



Isolation, Screening and Molecular Characterization of Cellulase producing *Trichoderma* Strains

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Cellulose is the most abundant polysaccharide on earth, and its crystalline structure is challenging to break down into sub-particles. To overcome the issue, cellulase enzyme plays a key role in the breakdown of cellulose into glucose monomers. Microorganisms, such as fungi, are known for the production of various commercially important enzymes. Among fungal genera, *Aspergillus* and *Trichoderma* are considered to have great potential for cellulase production. The present study was conducted to collect 75 soil samples from different districts in Punjab to isolate the *Trichoderma* strains. The isolated *Trichoderma* strains were then screened through double-phase screening methods. The highest cellulase-producing strain was characterized based on the internal transcribed spacer (ITS) region using the primer sets ITS1F and ITS4 and was identified as *Trichoderma asperellum*. Congo red dye and cellulase assay showed the maximum activity of 0.83 U/ml from *Trichoderma asperellum* and was used for cellulase production. Endoglucanase was produced using basal media with CMC as a substrate at 28°C for 72h. Crude extract was subjected to the Lowry assay and the total protein content was recorded. Cellulase was partially purified using various ammonium sulfate concentrations (30-80%). Partially purified enzyme characterized based on temperature and pH. The enzyme showed a maximum specific activity of 104.39 U/mg after dialysis. The maximum activity of the partially purified enzyme was observed at pH 4 and temperature 55°C

Keywords: Cellulase, Fungal Strains, Internal Transcribed Spacer Region, Ammonium Sulfate

Introduction:

Cellulase is a diverse group of three enzymes that catalyze the hydrolysis of β 1, 4-glycosidic bond in cellulose and other related oligosaccharides [1]. Endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91), and β -glucosidases (EC 3.2.1.21) constitute the cellulase complex and it takes the synergetic action of these three enzymes for cellulose to be completely hydrolyzed into glucose [2]. Cellulases are essential for bio stoning and bio polishing in the textile industry, for color enhancement and filth removal from color blend clothes in the detergent market, municipal waste management, fiber modification, and improving feed digestibility [3]. Recent research showed that cellulase is crucial to the breakdown of lignocellulosic material and leads to bioethanol production [4]. Cost-effectiveness and recyclability in cellulase enzyme production have been major concerns [5]. Cellulase enzyme has tremendous application in industrial as well as commercial sectors and is produced by a variety of microbes including fungi (*Aspergillus*, *Humicola*, *Penicillium*, and *Trichoderma*) and bacteria (*Cellulomonas*, *Actinomucur*, *Actinomycetes*, *Pseudomonas*, *Streptomyces* and *Bacilli*) [6]. Fungi are important in the decomposition of decaying organic matter and use lignocellulosic material as a substance for the production of cellulase. They spread widely in nature, particularly in soil, and exist in saprophytic mode producing cellulolytic enzymes in excess quantity. They can be isolated from soil, bark, wood, and other fungi, because of their adaptability to different conditions of the ecosystem [7]. The genus *Trichoderma* helps in the production of enzymes by a fermentation process. It helps in the degradation of polymers of carbohydrates. These fungi have dual activity, as they possess both biocontrol and plant growth-promoting properties and are therefore used in many crop varieties. Previously researchers focused on the production of cellulase from *Trichoderma harzianum* [8], *Trichoderma reesei* [9][10] and *Trichoderma viride* [1] but in the past few years, *Trichoderma asperellum* gained importance in cellulase production [11][12][13]. The current study is based on the isolation, screening, and characterization of the cellulase from locally isolated *Trichoderma asperellum*. This study is important due to the role of *Trichoderma asperellum* in the prevention of soil-borne diseases, improving plant resistance, and strengthening the efficiency of nutrient utilization.

Materials and Methods:

The research was conducted in the Fungal Pathology lab at Crop Disease Research Institute, National Agricultural Research Council (NARC), and Enzyme Biotechnology Lab, University Institute of Biochemistry and Biotechnology, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, to investigate cellulase-producing fungal strains.

