzymes, mainly because of its superior energy efficiency have witnessed.[7] Several studies have compared the capabilities of SSF and SmF, consistently demonstrating that SSF yields better hydrolytic performance and higher initial reaction rates, resulting in higher levels of extracellular enzymes.[8-9] In SSF, microorganisms grow on the surface of solid substrates without access to free water. This enables faster and more cost-effective downstream processing with reduced purification and extraction requirements[10] Additionally, SSF allows the utilization of low-cost agricultural waste or residues as substrates for enzyme production, thereby reducing production costs.[11] Numerous reports have demonstrated successful cellulase production through SSF using a variety of substrates, including wheat bran,[12] oil palm waste,[13] agro-waste,[14] waste paper,[15] agricultural kitchen waste residue,[16] *Jatropha curcas* seed cake,[17] sweet sorghum stalk,[18] sugarcane residue,[19] rice straw,[20] apple pomace,[21] corn straw,[22] and fruit wastes,[23] among others.

Fungi exhibit greater involvement in solid state fermentation (SSF) compared to bacteria due to the low water activity present in solid substrates.[24] In this study, various fungal isolates obtained from soil samples were investigated, and the fungal isolate displaying the highest hydrolytic potential was selected for SSF using two cost-effective substrates: municipal solid waste (MSW) and water hyacinth (*Eichhornia crassipes*) to study cellulases production. Municipal solid waste, which contains a high content of lignocellulosic material, was chosen as a promising substrate that has received limited attention in cellulase production studies. On the other hand, water hyacinth was selected due to its rapid and problematic growth in water-bodies, causing environmental issues,[25] but containing high cellulose and low lignin content, with approximately 60% cellulose, 8% hemicellulose, and 17% lignin which makes it a suitable and cost-effective substrate for bioethanol production.[26]

Materials and Methods

Isolation and Screening of Microorganisms

Fungi capable of producing cellulase were isolated from soil samples collected in the districts of Kurukshetra, Jind, Kaithal, Ambala, Rohtak, and Jhajjar in Haryana, India (27°37 to 30°35N and 74°28 to 77°36E). The isolation process involved serial dilution of the soil samples followed by culturing on Potato Dextrose Agar (PDA) media.[27] Each fungal isolate was spot inoculated on modified Mandel's and Sternburg (MS) basal agar medium[28] (composition: Proteose Peptone - 1.4g/l, KH_2PO_4 - 1gm/l, $(NH_4)_2SO_4$ - 2.0gm/l, $MgSO_4.7H_2O$ - 0.3gm/l, Urea - 0.3gm/l, $FeSO_4.7H_2O$ - 0.005gm/l, $CaCl_2$ - 0.3gm/l, $MnSO_4.H_2O$ - 0.0016gm/l, $ZnSO_4.7H_2O$ - 0.0014gm/l, $CoCl_2$ - 0.002gm/l) at pH 5.0, supplemented with 1% (w/v) CMC (Carboxymethylcellulose) in petriplates. The fungal cultures were then incubated at 30ºC for three days. Following incubation, every culture plate was exposed to a Congo red solution (0.1%) for a duration of 15 min., proceeded by rinsing with 1M sodium chloride to eliminate any surplus dye. The measurement of the diameter of the hydrolysis

zone surrounding the fungal colonies served as an indicator of cellulase activity.

Fungal isolates displaying larger hydrolysis zones were chosen for cellulase enzyme production. The screening of cellulase production through SSF was conducted using the untreated and dried lignocellulosic biomasses of *E. crassipes* as a carbon source. SSF was conducted in 500 mL Erlenmeyer flasks containing 5g of dried *Eichhornia crassipes* biomass with particle sizes ranging from 0.2 to 1.0 mm. To initiate SSF, the biomass was moistened first with 30 mL of MS basal medium and then autoclaved to ensure sterility for 20 minutes at 121°C. A 1.0 mL suspension containing $5x10^7$ spores was used to inoculate the sterilized flasks, which were incubated at 30°C with intermittent shaking. On the 4th day, the culture flasks were removed, and the enzymes were extracted by adding 10 mL of Citrate Buffer (0.05 M, with a pH of 4.8) per gram of substrate. The mixture was shaken on an incubator shaker with a rotation of 200 per minute (rpm), kept at a constant temperature (30°C) for an interval of one hour, and then filtered. The filtrate was then carefully subjected to centrifugation at a speed of 10,000 revolutions per minute for a duration of 10 minutes with a temperature of 4°C, which successfully yielded a vibrant supernatant. Subsequently, the cellulase activity of this supernatant solution was assayed and compared to that of *Trichoderma reesei* NCIM 992, a commercially available fungus strain. The reference strain was sourced from the "National Collection Centre of Industrial Microorganisms" located in Pune, India.

Identification of Potent Cellulolytic Fungal Isolates

The fungal isolate-130, analyzed for its strong cellulase production, underwent primary identification at the Institute of Microbial Technology (IMTECH) in Chandigarh. This identification was achieved through the analysis of the Translation Elongation Factor gene sequence. The obtained sequence was compared with the existing database sequences using a "Basic Local Alignment Search Tool (BLAST)" to examine its resemblance with the known one. For a more in-depth examination of its evolutionary relationships, a comprehensive phylogenetic tree was created using the "Mega 5 software".

Lignocellulosic Substrate

The dried and untreated lignocellulosic biomasses (LBs) of *E. crassipes* and Municipal Solid Waste (MSW) of particle sizes 0.2 to 1.0 mm were used as substrates for solid-state fermentation.

