

Bioprocessing of Proximally Analyzed Wheat Straw for Enhanced Cellulase Production through Process Optimization with *Trichoderma viride* under SSF

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Abstract—The purpose of the present work was to study the production and process parameters optimization for the synthesis of cellulase from *Trichoderma viride* in solid state fermentation (SSF) using an agricultural wheat straw as substrates; as fungal conversion of lignocellulosic biomass for cellulase production is one among the major increasing demand for various biotechnological applications. An optimization of process parameters is a necessary step to get higher yield of product. Several kinetic parameters like pretreatment, extraction solvent, substrate concentration, initial moisture content, pH, incubation temperature and inoculum size were optimized for enhanced production of third most demanded industrially important cellulase. The maximum cellulase enzyme activity 398.10 ± 2.43 $\mu\text{M/mL/min}$ was achieved when proximally analyzed lignocellulosic substrate wheat straw inoculated at 2% HCl as pretreatment tool along with distilled water as extraction solvent, 3% substrate concentration 40% moisture content with optimum pH 5.5 at 45°C incubation temperature and 10% inoculum size.

Keywords—Cellulase, Lignocellulosic residue, Process optimization, Proximal analysis, SSF, *Trichoderma viride*.

I. INTRODUCTION

LIFE on earth depends on photosynthesis, which results in production of plant biomass having cellulose as the major component. Lignocellulosic materials rich in lignin, hemicellulose and cellulose [1, 2] including wood, agricultural residues and paper wastes like rice straw, corncob, corn stover, wheat straw, rice husk, and bagasse etc. are particularly attractive in this context because of their relatively low cost and plentiful supply of desired product [2, 3-5].

In nature, cellulose is the most abundant component of plant biomass and found exclusively in plant cell walls. Despite great differences in the composition of cell walls across plant taxa, high cellulose content typically in the range of approximately 35 to 50% [6] however, the cellulose fibers are embedded in a matrix of other structural biopolymers,

primarily hemicelluloses and lignin, which comprise 20 to 35% and 5 to 30% of plant dry weight [6, 7]. An important feature of cellulose, relatively unusual in the polysaccharide world, is its crystalline structure [8]. Approximately 30 individual cellulose molecules are assembled into larger units known as elementary fibrils (proto fibrils), which are packed into larger units called micro fibrils, and these are in turn assembled into the familiar cellulose fibers [9, 10].

A wide variety of micro-organisms can produce the cellulolytic enzymes [11-13] under appropriate culture conditions [14] and among them *Trichoderma*, *Aspergillus*, *Penicillium*, and *Fusarium* genera are some of among them and *Trichoderma reesei* is one of the most efficient cellulases producer [15] has been extensively studied for the production of cellulolytic enzymes from various cellulosic materials such as wood, wheat bran and wheat straw [5, 16-18].

A cellulolytic enzyme system is a complex of three major types of enzyme composed of: (i) Endoglucanases or 1, 4-D-glucan-4-glucanohydrolases (EC 3.2.1.4), or C_1 (ii) Exoglucanases, including 1, 4-D-glucan glucanohydrolases or C_x (also known as cellodextrinases) (EC 3.2.1.74) and 1, 4-D-glucan cellobiohydrolases (cellobiohydrolases) (EC 3.2.1.91), and (iii) glucosidases or glucoside glucohydrolases (EC 3.2.1.21) [5, 13, 19]. The cellulase systems exhibit higher collective activity than the sum of the activities of individual enzymes, a phenomenon known as synergism, Han and Chen [20, 21]. The three enzymes act synergistically for the complete hydrolysis of cellulose into glucose. CMCase (endoglucanase) converts the polymeric form of cellulose into oligosaccharide form, and then exoglucanase separates cellobiose into glucose units [22, 23].

Pakistan being an agricultural country produced approximately 50 million tones of agriculture waste annually in the form of crop residue and wheat is one of the most important agricultural crops. Wheat straw contains 29-35% cellulose in it. Cellulase are being used in the field of cotton processing; paper recycling, in juice extraction, as detergent enzymes and animal feed additives [13] brewery, textile and laundry industries, animal feed industry, wine, agriculture as well as in the field of research and development.