Collection of Samples:

For the microbial investigation of cellulase-producing fungal strains, 75 rhizospheric soil samples were collected across 11 different cities (Sialkot, Gujranwala, Sargodha, Faisalabad, Jhang, Layyah, Multan, D.G Khan, Muzaffargarh, Bahawalpur and Rahim Yar Khan) of Punjab. Samples were collected from 2 points in each region from a depth of 15 cm and then mixed thoroughly to obtain a representative sample. Soil samples were stored at 10°C for up to 1 week until enzymatic analysis. The sampling location is shown in Table 1.

Table 1. Sampling sites of Punjab

S. No.	Punjab	No of samples
1	Sialkot	5
2	Gujranwala	2
3	Sargodha	3
4	Faisalabad	11
5	Jhang	14
6	Layyah	18
7	Multan	8
8	D.G Khan	3
9	Muzaffargarh	4
10	Bahawalpur	5
11	Rahim Yar Khan	2

Isolation of Fungi:

Fungi were isolated from the rhizosphere soil by serial dilution on potato dextrose agar (PDA) media. 1 g of soil sample was weighed and added to 9 ml of autoclaved distilled water to make a final volume of 10 ml and vortexed to obtain a homogeneous suspension. Serial dilutions up to 10^{-3} were made, and 0.1 ml was taken using a micropipette, spread onto the plates, and incubated at $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 72 hours. After three days, fungal colonies were purified into a new PDA plate and preserved for further use on slants.

Screening of Cellulase Producing Fungi:**Primary screening:**

Cellulolytic activity was determined by clear zone formation in carboxymethylcellulose medium (CMC). The plate screening medium (PSM) that contained Mendel's mineral salts in a concentration of 0.3 g/L of Urea, 2 g/L of KH_2PO_4 , 0.3 g/L of CaCl_2 , 1.4 g/L of $(\text{NH}_4)_2\text{SO}_4$, 0.3 g/L of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.75 g/L of Proteose peptone, 0.25 g/L of Yeast extract, 10 g/L of Carboxymethylcellulose (CMC) and 17.5 g/L of agar. The medium was autoclaved and poured into Petri plates. Fungal isolates were inoculated onto CMC medium and incubated at $28^{\circ}\text{C} \pm 2$ for 72 hours. After incubation, the culture plates were stained with a 0.1% Congo red solution for 15 minutes, and the plates were washed with 1 M NaOH for 10 minutes. The creation of clear zones around microbial colonies indicates the hydrolysis of cellulose or CMC [2]. The hydrolytic index value is the ratio of the clear zone / Colony diameter that was determined according to the standard protocol of CLSI [6].

Secondary screening:

Potential fungal strains that showed a large clear zone in primary screening were selected for enzyme production on Minimal basal media with 1% cellulose. Basal media (100 ml) was filled into the Erlenmeyer flask (250 ml), autoclaved, and inoculated with the 4 discs (2mm diameter) from a 7-day-old culture plate. The flasks were then incubated at 30°C in a shaking incubator for 4 days. The contents were filtered with the Whatman number 1 filter paper and further analyzed for enzyme activities.

Molecular Characterization:

DNA was extracted from a 7-day-old culture using the modified 2% CTAB method. The purity and concentration of extracted DNA were determined using a Thermo Scientific™ μ Drop™ plate with a Multiskan SkyHigh microplate reader. The internal transcribed spacer (ITS) regions amplified in a Thermal Cycler (Applied Biosystems® Veriti®) using primers sets ITS1F and ITS4. Moreover, the amplified products were sequenced by Macrogen and analyzed through BLAST analysis on the NCBI database (<http://blast.ncbi.nlm.gov/Blast.cgi>).