Preparation of Inoculum

The fungal slants were prepared by dispensing a sterile solution of normal saline mixed with 0.1% (v/v) Tween-80 into each tube, with volumes ranging from 20 to 40 mL. Using a sterile loop, the spores were gently dislodged from the fungal culture and introduced into the saline solution. To ensure an even distribution of spores, the liquid was vigorously shaken. For a precise determination of spore concentration, a hemocytometer was employed, and 1 mL aliquot of the suspension estimated to contain approximately $1x10^7$ spores was utilized for spore count procedure.

Solid State fermentation

In the experiment, Erlenmeyer flasks with volumes of 250mL and 500mL were used. The flask of 250mL contained 5g of municipal solid waste (MSW), while the 500mL flask contained 5g of *Eichhornia crassipes* biomass. These materials were combined with a specific quantity of Mandels and Sternburg (MS) basal medium,[29] which consisted of the following components per liter: protease peptone-1g, $(NH_4)_2SO_4$-1.4gm, KH_2PO_4-2.0gm, Urea-0.3gm, $MgSO_4 .7H_2O$ - 0.3gm, $CaCl_2$-0.3gm, $FeSO_4.7H_2O$ - 0.005gm, $ZnSO_4$.7H2O-0.0014gm, $CoCl_2$ -0.002gm, $MnSO_4.H_2O$-0.0016gm and Tween-80 (0.1% v/v). Additionally, glucose (0.1% w/v) was supplemented into the medium. The entire mixture was sterilized using an autoclave (at 121°C) for 20 minutes before inoculation with 1.0mL of a suspension containing $5x10^7$ spores. To sustain the cultures, all flasks then were incubated with periodic shaking (at 30°C temperature). At predetermined intervals, the culture was removed for cellulase activity analysis, focusing on the measurement of three enzymes: FPase (filter paperase), CMCase (carboxymethylcellulase), and BGL (β-glucosidase).

Enzyme Extraction

To extract the contents of the flasks, a substrate-to-citrate buffer ratio of 1:20 was employed, with the buffer consisting of a 0.05M solution at pH 4.8. The flasks were placed in an incubator and shaken for

one hour at 200 rpm (30°C temperature). Afterward, the entire mixture was filtered to separate the solid and liquid components using a muslin cloth. To eliminate any remaining spores, the obtained filtrate underwent 10,000 rpm centrifugation at 4°C for 15 minutes. The subsequent clear supernatant obtained was then used for further experiments.

Enzyme Assays

To calculate FPase activity, we used a conversion factor indicating 0.37 FPUs of enzyme yields 2.0mg of glucose when assessed during the specified assay conditions.[30] For estimating CMCase activity (U/mL), we utilized the fact that 0.185 units of enzymes release 0.5mg of glucose during the established conditions.[30] The assessment of BGL activity was based on the utilization of P-Nitrophenyl-D-Glucopyranoside (PNPG) as a substrate.[31] In the context of SSF, the quantity of β-glucosidase was quantified in units per gram of dry substrate (U/g). Here, 1 U represented the specific volume of enzyme needed to produce one mole of p-Nitrophenol in one minute, provided that the enzyme is operated under standardized assay conditions.

Optimization of fermentation conditions in solid state

The investigation into cellulase production involved assessing the impact of various factors using an OFAT (One-Factor-At-A-Time) design. The factors under scrutiny included moisture content, incubation time, pH, and nitrogen sources. To determine the impact of initial moisture level on cellulase synthesis, the substrate-to-media ratio (w/v) was modified from 1:1 to 1:3 for municipal solid waste (MSW) and from 1:4 to 1:7 for *Eichhornia crassipes*. Further analysis of cellulase production was conducted at the optimized substrate moisture ratios of 1:2 for MSW and 1:6 for *Eichhornia crassipes*. The fermentation process spanned a duration of 7 days, with cellulase production monitored at 24-hour intervals from the 3rd to the 7th day. To examine the influence of the initial pH of the culture on cellulase activity during the optimal incubation time, the pH of the MS medium was regulated by introducing 1N HCl or 1N NaOH, covering a range of 4.0 to 8.0. Additionally, the impact of diverse nitrogen sources on cellulase production was investigated using a complex MS medium. The medium incorporated varying amounts and combinations (I to VI) of nitrogen sources of both organic and inorganic origin, maintaining the optimal incubation period and starting pH of the moistening medium.

I: 0.1% PP + 0.03% Urea + 0.14% AS
II: 0.05% PP + 0.03% Urea+ 0.14% AS
III: 0.1% PP+ 0.03% Urea + 0.28% AS
IV: 0.1% PP+ 0.03% Urea + 0.05% YE + 0.21% AS
V: 0.05% PP + 0.03% Urea+ 0.1% YE + 0.21% AS
VI: 0.2% PP+ 0.03% Urea + 0.28% AS
AS = Ammonium Sulphate, PP = Protease Peptone, YE = Yeast Extract

StatisticalTool

All experiments were conducted in triplicate to confirm the accuracy of the results. The experiments data were analyzed and presented as mean value accompanied by the standard error of the mean to give a clearer understanding of the variability. Calculation of the statistical analyses were performed using MS Excel.

Table 1: Hydrolytic zones of different fungal isolates

Name/ No. of Fungal Isolates	Zone of hydrolysis (cm)	Name/ No. of Fungal Isolates	Zone of hydrolysis (cm)
2	9.0±0.3	81	8.8±0.2
7	8.7±0.3	87	9.3±0.3
44	8.8±0.2	130	9.4±0.2
55	9.1±0.2	148	8.7±0.3

Results

Identification of Fungal Strains

In this comprehensive study, a remarkable collection of 199 fungal isolates was meticulously procured from a diverse array of 50 soil samples spanning various regions of Haryana. To ascertain their cellulase production capabilities, a meticulous qualitative screening was carried out employing the plate assay

method. Remarkably, out of the myriad fungal isolates, a select group of eight strains (isolates 2, 7, 44, 55, 81, 87, 130, and 148) exhibited significantly augmented zones of hydrolysis, thereby indicating a promising cellulase activity (Table 1). These exceptional fungal isolates, which displayed immense potential, were subsequently subjected to a rigorous secondary screening process aimed at assessing cellulase production under solid-state fermentation (SSF) conditions.