This paper reports the results of a study which was carried out to get an enhanced cellulase production through SSF media optimization from proximally analyzed lignocellulosic wheat straw as media supported substrate for microorganism,

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which is renewable, efficient, safe, ideally an inexpensive and abundantly available natural source.

II. MATERIALS AND METHODS

Place of work

All the experimental work was carried out in Enzyme Biotechnology Laboratory, Department of Chemistry and Biochemistry, University of Agriculture Faisalabad, Pakistan.

Chemicals and lignocellulosic Substrate

All the chemicals used were of analytical grade and purchased from Fluka (France), Merk (Germany) and Scharlau (Spain). Lignocellulosic agro-industrial waste, wheat straw was obtained from Student research Farms, University of Agriculture, Faisalabad (UAF), Pakistan. The substrate was crushed into pieces, sun and oven dried (60°C) and ground to 40 mm mesh size and stored in air tight plastic jars.

Fungal Culture

The pure culture of *Trichoderma viride* was obtained from Molecular Biotechnology Laboratory, Department of Chemistry and Biochemistry, University of Agriculture Faisalabad, Pakistan.

Maintenance and Inoculum development

Spores of *Trichoderma viride* were grown and maintained on Potato Dextrose Agar (PDA) slants. Spores were cultivated in an Erlenmeyer flask (250 mL) capacity containing 30 mL of Potato Dextrose broth at 30±1 °C for 7 days after sterilizing the potato dextrose broth at 15 lbs/inch² pressure and 121°C in laboratory scale autoclave (Sanyo, Japan) for 15 minute, pH was adjusted before sterilization, and incubated under stationary conditions for the development of fungal spore suspension [24].

Pretreatment of wheat straw

Powdered wheat straw (10g) was pretreated with 1 to 4% HCl in an Erlenmeyer flask (250 mL). The pretreated substrate was stayed at room temperature for 2 hrs, autoclaved at 121°C and 15 lb/inch² pressure for 15min. After this slurry of substrate was filtered through four layers of muslin cloth, both filtrate and residue were saved. Residue was washed 4 to 5 times with distilled water and filtrate was used for analysis of total sugars and reducing sugars while the residue was used for production of cellulase and further proximate analysis.

Fermentation Protocol

Basel fermentation media was used to moist the lignocellulosic substrate (10g) in an Erlenmeyer flask (250 mL) for cellulase production as reported by [25]. The major constituents of media were: (NH₄)₂SO₄ 10; KH₂PO₄ 4; MgSO₄·7H₂O, 0.5 and CaCl₂ 0.5 (g l⁻¹). The initial pH value of the medium was adjusted to 6 before sterilization at 121 °C and 15.0 lbs/inch² pressure for 15 min. The autoclaved medium was inoculated with 5 mL of freshly prepared spore suspension of fungus and incubated at 30±1°C in a temperature controlled incubator for stipulated time period under still culture conditions.

Proximate Analysis

Determination of % Moisture content

To determine the % moisture contents of wheat straw 1 g sample was taken in a moisture determination bottle (W₁) and kept in an oven at 105 °C for 2 hrs. The sample was then kept in desiccators to cool and weight, again it was kept in an oven until constant weight after drying was obtained (W₂) and % moisture was determined by following the formula:

$$(\%) \text{ Moisture} = \frac{(W_1) - (W_2)}{(W_1)} \times 100$$

Determination of % Ash content

1 g of wheat straw (W₁) was taken in a crucible with 5 mL HNO₃ and heated continuously at low flame until the material begins to char. After charring the sample, kept in muffle furnace at 650 °C for 4 hrs and weighed (W₂) to determine % ash contents of substrate sample.

$$(\%) \text{ Ash} = \frac{(W_1) - (W_2)}{(W_1)} \times 100$$

Determination of % Fat content

Sample of 5 g (W₁) was taken in a pre-weight thimble and oil was extract with hexane for 16 hrs. The thimble along with extracted sample was dried and thus from loss in weight of initial sample the value for oil was determined (W₂).

$$(\%) \text{ Fat} = \frac{(W_1) - (W_2)}{(W_1)} \times 100$$

Determination of % Lignin content

1 g (W₁) of sample was refluxed with 70 mL of 1.25 % (V/V) H₂SO₄ solution for 2 hrs in order to hydrolyze the cellulose and hemicellulose. The remaining suspension after the above treatment was filtered through filter paper with hot water. Then 30 mL of 72% H₂SO₄ was added into the mixture and the solid residue was dried at 105 °C for 24 hrs and weighed (W₂). The residue was then transferred to a pre-weighed dry porcelain crucible and heated at 650 °C for 4 hrs. After cooling down, it was weighed (W₃) and lignin content (%) was determined.