Enzyme Production and Extraction:

A high cellulase-producing fungal strain was selected for cellulase production and grown in liquid medium containing carboxymethylcellulose as a carbon source and incubated at 28°C for one week. The stock suspension was diluted to 4.5×10^6 spores/ml using a hemocytometer and this dilution was used as inoculum. 2ml of inoculum was poured into 250 ml of production media to produce cellulase enzyme and incubated at 28°C for 96 hours. The fermented culture was centrifuged at 10000 rpm for 10 minutes. The fungal cells were pelleted while the supernatant was taken as crude extract [1].

Enzyme Activity and Protein Estimation:

The enzyme activity of the supernatant was determined using a Thermo Scientific™ μ Drop™ plate with a Multiskan SkyHigh microplate reader by the method described by Ghose [14]. The substrate which is 0.5 ml of 1% carboxymethylcellulose (CMC) and 0.5 ml of enzyme sample prepared in citrate buffer (pH 4.8) made up the reaction mixture. For 30 minutes, the reaction mixture was incubated at $50^\circ\text{C} \pm 2$. To stop the reaction after the incubation period, 3 ml of DNS reagent was added, and the mixture was heated for 5 minutes before cooling to check the absorbance at 540 nm. Enzymatic activity is a measure of micromole (μmol) released / min/ml. Protein estimation was done by using Lowry's method and bovine serum albumin (BSA) was taken as standard [15].

Partial Purification of Cellulase Ammonium Sulfate Precipitation:

To purify the protein, the first process that followed was ammonium sulfate $[(\text{NH}_4)_2\text{SO}_4]$ precipitation. This technique partially purified the protein in the crude extract partially. Precipitation makes it easy for protein to get concentrated and partially purified. To start the procedure, 30%, 40%, 50%, 60%, 70%, and 80% of ammonium sulfate were added to the enzyme solution and the mixture was vortexed until the ammonium sulfate dissolved. Then it was allowed to stir at 4°C for 6 hours. After the ammonium sulfate was completely dissolved in crude extract, it was centrifuged at 10000rpm for 15 minutes at 4°C [6]. The serial fraction that was able to produce more pellets was selected for further precipitations. The supernatants and pellets of all ammonium sulfate fractions were analyzed for enzyme assay. In further precipitations, the supernatant was discarded and the pellet was dissolved in a phosphate buffer of pH 8. The concentration of partially purified protein is determined by the Lowry method as mentioned above [15].

Dialysis and Concentration: The dissolved pellet in phosphate buffer was transferred into cellulose dialysis tubing against distilled water. It was allowed to stir for 24 hours. The water

was changed occasionally. The dialysate was further used in the Lowry assay for the determination of protein contents.

Characterization of Enzyme:

Endoglucanase was characterized based on different pH values (3-9), with the acetate buffer (pH3-5), citrate buffer (pH 6), and phosphate buffer (pH 7-8). The enzyme activity was characterized at different temperatures ranging from 30 to 80°C

Statistical Analysis:

All experiments were run in triplicate. The data were expressed as mean ± Standard error, followed by one-way ANOVA with Tukey HSD at p=0.05 test using statistical tool statistics version 8.1.

Results:

Screening of Fungal Strains:

Primary screening (plate screening method):

Primary screening was performed with the Congo red dye and the results revealed that the cellulase-producing fungal strains showed a large clear zone as compared to the less cellulase-producing fungal strains. Five strains showed no clear zone which means that they could not produce cellulase Figure 1. Among the 15 fungal strains, eight showed maximum clearance zones. The high zone of clearance was observed with 1SR (8.7cm), K.R.S (8.3cm), TV (9cm), T.J (8.1cm) and N.S (7.8cm). The index of relative enzyme activity in Table 2 was determined as hydrolysis zone diameter/colony diameter and distinguished high cellulase-producing species from low cellulase-producing species.