A comparative analysis of cellulase production under SSF was performed, pitting selected fungal isolates against the commercial strain *Trichoderma reesei* NCIM 992 (Figure 1). Remarkably, fungal isolates 55, 81, 87, 130, and 148 displayed significantly higher activity levels of FPase, CMCase, and BGL in comparison to *T. reesei* NCIM 992. Conversely, fungal isolates 2, 7, and 44 exhibited lower production of FPase and CMCase, although their BGL activities were mostly comparable, except for fungal isolate 2, which showcased higher β-glucosidase activity than *T. reesei* NCIM 992. Among all the fungal isolates, isolate 130 exhibited the maximum values of FPase, CMCase, and BGL, measuring at 38.57, 103.49, and 23.08 U/g, respectively, in contrast to 28.91, 82.51, and 6.11 U/g observed in *Trichoderma reesei* NCIM 992. Due to its exceptional cellulase activity, fungal isolate-130 was chosen as the prime candidate for subsequent trials among all the fungal isolates.

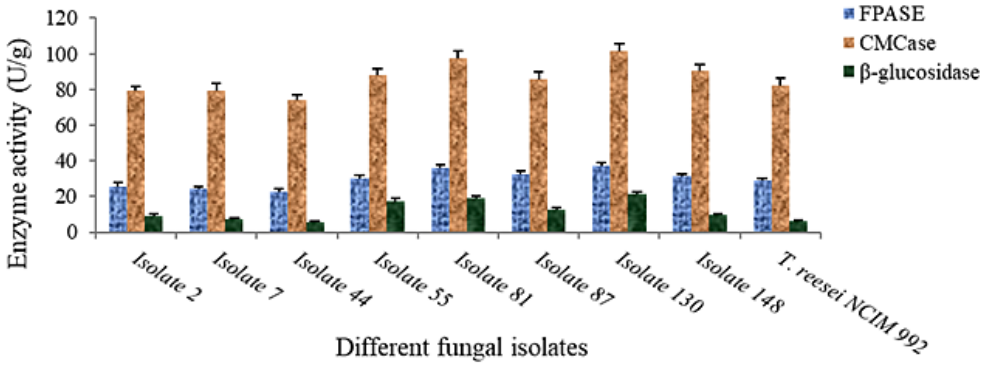

Fig. 1 : Cellulase production on *E. crassipes* biomass under solid state fermentation by various selected fungal isolates as compared to *T. reesei* NCIM 992

Identification of Fungal Isolate

Based on an initial microscopic examination of morphological features, the fungus in question was identified as a member of the *Trichoderma* genus. To further confirm the molecular identity of fungal isolate-130, 19 nucleotide sequences for Translation Elongation Factors were utilized and subjected to rigorous analysis. To ensure robust and accurate identification, a phylogenetic tree was constructed using the Kimura-2-parameter (K2P) model, and the gene sequence database and MEGA 5 software were employed (Figure 2). Notably, the selected strain exhibited a close affiliation with *Trichoderma atroviride* (AF456891), a previously reported deuteromycetes fungus, as evident from their shared clade. Fusarium tricinctum (AB674263) served as the outgroup species. Consequently, the chosen strain was officially designated as *T. atroviride* AD-130, as per GenBank Accession KJ997913, and subsequently employed in solid state fermentation experiments, utilizing *E. crassipes* and municipal solid waste (MSW) as substrates, to explore its potential for producing desired enzymes.

> *Trichoderma atroviride,* Translation Elongation Factor gene sequence data

GTCGTCAGCGGCATTCTCTCTGCCGTTGGCACTGATGTGTATGACATTT
TGCTGACCATCATCGTCTAGGGGTTCGTATTTCTCCATCAGACAGCTTG
CGACCGACTCCAATACTGACTTGCTATAACAGCCACGTCGACTCCGGCA
AGTCTACCACCGTGAGTAACTCCCAATTCCTCGAGCCCTGCTGCCATCG
ACTCTATCGGTCGGCGCGGGGTATCATCATCATGAACGCATCCGGCTGA
CATTTTCCCAACAGACTGGTCACTTGATCTACCAGTGCGGTGGTATTGA
CAAGCGTACCATCGAGAAGTTCGAGAAGGTGAGCTCATTTCTGCTTTTT
CACAATCGTGCCCGACAATTCTGTCCTCAGTCTTGTCTTTTTTCTCTCGC
AGCATCACACCCCGCTTTACCTGTCTACCCCTCCTTTGGCACAGCAAAA
ATTTTCTGGCTGCCTTGCTTGGCTTTTAGTGGGGTGCCAACTTTTTTTTT
GTTGTGGCCGGAACCCCGCTATCGCCACTGTCCCTCACTCAATTGCATC
GTCTTCTGCTGGTTCATTGTGCTAATCATGCTTCAATCAATAGGAAGCC
GCCGAACTCGGCAAGGGTTCTTTCAAGTATGCGTGGGTTCTTGACAAGC
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TTTCCTCATTGACCCT