$$\% \text{ Lignin} = \frac{(W_2) - (W_3)}{(W_1)} \times 100$$

Determination of % Cellulose content

1g (W₁) of sample was refluxed with 10 mL of 80% acetic acid and 1.5 mL HNO₃ for 20 min. The mixture was oven dried at 105°C (W₂). The residue was then transferred to a pre-weighed dry porcelain crucible and heated at 650°C for 6 h. After cooling down, it was weighed (W₃) and ash content (%) was determined.

$$\% \text{ Cellulose} = \frac{(W_2) - (W_3)}{(W_1)} \times 100$$

Analytical Methods

Estimation of total sugar

Phenol sulfuric acid method [26] was used to measure the total sugar in the medium. 1mL sample was mixed with 1mL 5% phenol and 5mL Conc. H₂SO₄ and stayed for 10-15 minutes. Optical density was checked at 470 nm to measure color intensity.

Estimation of Reducing Sugar

Reducing sugar was measured using 3, 5-dinitrosalicylic acid (DNS) by the method of Miller, [27]. 1mL sample was mixed with 3 mL DNS and boiled for 10 minutes. Optical density was checked at 550 nm to measure color intensity.

Protein Determination

Protein in the medium was determined by the method of Lowery, [28] with bovine serum albumen as standard.

Enzyme extraction

The enzyme was extracted by a simple contact method [29]. Citrate buffer 0.05 M of pH 4.8 was introduced into each fermented flask in 1:10 (w/v) ratio kept in shaker at 120 rpm for 30 minutes and filtered using muslin cloth. The filtrate was centrifuged at 10,000 rpm (4°C) for 15 min to obtain clear supernatant that was used for further enzyme analysis.

Enzyme activity

Cellulase activity was determined by the method of Ghose, [30]. The reaction mixture contained 0.5 mL of 1 % carboxymethyl cellulose in 0.05 M Na-citrate buffer of pH 4.8 and finally 0.5 mL of diluted crude enzyme and incubated at 50 °C for 30 min. An appropriate control was also run along with the test. At the end of the incubation period, tubes were removed from the water bath, and the reaction was stopped by addition of 3 mL of 3, 5-dinitrosalicylic acid reagent per tube. The tubes were incubated for 5 min in a boiling water bath for color development and were cooled rapidly. The activity of reaction mixture was measured against a reagent blank at 540 nm in a UV-VIS spectrophotometer. The concentration of glucose released by enzyme was determined by comparing against a standard curve constructed similarly with known concentrations of glucose. One unit of enzyme activity was defined as the amount of enzyme required for liberating 1μM of glucose per milliliter per minute and was expressed as μM/mL/min.

Statistical analysis

All the data was statistically evaluated according to Steel and Torrie [31]. The means and standard errors of means (Mean ± S.E) were calculated for each treatment.

III. RESULTS AND DISCUSSION

Chemical Composition of Substrate

To investigate the suitability of wheat straw for cellulase production under solid state fermentation condition, chemical composition of wheat straw was determined through proximal analysis under standard experimental conditions. Results

obtained after analysis showed moisture, ash, fat cellulose, lignin and protein content of wheat straw were 5.074 %, 7.30 %, 1.854 %, 39.4 %, 6.4 % and 3.50 % respectively while, the amount of total sugar and reducing sugar were 0.552 % and 0.535 % respectively (Table I). Almost similar amount of moisture, ash and fat content was also reported by [32]. In structural composition, lignocellulosic wheat straw contained almost 29-35% cellulose reported by Rowell [33]; McKendry [34]. Prasad et al. [35] also reported that common agricultural residue (wheat straw) contained Cellulose, hemicellulose and lignin content in % of 33–40, 20–25 and 15–20 respectively.