Table 2. Clear zone diameter and measurement of hydrolysis activity index

Sr. no.	Fungal Isolates	Colony diameter (cm)	Hydrolysis zone (cm)	Hydrolysis activity index Hydrolysis zone/ Colony diameter (cm)
1	SY	7.1 ± 0.12	0.0 ± 0.00	0 ± 0.00
2	VGL	5.5 ± 0.07	0.0 ± 0.00	0.0 ± 0.00
3	AUS-127	6.8 ± 0.07	0.0 ± 0.00	0.0 ± 0.00
4	Tm	6.2 ± 0.12	0.0 ± 0.00	0.0 ± 0.00
5	G-3	5.7 ± 0.05	0.0 ± 0.00	0.0 ± 0.00
6	G-35	7.5 ± 0.08	1.5 ± 0.06	0.2 ± 0.01
7	A.S	6.3 ± 0.10	4.0 ± 0.03	0.63 ± 0.01
8	6SR	6.7 ± 0.05	5.3 ± 0.06	0.79 ± 0.01
9	TL	8.5 ± 0.07	5.6 ± 0.03	0.66 ± 0.01
10	CPF(R)	8.0 ± 0.07	5.3 ± 0.09	0.92 ± 0.02
11	N.S	8.1 ± 0.10	7.8 ± 0.06	0.96 ± 0.02
12	T.J	8.4 ± 0.07	8.1 ± 0.03	0.96 ± 0.01
13	K.R.S	8.6 ± 0.05	8.3 ± 0.09	0.96 ± 0.02
14	1SR	8.8 ± 0.05	8.7 ± 0.06	0.98 ± 0.01
15	TV	9.0 ± 0.00	9.0 ± 0.00	1.00 ± 0.00