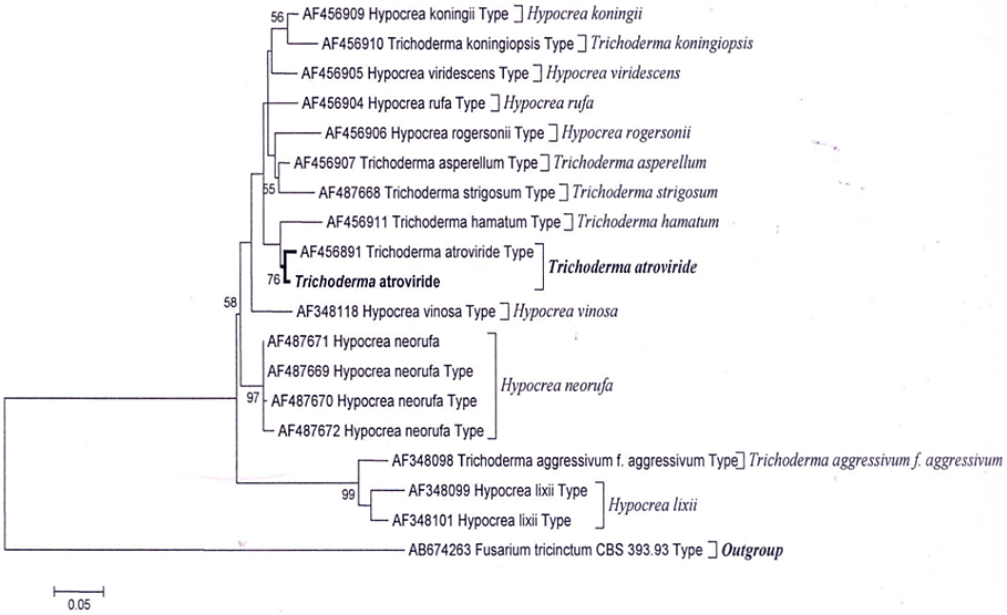

Fig. 2: Phylogenetic tree of Trichoderma atroviride

Impact of Moisture Level on Cellulase yield

In the present work, the effect of moisture level on enzyme activity was examined at the 5.0. pH of the fermentation medium while setting the temperature at 30°C. The enzyme activities were measured on the 5th day of fermentation. Figures 3 and 4 depict the impact of the primary moisture level on cellulase yields by *T. atroviride* AD-130 cultivated through SSF, using *E. crassipes* biomass and MSW residue as substrates, respectively. When *E. crassipes* was utilized as the substrate in SSF, the maximum activity of FPase was recorded at 41.17U/g, followed by CMCase at 141.83U/g, and BGL at 25.16U/g, all achieved at a substrate-moisture ratio of 1:6. On the other hand when MSW was employed as the substrate, the maximum production of all enzymes was found at a substrate to moisture ratio of 1:2, with FPase reaching 42.12U/g, CMCase at 79.54U/g, and β-glucosidase at 39.83U/g. However, it should be noted that a reduction in cellulase activity was observed in all other substrate-moisture ratios. Consequently, further investigations were carried out to assess the enzyme production capabilities of *T. atroviride* AD-130 through solid-state fermentation utilizing *E. crassipes* and MSW as carbon sources with the substrate-to-moisture ratios of 1:6 and 1:2, respectively.

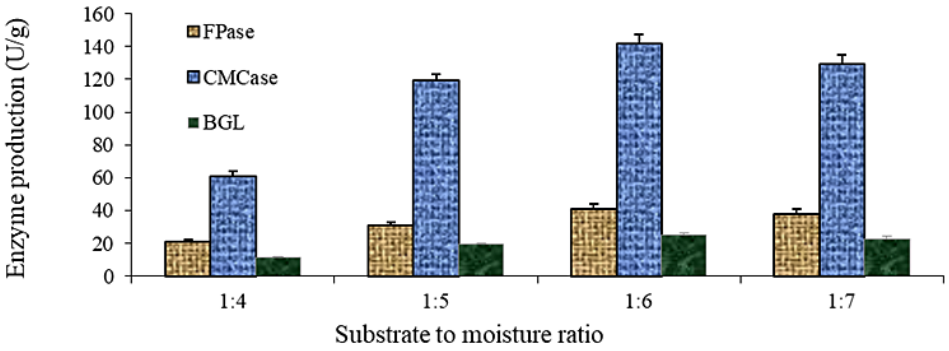

Fig. 3: Effect of substrate moisture ratio on cellulase production under SSF by *T. atroviride* AD-130 on *E. crassipes* biomass

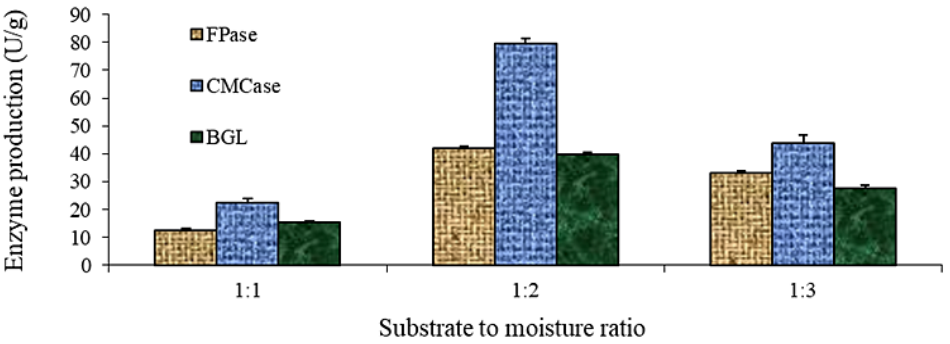

Fig. 4: Effect of substrate moisture ratio on cellulase production under SSF by *T. atroviride* AD-130 on MSW residue