TABLE I
CHEMICAL COMPOSITION OF WHEAT STRAW*

Treatment	Constituent	Value (%)
1	Moisture	5.07 ± 0.24
2	Ash	7.30 ± 0.42
3	Fat	1.85 ± 0.02
4	Cellulose	39.40±0.72
5	Lignin	6.40 ± 0.19
6	protein	3.50 ± 0.13
7	Total sugar	0.552 ± 0.015
8	Reducing sugar	0.535 ± 0.009

*Proximal Analysis

Effect of Pretreatment on Cellulase Production

In the present study pretreatment to substrate was given with HCl in an Erlenmeyer flask (250 mL) in order to make cellulose degradation efficient and optimized to get best enzyme activity at different concentration ranging from 1 to 4 % for cellulase production and subsequent experiment work. After pretreatment process of wheat straw it was observed that pretreatment cause the removal of lignin and hemicelluloses effectively, while at the same time loosening the structure of lignin and decreasing the crystallinity of digestible cellulose that improves the porosity characteristic of substrate. A significant difference in enzyme activity was observed with the change in acid concentration and became maximum (162±2.78μM/mL/min) at 2% while, Further increase in concentration did not enhance the enzyme activity (Table II). The acid catalyzed pretreatment significantly effective for hemicellulose removal from the biomass and this removal in turn displays the cellulose to enzymatic attack [36, 37]. Dahot and Noomrio [38] reported that the rate of β-glucosidase and CM-cellulase production by *Aspergillus fumigatus* in 0.6N H₂SO₄ and HCl pretreated wheat straw mineral medium reached maximum. It has been reported previously that glucose yield subsequent to complete removal of hemicellulose can reach as high as 100% [39]. During acid pretreatment, two reactions occur simultaneously relative to lignin: degradation and accumulation; therefore, the changes in lignin content greatly depend on which reaction is stronger [40].

TABLE II

EFFECT OF ACID PRETREATMENT ON CELLULASE ACTIVITY PRODUCED FROM *TRICHODERMA VIRIDE* UNDER SOLID STATE FERMENTATION OF PROXIMALLY ANALYZED WHEAT STRAW

Treatment	HCl (%)	Enzyme Activity ($\mu\text{M/mL/min}$)
1	1	112 \pm 1.38
2	2	162 \pm 2.78
3	3	82 \pm 2.95
4	4	72 \pm 3.18
5	5	61 \pm 2.28

Effect of Different Solvents on the Enzyme Recovery

The effectiveness of extraction is necessary towards the recovery of enzyme from the fermented biomass. Thus different solvents were optimized for the recovery of enzyme which were distilled water, tap water, tween 81, citrate and phosphate buffer (pH 4.8). The enzyme showed its maximum activity of 196 \pm 2.10 $\mu\text{M/mL/min}$ (Table III) when was recovered with distilled water; hence distilled water was found to be optimum for the extraction of enzyme while minimum enzyme activity (135 \pm 3.20 $\mu\text{M/mL/min}$) was obtained with phosphate buffer. Chandra *et al* [41] reported a single wash with 20 mL distilled water gave maximum yield (10.87 Ug^{-1}) among different solvents used for the recovery of enzyme. Distilled water is a commonly available, save and low cost extractant used by other workers for extraction of alkaline protease from solid state fermentation [42, 43]. Da-Silva *et al.* [44] extracted xylanase and CMCase enzymes from different solid substrates with distilled water.

TABLE III

EFFECT OF SOLVENT ON CELLULASE ACTIVITY PRODUCED FROM *TRICHODERMA VIRIDE* UNDER SOLID STATE FERMENTATION OF PROXIMALLY ANALYZED WHEAT STRAW

Treatment	Solvents	Enzyme Activity ($\mu\text{M/mL/min}$)
1	Distilled water	196 \pm 2.43
2	Tap water	144 \pm 4.31
3	Tween 81	172 \pm 2.14
4	Citrate buffer	169 \pm 3.36
5	Phosphate buffer	135 \pm 3.20

Effect of Substrate Concentration on Cellulase Production

In order to optimize substrate concentration for production of cellulase different concentrations of substrate (wheat straw) were optimized under same conditions ranging from (1-5%). Enzyme activity increased with the increase in substrate concentration. It was observed that maximum cellulase activity (248 \pm 1.66 $\mu\text{M/mL/min}$) was obtained at 3% substrate concentration (Table IV). Substrate concentration is an influencing factor that affects the yield and initial rate of hydrolysis of cellulose [45]. At low substrate concentration normally results in an increase of the yield and reaction rate of the hydrolysis [46]. However, high substrate concentration

can cause substrate inhibition, which substantially lowers enzyme production [47, 48]. The present strain of *Trichoderma viride* efficiently able to utilize lignocellulosic pretreated wheat straw with high volumetric levels of cellulase, up to 248 \pm 1.66 $\mu\text{M/mL/min}$, in contrast to 1.7 FPU/mL of cellulase in Czapek-Dox medium containing 1% cellulose [49, 50].