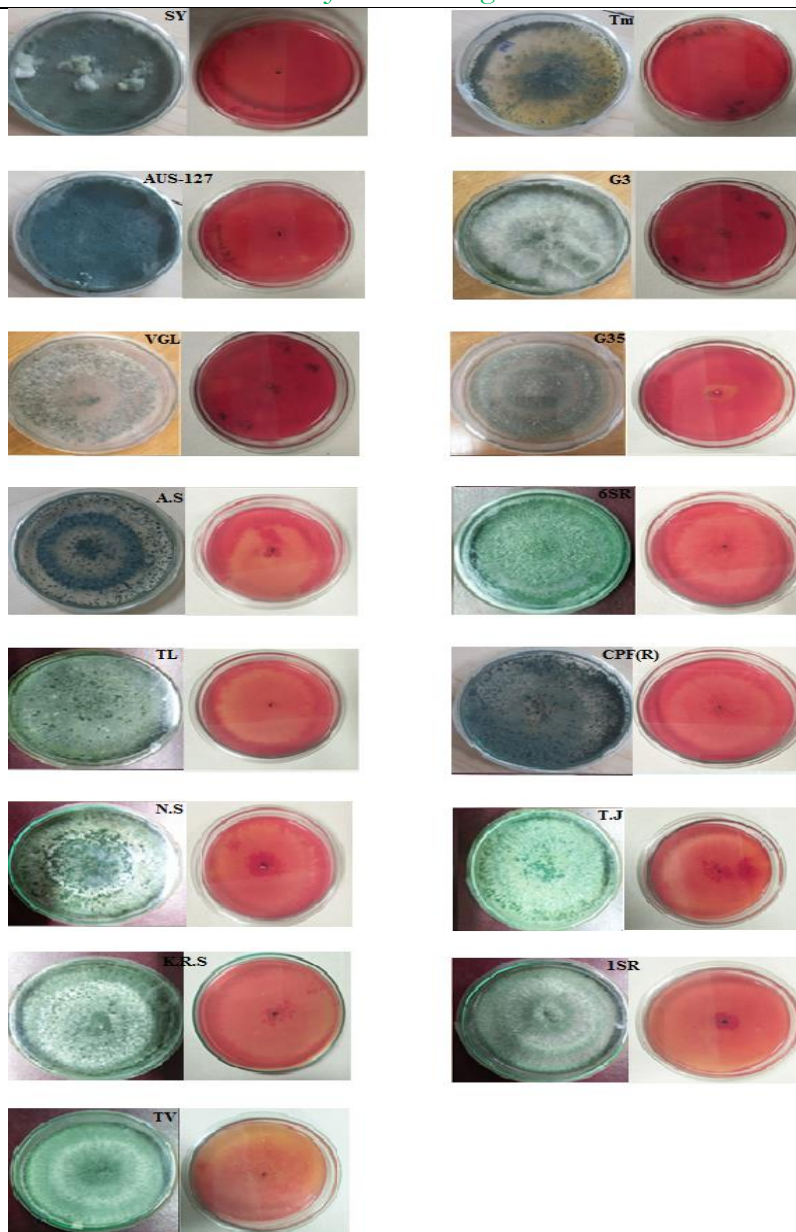


Figure 1. Primary screening: fungal strains SY-G3 indicates no hydrolysis zone G35-CPF(R) showed low to medium hydrolysis zone N.S –TV showed highest hydrolysis zone

Secondary screening:

Secondary screening of eight isolates that showed a large clear zone was performed with the DNS reagents. The results revealed that the isolates that showed high cellulase activity were TV, T.J, 1RS, N.S and K.R.S and comparatively low activity were TL, 6SR, GM Figure 2.

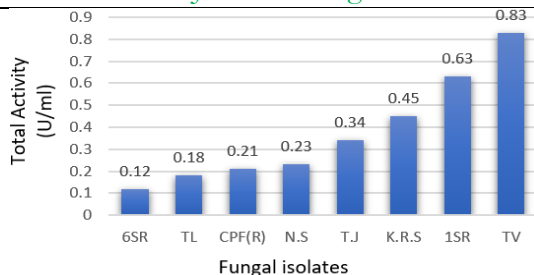


Figure 2. Secondary screening of fungal isolates for Cellulase activity.

Molecular Characterization and Phylogenetic Analysis:

The cellulase produced in the present research work from isolated fungal strains was identified as genera of *T. asperellum* based on similar morphological and molecular characteristics. From eight fungal isolates, five isolates with high cellulase activity were characterized for the ITS region. The blast analysis of the ITS region indicated that TV and 1SR resemble *Trichoderma asperellum* and K.R.S, T.J, and NS resemble *Trichoderma barzianum* and *T. longibrachiatum* (Table 3). A band size of 600 was observed in the gel (Figure 3). *Trichoderma asperellum* showed high cellulase activity and was used for cellulase production and characterization. The Phylogenetic tree of TV was constructed using the software MEGA11 by the Neighbor-joining method. The sequence showed an evolutionary relationship with the 17 related sequences and our sequence is located in the same clade as *Trichoderma Asperellum* (Accession No. OP077119) as shown in Figure 4.

Table 3. List of *Trichoderma* isolates used in molecular studies

Sample no.	Location/ code	Sample name	Sequence identity (%)	Nearest strains
X01	Sarghodha (1SR)	<i>T. asperellum</i>	99.28	<i>T. asperellum</i> strain Tasp-SH14
X04	Jhang (T.V)	<i>T. asperellum</i>	99.84	<i>T. asperellum</i> strain Tasp-STG25
W1	Jhang (T.J)	<i>T. barzianum</i>	99.39	<i>T. barzianum</i> strain S2611
W2	Muzaffargarh (N.S)	<i>T. longibrachiatum</i>	99.81	<i>T. barzianum</i> strain
W3	Faisalabad (K.R.S)	<i>T. barzianum</i>	99.83	<i>T. barzianum</i> strain PB