Impact of Incubation Period on Cellulase Production

The impact of incubation time, from 3 days to 7 days, on fungal cellulases production was investigated using *T. atroviride* AD-130 under SSF with *E. crassipes* and MSW as carbon sources in substrate to moisture ratio of 1:6 and 1:2, respectively. The results, illustrated in Figure 5, exhibited that the highest enzyme production occurred on the 5th day. Maximum yields of FPase, CMCase, and BGL were achieved with MSW: 42.12U/g, 79.54U/g, and 39.83U/g, respectively. On the other hand, *E. crassipes* demonstrated maximum yields of FPase (41.17U/g), CMCase (141.83U/g), and BGL (25.16U/g). Notably, a comparison between the 3rd and 7th day revealed significant differences in enzyme production. On the 3rd day, the activities were 18.96U/g, 51.87U/g, and 12.96U/g for FPase, CMCase, and BGL on *E. crassipes*, respectively, and 19.32U/g, 26.87U/g, and 14.53U/g on MSW, respectively. However, on the 7th day, higher enzyme activities were observed: 34.11U/g, 121.71U/g, and 19.56U/g for FPase, CMCase, and BGL on *E. crassipes*, and 35.16U/g, 65.27U/g, and 31.14U/g on MSW, respectively.

Impact of Initial pH on Cellulase Production

Fungi generally exhibit a broad pH tolerance, ranging from 4.0 to 8.0 (1998), and they have specific pH preferences for optimal growth and enzyme activity. To investigate the effect of initial pH on the cellulase activity using *T. atroviride* AD-130, the culture medium's pH was adjusted within the range of 4.0 to 8.0. Figure 6 illustrates that the highest yields of FPase (50.09U/g), CMCase (166.21U/g), and BGL (40.49U/g) were achieved at pH 7.0 during SSF of *E. crassipes*, using a substrate moisture ratio of 1:6 at 30°C, on the 5th day. At pH 4.0 and 8.0, the FPase yields were 40.06U/g and 42.19U/g, respectively. Likewise, CMCase activities at pH 4.0 and pH 8.0 were 147.57U/g and 79.04U/g, respectively, while the corresponding BGL yields were 28.09U/g and 31.03U/g.

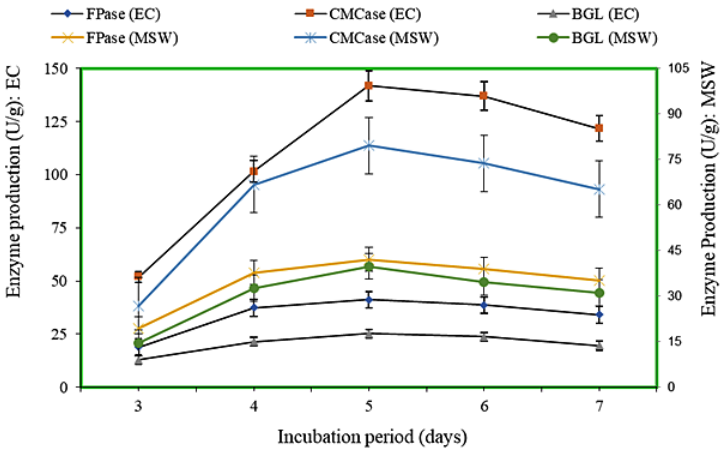

Fig. 5: Effect of incubation periond on cellulase production under SSF by *T. atroviride* AD-130 on *E. crassipes (EC) and* municipal solid waste (MSW) biomass

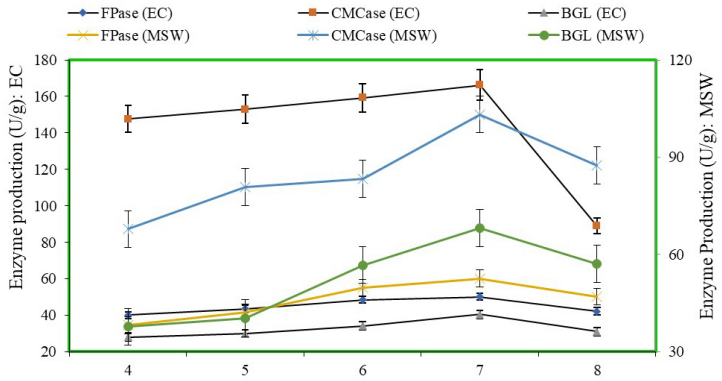

Fig. 6: Effect of initial pH of moistening medium on cellulase production under SSF by *T. atroviride* AD-130 on *E. crassipes (EC) and* municipal solid waste (MSW) biomass

Figure 6 also illustrates that the peak activities of FPase (52.57U/g), CMCase (103.21U/g), and BGL (68.12U/g) were attained at pH 7.0 during MSW solid state fermentation (SSF), employing a substrate moisture ratio of 1:2 at 30°C, on the 5th day. At pH levels of 4.0 and pH 8.0, the respective activities of FPase, CMCase, and BGL were 38.17U/g, 67.82U/g, and 37.68U/g, as well as 47.04U/g, 87.51U/g, and 57.07U/g, respectively.

Impact of Nitrogen Sources on Cellulase Yield

Cellulase enzyme synthesis during SSF is influenced by the nature and concentration of nitrogen sources in the medium,[41] as the fermentation process is significantly influenced by the pH of the solution, which can be altered by the nitrogen source employed.[42] Therefore, the current work aimed to evaluate the cellulase production of *T. atroviride* AD-130 during SSF, conducted at optimized 30°C and pH 7.0, under different combinations of organic and inorganic nitrogen sources (referred to as combinations I to VI in Table 2) with *E. crassipes* biomass and MSW residue serving as the substrates for a period of five days.