TABLE IV

EFFECT OF SUBSTRATE CONCENTRATION ON CELLULASE ACTIVITY PRODUCED FROM *TRICHODERMA VIRIDE* UNDER SOLID STATE FERMENTATION OF PROXIMALLY ANALYZED WHEAT STRAW

Treatment	Wheat straw/flask (%)	Enzyme Activity ($\mu\text{M/mL/min}$)
1	2	185 \pm 1.59
2	2.5	212 \pm 2.47
3	3	248 \pm 1.66
4	3.5	199 \pm 2.68
5	4	172 \pm 1.71

Effect of Moisture Content on Cellulase Production

Moisture content in the solid state fermentation is a key factor for enzyme production. In order to optimize moisture content, different % ages of moisture were employed ranging from (10-50%) to lignocellulosic substrate wheat straw. The present study indicates that maximum cellulase (301 \pm 1.16 $\mu\text{M/mL/min}$) production was observed at 40% moisture level (Table V) while, further increase in moisture influenced the enzyme production negatively. These results indicated a positive relationship between cellulase production and moisture content. Higher and lower water contents adversely affect the primary metabolic activities of microbes leading to slower enzyme formation [51, 52]. Optimal water fractions in the solid substrate appear to be 40 to 60 % (by mass) under solid-state fermentation [53]. Singhania *et al.* [54] reported that optimization of incubation temperature and initial moisture content of the medium resulted in a 6.2 fold increase in production from 0.605 to 3.8 U/gds of cellulase.

TABLE V

EFFECT OF MOISTURE CONTENT ON CELLULASE ACTIVITY PRODUCED FROM *TRICHODERMA VIRIDE* UNDER SOLID STATE FERMENTATION OF PROXIMALLY ANALYZED WHEAT STRAW

Treatment	Moisture (%)	Enzyme Activity ($\mu\text{M/mL/min}$)
1	10	235 \pm 1.16
2	20	261 \pm 1.55
3	30	282 \pm 2.74
4	40	301 \pm 0.41
5	50	270 \pm 0.52

Effect of initial pH on cellulase production

The effect of initial pH on enzyme production ranging from 3 to 7 was determined and maximum cellulase activity (348 \pm 1.29 $\mu\text{M/mL/min}$) was recovered at pH 5.5 (Table VI). Further increase or decrease in pH from this level retarded the enzyme activity. Ahmed *et al* [55] reported that optimum pH

for the fungal growth for maximal EXG, EG and BGL production was found to be 5.5 at 28°C. An optimum pH for fungal cellulases varies from species to species; though in most cases the optimum pH ranges from 3.0 to 6.0 [56, 57]. Similarly it was found earlier that maximum induction of endoglucanase was achieved at pH 5.5 as there was a correlation between the initial pH of the medium and cellulase titers by *Trichoderma reesei* Rut C-30 [58, 59]. Maximum production of β -glucosidase by *Aspergillus terreus* was achieved with the pH in the range of 4.0-5.5 [60]. Similar results are also reported by Sami *et al* [61] and found that the CMCase was more active on substrate in the pH 5.8.

TABLE VI
EFFECT OF DILUENTS pH ON CELLULASE ACTIVITY PRODUCED FROM
TRICHODERMA VIRIDE UNDER SOLID STATE FERMENTATION OF PROXIMALLY
ANALYZED WHEAT STRAW

Treatment	pH	Enzyme Activity ($\mu\text{M/mL/min}$)
1	4.0	220 \pm 2.37
2	4.5	298 \pm 2.55
3	5.0	315 \pm 1.79
4	5.5	348 \pm 1.29
5	6.0	322 \pm 3.29
6	6.5	299 \pm 2.63
7	7.0	261 \pm 2.34