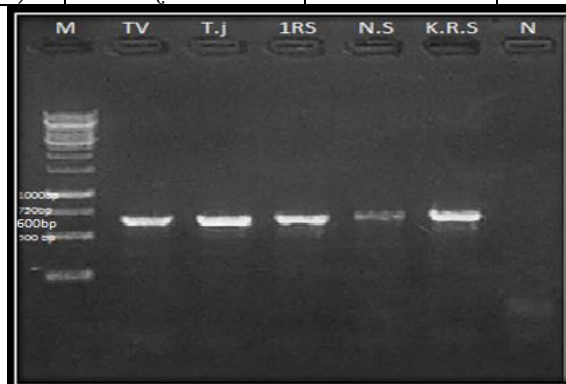


Figure 3. Native gel electrophoresis of different *Trichoderma* strains (TV, T.J, 1RS, N.S and K.R.S) M: Marker, N: No template control

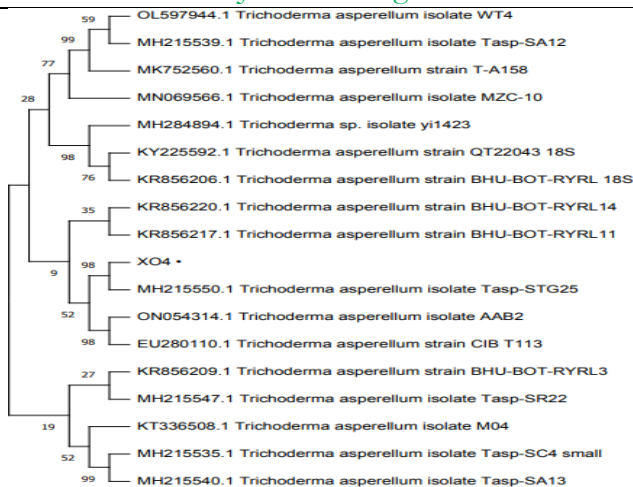


Figure 4. The phylogenetic tree of *Trichoderma asperellum* (TV) was constructed by the Neighbor-joining method showed an evolutionary relationship with the 8 related sequences
Cellulase activity:

T. asperellum inoculum was added into the liquid medium with CMC as a substrate and incubated for 2-7 days, and enzyme activity was determined after each day, as shown in Figure 5. It found that cellulase production increased at first and then decreased. The maximum cellulase activity after 4 days was 0.84 U/ml (0.36 U/mg). After the optimization of incubation time, *T. asperellum* was used to produce cellulase for the characterization of CMCcase.

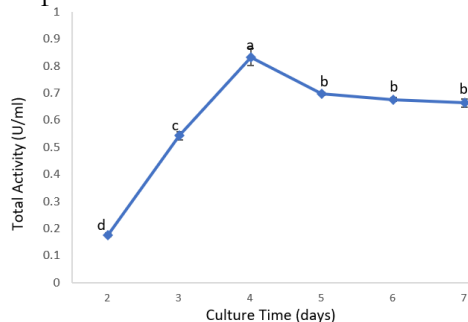


Figure 5. *T. asperellum* incubated for 2-7 days, total enzyme activity was determined after each day, and different letters (a-d) represents the significant difference at $p < 0.05$ (Tukey test)

Partial Purification and Dialysis:

The crude enzyme solution was used as a supernatant, which had a cellulase activity of 28.34U/25mL and a specific activity of 48.27U/mg. The crude enzyme was purified 1.23-fold and precipitated at 80% saturation with a specific activity of 60.29U/mg. The precipitate (pellet) was dissolved in a small amount of 0.2M Tris-HCl buffer (pH 8) and dialyzed against distilled water with four equal changes of water every six hours to remove the excess salt. The specific activity was 104.39 U/mg following dialysis. (Table 4).