Table 2 illustrates the maximum cellulase production in term of FPase, CMCase and BGL activities by the fungus *T. atroviride* AD-130 as 52.57U/g, 103.03U/g, and 68.12U/g on MSW residue, and 50.09U/g, 166.21U/g, and 40.49U/g on *E. crassipes* biomass, respectively. These results were achieved during SSF when using a combination of 0.1% protease peptone, 0.14% ammonium sulfate, and 0.03% urea (referred to as combination I). Interestingly, altering the concentration of organic and inorganic nitrogen sources did not significantly contribute to

the enhancement of cellulase production under SSF conditions. The lowest activities (U/g) of FPase, CMCase, and BGL, when using MSW residue and *E. crassipes* biomass as substrates, were observed in the presence of combination VI, with 0.2% protease peptone, 0.28% ammonium sulfate, and 0.03% urea, resulting in values of 40.82, 77.63, and 41.29, and 36.03, 118.71, and 27.09, respectively.

Table 2: Effect of various Nitrogen Sources on cellulase yields by *T. atroviride* AD-130 at 30°C and pH 7.0

Combi-nation	Municipal solid wastes			*E. crassipes*		
	FPase Activity (U/g)	CMCase activity (U/g)	BGL activity (U/g)	FPase Activity (U/g)	CMCase activity (U/g)	BGL activity (U/g)
I	52.57±2.47	103.21±5.03	68.12±3.41	50.09±2.25	166.21±6.28	40.49±1.28
II	45.03±2.38	87.73±3.29	51.38±2.12	43.40±2.22	148.07±5.80	32.19±1.21
III	41.63±1.67	86.98±3.70	49.31±1.82	41.82±1.94	143.15±3.78	30.91±1.27
IV	49.42±2.56	97.83±5.09	63.05±2.27	47.15±2.87	157.47±6.21	38.03±1.37
V	38.03±1.51	84.07±3.51	60.39±2.23	42.51±2.12	137.15±5.02	37.60±1.73
VI	40.89±1.63	77.63±2.29	41.29±1.71	36.03±1.71	128.71±6.04	27.09±1.08

Combinations (I to VI)
I: 0.1% PP + 0.03% Urea + 0.14% AS
II: 0.05% PP + 0.03% Urea + 0.14% AS
III: 0.1% PP + 0.03% Urea+ 0.28% AS
IV: 0.1% PP + 0.03% Urea+ 0.05% YE + 0.21% AS
V: 0.05% PP + 0.03% Urea+ 0.1% YE + 0.21% AS
VI: 0.2% PP + 0.03% Urea+ 0.28% AS
AS: Ammonium Sulphate; PP: Protease Peptone; YE: Yeast Extract

Characteristics of Partially Purified Enzymes
Impact of pH on Enzyme Activity
The activity of enzymes is significantly influenced by the pH of the medium, as it affects the distribution of charges on both the substrate and enzyme molecules, thereby impacting catalysis through substrate binding to the enzyme's active site. Figure 7 illustrates how pH affects the performance of CMCase, FPase, and BGL. The optimal pH ranges for CMCase, FPase, and BGL were, 4.0 to 5.0, 5.0 to 7.0, and 4.5 to 5.5, with their respective optimum pH values being 4.5, 6.0, and 5.0. A marked reduction in relative enzyme activity was detected when the pH level ranged below and above the optimum level. The relative activities of FPase at pH 7.5 and 4.5 were 78.96% and 82.90%, respectively, compared to the optimum pH, while it was 73.84% at pH 3.5. CMCase exhibited relative activities of approximately 78% at pH 3.5 and 5.5, with a minimum relative activity of 18.79% at pH 7.5. The relative activities retained by β-glucosidase were 56.90% at pH 4.0, 63.59% at pH 6.0, and a minimum of 8.16% at pH 7.5.

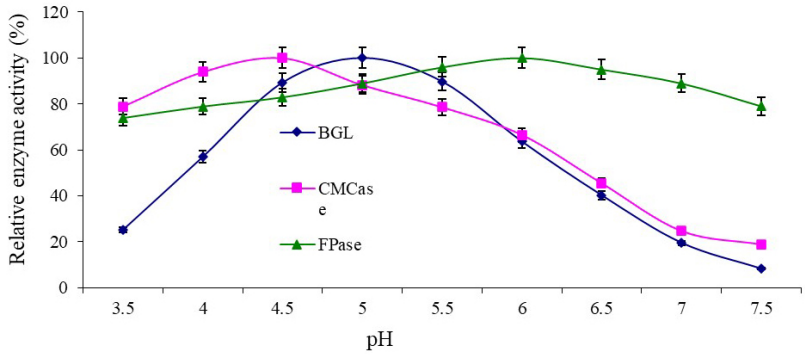

Fig. 7: Effect of varying pH on the activity of partially purified cellulases of *T. atroviride* AD-130

Impact of Temperature on Enzyme Activity

Cellulases typically exhibit optimal activity at temperatures between 40°C to 60°C.[52] To determine the optimum temperature for FPase, CMCase, and BGL, enzyme samples were incubated with their respective substrates at temperatures ranging from 30°C - 80°C. The temperature dependence of FPase, CMCase, and BGL activities is presented in Figure 8. In this study, the optimum temperatures for FPase, CMCase, and BGL activities was investigated as 60°C, 50°C, and 70°C, respectively. The respective optimal temperature ranges for FPase, CMCase, and BGL were found to be 40 to 70°C, 40 to 60°C, and 60 to 70°C. Significantly, at 80°C, there was a noticeable reduction in FPase and BGL activity, whereas CMCase activity decreased at 70°C, resulting in residual activities of 62.17%, 41.88%, and 48.68%, respectively, at these temperatures.