Effect of Incubation Temperature on Cellulase Production

Incubation temperature plays an important and significant role in the metabolic activities of microorganism. To elucidate the effect of different temperatures on the production of cellulase from *Trichoderma viride* on pretreated wheat straw by solid-state fermentation experiments were conducted at various temperatures between (25-45°C). It was observed that enzyme activity was increased with increase in temperature up to 40°C with maximum activity of 348 \pm 1.66 $\mu\text{M/mL/min}$ (Table VII), while further increase in temperature showed decreasing trend in enzyme activity. Similar finding of incubation temperature have also been reported by [62]. Our results are in good agreement with the study of Gomes *et al* [63] who reported that at 45°C and 50°C, β -glucosidase was the most stable enzyme, retaining 81-100% activity in 72 h, whereas FPase and xylanase retained 62-67% and 56-77% activity respectively at these temperatures. Omojasola *et al* [64] reported that optimum glucose from the waste substrates using *T. longibrachiatum*, was produced at 10% inoculum size at 45°C on Day7 but at pH 5.0 and 3% substrate concentration for pineapple peel and pH 4.5 and 1% substrate concentration for pineapple pulp.

TABLE VII
EFFECT OF INCUBATION TEMPERATURE ON CELLULASE ACTIVITY PRODUCED
FROM *TRICHODERMA VIRIDE* UNDER SOLID STATE FERMENTATION OF
PROXIMALLY ANALYZED WHEAT STRAW

Treatment	Incubation Temperature (°C)	Enzyme Activity ($\mu\text{M/mL/min}$)
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1	25	227 \pm 2.39
2	30	288 \pm 2.40
3	35	335 \pm 2.22
4	40	371 \pm 3.14
5	45	352 \pm 2.75
6	50	313 \pm 3.85

Effect of Inoculum size on Cellulase Production

Effect of different inoculum sizes 5-25% (v/v) were studied for the production of cellulase enzyme from *Trichoderma viride* on pretreated wheat straw in SSF under temperature controlled still culture condition. Maximal cellulase activity (417 \pm 1.45 $\mu\text{M/mL/min}$) was noted at 15% inoculum size while, further increase in an inoculum's size showed a decline in enzyme activity which became least (74.02 \pm 1.93 $\mu\text{M/mL/min}$) at 25% inoculum size (Table VIII). Hence 15% inoculum size was found to optimum for the production of cellulase enzymes. Optimum spore density (number of spores per unit weight of substrate) is important for SSF process. Lower inoculum's level may not be sufficient to initiate the growth of microorganism resulting in longer lag phase and slower enzyme formation. Fadel [65] reported maximum enzyme activity (216.2 IU/g) at 10% inoculum size with wheat straw as substrate. Omojasola and Jilani [62] coated maximum cellulase activity with 8% inoculum size. Inoculum size controls and shortens the initial lag phase [66] where as larger inoculum's size increased the moisture content to a significant extent. The free excess liquid prevents additional barrier with that imposed by the solid nature of the substrate and leads to a decrease in growth and enzyme production [67].

TABLE VIII
EFFECT OF INOCULUM SIZE ON CELLULASE ACTIVITY PRODUCED FROM
TRICHODERMA VIRIDE UNDER SOLID STATE FERMENTATION OF PROXIMALLY
ANALYZED WHEAT STRAW

Treatment	Inoculum size (%)	Enzyme Activity ($\mu\text{M/mL/min}$)
1	5	382 \pm 1.73
2	10	417 \pm 1.45
3	15	392 \pm 1.84
4	20	341 \pm 1.93
5	25	299 \pm 1.56

IV. CONCLUSION

This paper work indicates remarkable cellulose production potential of *Trichoderma viride* from wheat straw, an agricultural by-product which has sufficient amount of cellulose (39.4 %), used as an alternative energy source for microorganism, which is renewable, efficient, safe, ideally an inexpensive and abundantly available carbon source. Cellulose is degraded to fermentable sugar through cellulase enzyme produced under still culture solid state fermentation process. In conclusion attempt was made, to find the optimum fermentation conditions for successful cultivation of

Trichoderma viride, and also towards an enhanced production of third most demanded industrially important cellulase. However, the suitability of the enzymes for biotechnological applications can be investigated through kinetic characterization of the purified enzymes as thermo-stability is a desired characteristic of an enzyme for its possible use in industry.

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