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Sample	Volume (ml)	Total Protein (mg)	Activity (Units)	Specific Activity (units/mg)	Purification Fold	% Yield
Crude Extract	25	0.587	28.34	48.27	1	100
Ammonium Sulphate Precipitation (80%)	10	0.435	26.23	60.29	1.23	92.5
Dialysis	5	0.223	23.28	104.39	2.2	88.7

Characterization of Enzyme:

Effect of pH on cellulase activity:

Enzymes are characterized based on different pH using different pH buffers, citrate buffer (3-5), and phosphate buffer (6-8). Enzyme activity was determined from 3-8 and results showed that maximum activity (41.8U/mg) was observed at pH 4. But activity decreases drastically at high pH (6-8) different letters (a-e) on the bar represent the significant difference at $p < 0.05$ (Tukey test). There were 5 groups (A, B, C, D, E) in which the means were not significantly different from one another as in Figure 6.

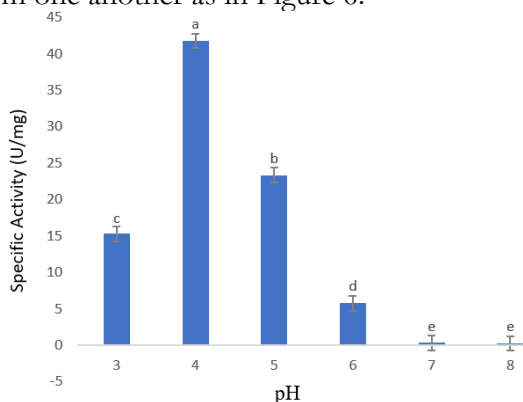


Figure 6. Characterization cellulase based on pH: enzyme activity was determined from 3-8 pH and results showed that maximum activity was observed at pH 4 but activity decreases drastically at high pH (6-8) different letters (a-e) on bar represent the significant difference at $p < 0.05$ (Tukey test)

Effect of temperature on cellulase activity:

Enzymes are characterized based on different temperature ranges (35-80°C). Enzyme activity was determined from 30-80°C and results showed that maximum activity 1.7 U/ml (8.2U/mg) was observed at 55 °C but activity decreases drastically at high temperatures (60-80 °C) different letters (a-d) on the bar represents the significant difference at $p < 0.05$ (Tukey test) as shown in figure 7 This strain was found to produce higher activity level (48.27 U/mL for CMCase under the optimized condition of a temperature of 28°C, pH 4.5 and incubation time of 72 h).

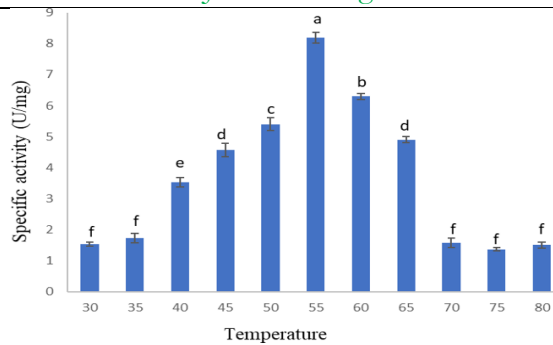


Figure 7. Characterization cellulase based on Temperature: enzyme activity was determined from 30-80°C and results showed that maximum activity was observed at 55 °C but enzyme activity decreases drastically at high temperatures (60-80 °C) different letters (a-f) on the bar represents the significant difference at $p < 0.05$ (Tukey test).

Discussion:

This research was designed to isolate *Trichoderma* strains from soil samples collected from different regions of Punjab to investigate cellulase enzyme production. *Trichoderma* strains that were able to produce cellulase enzyme utilized the Carboxymethylcellulose as a sole source of carbon. A total of 15 isolates of *Trichoderma* were isolated from different locations and screened for cellulase activity. Among 15 fungal strains, eight strains showed maximum clearance zone but 5 showed no such zone. The hydrolytic index value, shown in Table 2, was calculated as the ratio of clear zone diameter to colony diameter [6]. The study conducted in Malaysia identified *Trichoderma* on the basis of morphological features, lignocellulolytic activities and multilocus gene sequences of rDNA. The results indicated the successful cultivation of *Trichoderma* species [1]. A study was conducted to detect important cellulase genes. The results demonstrated the greater CMCase, Xylanase, and FPase activities [16]. Agriculture waste materials mainly lignocellulosic waste materials are decomposed by fungi [17]. For the past few decades, cellulase production from fungi was preferred from soil samples [18]. A study was conducted to determine the isolates of *Trichoderma* possessing cellulase activity. Molecular approaches and morphological characteristics were used to characterize the isolates. The results indicated the diversity of *Trichoderma* strains [3]. These findings relate to the previous study as reported in the literature [13][19][20][21] that CMCase produced by the *Trichoderma asperellum* PQ34 strain showed maximum activity at pH 4 and temperature 55 °C. Previously researchers focused on the isolation and characterization of CMCase from *T. harzianum* [8], *T. reesei* [9], and *T. viride* [1] but in the past few years, the study of cellulase production from *T. asperellum* has gained importance because of usage in bioproduction [11][13]. The particular activity of the hydrolytic enzymes generated by *T. asperellum* during interaction with *A. rolfsii* was ascertained by biochemical analysis. Our results showed that isolate A10 produces more hydrolytic enzymes than isolates A17 and A11, with specific activities of 174.68 IU/mg of β -1, 3 glucanase, 183.48 IU/mg of β -1, 4 glucanase, and 106.06 IU/mg of protease, respectively. The results demonstrated that *Trichoderma* has great potential to control collar rot disease [22]. Cellulase produced from some strains of

Trichoderma sp. indicated relatively less enzyme activity in the Solid-State Fermentation (SSF) with optimized conditions of temperature of 32°C, pH 4.5, and with the incubation time of 72 h. However, cellulase produced from the other *Trichoderma* strain showed maximum cellulase activity at comparatively low temperatures. The maximum cellulase activity for *T. viride* was reported at 50 °C, whereas in this study the optimum temperature was 55 °C [1]. In another study, *Trichoderma asperellum* was reported as an interesting source of antimicrobial compounds [10]. The study concluded that the cell-free filtrate of *Trichoderma asperellum* has a profound antagonistic effect [8]. Another attempt was done to evaluate the encapsulation of *Trichoderma* with *ca*-alginate matrix. The results showed that alginate beads support the viability of *Trichoderma asperellum* of *Trichoderma asperellum* [23] such as the cellulase from *T. reesei* NRRL 3652 showed the maximum activity at pH 5 while in this study pH was 4.5 while from *T. longibrachiatum* showed activity at pH 4.8 [24]. The mutated strain of *Trichoderma reesei* 2414 showed activity at pH 4.5 [25], while CMCase from *T. viride* showed activity at pH 4 [26], and that from the *T. reesei* showed its activity even at pH 3 [9] Some *Trichoderma* strains were found to produce cellulase with maximum activity at higher optimal pH such as *T. viride* showed at pH 6.5 and *T. harzianum* showed at pH 6 [1][8]. The highest cellulase-producing strain, characterized based on the internal transcribed spacer (ITS) region using primer sets ITS1F and ITS4, was identified as *Trichoderma asperellum*. The Cellulase was partially purified using various ammonium sulfate concentrations (30-80%) and characterized based on temperature and pH. The enzyme showed a maximum specific activity of 104.39 U/mg after dialysis. The maximum activity of the partially purified enzyme was observed at pH 4 and temperature 55°C.

Conclusions:

In conclusion, it could be said that incubation time, pH, and temperature are the important factors for enzyme activity. The present study suggested that apart from fungal species *Trichoderma reesei*, *T. harzianum*, and *T. viride*, *T. asperellum* has considerable potential for cellulase production. The results indicated that the maximum activity was observed at pH 4.8 and temperature 55 °C as compared with other species. This genus can be used for industrial production and the management of phytopathogenic diseases.

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