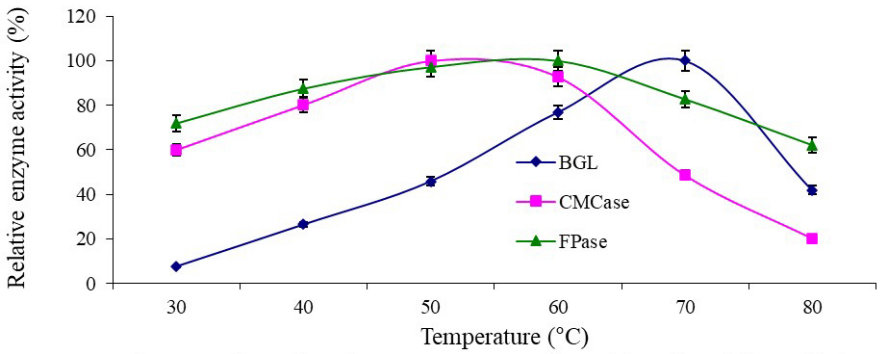

Fig. 8: Effect of varying temperatures on the activity of partially purified cellulases of *T. atroviride* AD-130

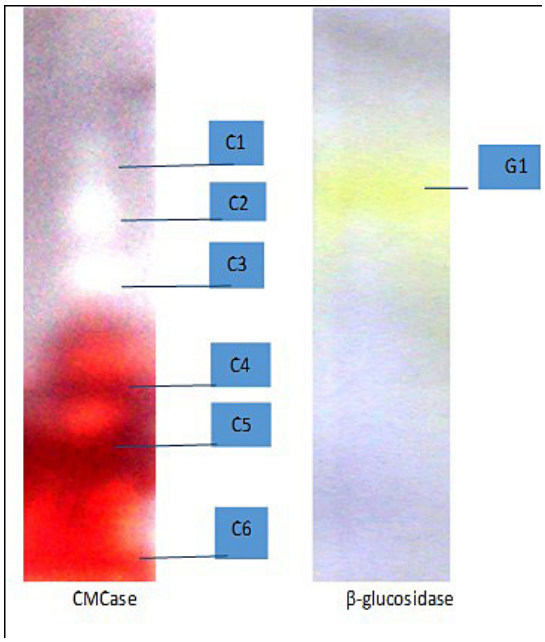

Fig. 9: Zymogram staining on native PAGE of partially purified CMCase and β-glucosidase

Detection of CMCase and β-glucosidase Activity by Zymogram Staining

In the current study, the zymograms of CMCase and BGL were prepared by incorporating 0.2% carboxymethylcellulose (CMC) and 0.5% p-nitrophenol-D-glucopyranoside into 10% native PAGE respectively as shown in Figure 9. Zymogram analysis of partially purified cellulases from *T. atroviride* AD-130 exhibited six distinct bands (C1, C2, C3, C4, C5, and C6) corresponding to different isoforms of CMCase, along with a single active band (G1) for β-glucosidase.

Discussion

Different studies reported varying optimal substrate-to-water ratios and moisture levels for maximizing enzyme production. Researchers[12] found a 1:1.5 ratio suitable for highest CMCase and FPase production by *Aspergillus niger*, while a 1:1.75 ratio yielded the highest β-glucosidase yield. A study demonstrated that *Trichoderma* asperellum achieved maximum FPase production (26.03U/g) at 60-80%

moisture content during SSF of oil palm frond leaves.[32] Another study reported the maximum cellulase production at a 70% moisture level using *A. niger*.[17] Similarly, the highest FPase yield using *A. niger* and *T. reesei* at 70% moisture level was obtained.[33] Talaromyces stipitatus exhibited highest FPase (4.51FPU/gds), endoglucanase (53.3IU/gds), and BGL (62.6IU/gds) activities with *Parthenium hysterophorous* as substrate under SSF conditions (pH 6.0, 30°C) with 80% moisture level.[34] Thus, multiple studies have emphasized the impact of moisture levels on cellulase enzymes across various fungal strains and substrates. In this study, deviations from the optimal moisture content, whether higher or lower, negatively affected enzyme production because the increased moisture content might have led to decreased porosity, changes in substrate structure, reduced gas volume, and hindered inter-particle spaces.[35] while the lower moisture content could have reduced nutrient solubility, impeded swelling, and increase water tension within the solid substrate.

The cellulase production in the present study exhibited a pattern where the minimum production was observed on the 3rd day, followed by a peak on the 5th day, and a subsequent decline. This decline in enzyme yields could be attributed to the release of proteases and a decrease in the pH of the medium, which, as suggested by the previous study,[36] might have been influenced by various factors, including the ratios of amorphous to crystalline cellulose. Herewith, it is also noteworthy that some of the studies testified the highest cellulases production by *A. niger* after 120 hours of incubation,[17] whereas some work contradicts the results and achieved significant cellulases production within a three-day fermentation period using Aspergillus and *Trichoderma* spp.[37,38] Likewise, researchers reported maximum FPase and β-glucosidase (BGL) production by *A. niger* after 96 hours of fermentation[39] while some authors observed the highest FPase and CMCase production by *T. harzianum* on the 4th day of fermentation.[40] These contrasting results highlight the variability in cellulases production kinetics among different fungal strains and underscore the importance of optimizing fermentation conditions for each specific strain to achieve maximum enzyme yields.

The obtained results align with previous works, which also observed that the highest yields of CMCase, FPase, and BGL were achieved at pH 7.0.[12,13] Contrarily, a study reported that *A. niger* exhibited the highest cellulase production at an initial pH of 5.0.[17] Similarly, rediscovered that *A. niger* displayed the highest production of FPase and β-glucosidase at an initial pH of 4.0.[39] At the same time, maximum production of FPase and CMCase at an initial pH of 6.0 was observed by *T. harzianum*.[40]

The study demonstrated that the maximum yield using A. flavus was achieved via incorporating 2 g/L sodium nitrate (NaNO3T) and 2 g/L protease peptone as nitrogen sources in the fermentation medium [43]. In contrast, highest cellulase yield by A. terreus when fermentation medium contained 1% (w/v) yeast extract.[44] Additionally, studies have found that inorganic nitrogen sources to be optimal for cellulase production. For instance, researcher reported that, *T. harzianum* exhibited maximum enzyme production of CMCase (26.22IU/gds) and total cellulase (7.42FPU/gds) in the presence of 0.02M ammonium ferrous sulfate.[40] Similarly, a study found that incorporating 0.2% (w/w) urea in the fermentation medium resulted in maximum cellulase production by *A. niger*.[39] Study reveals that, addition of urea and ammonium sulfate significantly increased cellulase production by *P. funiculosum*.[41] In optimizing the medium components for cellulase synthesis by *Melanocarpus* sp. under SSF, study found that the addition of urea to rice straw, used as the carbon source, was beneficial.[45] However, one study observed no significant increase in cellulase activity in *A. niger* when using any of the investigated organic or inorganic nitrogen sources.[17]

Previous studies have also reported optimal pH values for CMCase and FPase produced by *T. atroviride* to be 4.0 and 5.0, respectively.[46] In another study pH 5.5 was found to be the most favorable for CMCase activity in *Trichoderma* sp.,[47] while pH 5.0 was identified as the optimal condition for FPase activity in another strain of *T. atroviride*.[48] Although endoglucanase and β-glucosidase produced by A. terreus exhibited higher activity at pH 2.0 and pH 3.0, respectively,[44] but pH 7.0 found to be the most favorable for CMCase activity.[49] *A. niger*'s CMCase exhibits its most effective performance in the pH of 6.0 to 7.0.[50] The optimal pH values for CMCase, β-glucosidase, and FPase produced by *Aspergillus fumigatus* were determined to be pH 3.0, 4.0, and 5.0, respectively.[51] In another work, pH 4.0 reported as the

most favourable for cellulase activity in *A. niger.*[17] However, the optimal pH values for CMCase and FPase produced by *T. harzianum* to be 5.5 and 5.0, respectively.[40]

CMCase and FPase produced by *T. atroviride* displayed optimal activity at 60°C and 50°C, respectively (47). Most endoglucanases were found to be optimally active at temperatures close to 50°C (50). However, maximum FPase activity was reported from *T. atroviride* at 50°C,[46] while the optimal temperature for FPase activity identified as 60°C in *Trichoderma* sp. A-001.[53] CMCase and BGL were also found to be optimally active at 70°C, while FPase exhibited optimal activity at 60°C.[54] Both endoglucanase (CMCase) and β-glucosidase from A. terreus displayed maximum activity at 70°C.[44] Cellulase produced by *A. niger* demonstrated peak activity at 65°C.[17] In line with these findings, a study was reported the optimal temperatures for CMCase and FPase synthesized by *T. harzianum* to be 60°C and 55°C, respectively.[40]

Similarly, zymogram analysis of *A. niger* enzyme extracts revealed six active bands for CMCase.[17] In contrast, researchers identified two active bands for CMCase in *T. atroviride* zymogram staining, indicating the presence of two isoforms,[46] while team of authors observed three active bands for CMCase and one band for β-glucosidase in cellulases produced by *T. viride*.[54] Zymogram analysis of *A. fumigates* cellulases indicated the presence of three CMCase isoforms and two β-glucosidase isoforms.[51] Additionally, native PAGE for characterization of partially purified enzymes from A. flavus observed a single activity band for CMCase.[43]

Conclusion

To optimize cellulase production during SSF, the newly isolated *T. atroviride* AD-130 was cultivated using untreated and dried lignocellulosic biomasses, namely *E. crassipes* and municipal solid waste, at 30°C. The highest cellulase production was attained on the 5th day, with a substrate-moisture ratio of 1:6 for *E. crassipes* and 1:2 for MSW, with pH attuned to 7.0. Optimized medium contained 0.1% protease peptone, 0.14% ammonium sulfate, and 0.03% urea, with a pH range of 6.0-6.5 for FPase, 4.5-5.0 for CMCase, and 5.0-5.5 for β-glucosidase activity. The optimum temperatures for FPase, CMCase, and BGL were determined as 60°C, 50°C, and 70°C, respectively. Zymogram of partially purified cellulases from *T. atroviride* AD-130 exhibited six isoforms (C1, C2, C3, C4, C5, and C6) of CMCase, along with a single active band (G1) for β-glucosidase. Despite the limited β-glucosidase production in numerous *Trichoderma* spp., *T. atroviride* AD-130 demonstrated a well-balanced proportion of both FPase and BGL. This balanced enzyme profile will be quite conducive for efficient cellulose hydrolysis into glucose by overcoming cellobiose inhibition.

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Conflict of Interest

The author(s)do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Author Contributions

- **Anil Kumar:** Ph.D. student and Co-investigator, of Research Project.
- **Dr. Meenakshi Suhag:** helped draft, edit, and corresponding author manuscripts.
- **Dr. Joginder Singh:** Principal Investigator of Research Project, Conceptualization, Methodology, Designing and Editing
- **Dr. Ritu Nandal:** Assist in Experimental Work
- **Dr. Naveeta Dhaka:** Assist in Experimental Work
- **Dr. Rajesh Dhankhar:** Supervisor of Co-investigator.

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