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# Efficient biomass saccharification using a novel cellobiohydrolase from *Clostridium clariflavum* for utilization in biofuel industry†

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The present study describes the cloning of the cellobiohydrolase gene from a thermophilic bacterium *Clostridium clariflavum* and its expression in *Escherichia coli* BL21(DE3) utilizing the expression vector pET-21a(+). The optimization of various parameters (pH, temperature, isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration, time of induction) was carried out to obtain the maximum enzyme activity ($2.78 \pm 0.145$ U ml$^{-1}$) of recombinant enzyme. The maximum expression of recombinant cellobiohydrolase was obtained at pH 6.0 and 70 °C respectively. Enzyme purification was performed by heat treatment and immobilized metal anionic chromatography. The specific activity of the purified enzyme was 57.4 U mg$^{-1}$ with 35.17% recovery and 3.90 purification fold. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) showed that the molecular weight of cellobiohydrolase was 78 kDa. Among metal ions, Ca$^{2+}$ showed a positive impact on the cellobiohydrolase enzyme with increased activity by 115%. Recombinant purified cellobiohydrolase enzyme remained stable and exhibited 77% and 63% residual activity in comparison to control in the presence of *n*-butanol and after incubation at 80 °C for 1 h, respectively. Our results indicate that our purified recombinant cellobiohydrolase can be used in the biofuel industry.

## Introduction

Due to the depletion of fossil fuels and increased prices of crude oils, access to renewable energy sources has become crucial to maintain the equilibrium of modern economies.[1] Lignocellulose being the most abundant renewable energy reservoir on earth, has the great potential to be utilized as a substrate for biofuel production in the biofuel industry. But the main limitation occurs in its conversion to fermentable sugars due to its rigid and compact structure.[2,3] The enzymatic hydrolysis of lignocellulosic biomass into simple sugars that can be

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fermented into biofuel, requires a complete set of enzymes that includes cellulase, hemicellulases and ligninases.[4] To depolymerize the lignocellulosic plant biomass, these enzymes act synergistically and specifically and convert it into simple fermentable sugars for biofuel production at industrial scale.[5] Based on their mode of action, the cellulases are divided into three groups: β-glucosidases, cellobiohydrolase and endoglucanase.[6] Endoglucanases randomly cleaves amorphous cellulose regions and generates oligosaccharides of various sizes.[7,8] Cellobiohydrolases act on the terminals of the cellulose chains and produces either glucose or cellobiose. Lastly, β-glucosidases act on the cellobioses and convert them into glucose.[9]

Cellobiohydrolases are important component of cellulases system and play important role in the production of biofuel from plant biomass. Cellobiohydrolases are also called *exo*-1-4-*p*-D-glucanases or cellobiohydrolases due to the generation of cellobioses. These enzymes include both 1,4-*p*-D-glucan glucohydrolases (EC 3.2.1.74) as well as 1,4-*p*-D-glucan cellobiohydrolase (EC 3.2.1.91).[10] Cellobiohydrolases generally cleave the cellulose strand by attaching to the ends of microfibrils and results in the production of cellobiose. Mostly these enzymes belong to glycoside hydrolase family six, seven and nine, however, some of these enzymes also found in GH-48 and GH-5.[11] Cellobiohydrolases typically acts in a directional preference which usually comes from the structural arrangements of the

active site of enzyme as well as initial recognition of specific cellulose chain and preferential binding of carbohydrate binding module of the cellulose.[12]

Cellobiohydrolases are mostly produced by fungal species but some bacteria also contain the genes for the production of exoglucanases. Among bacteria, Clostridia are attracting considerable attention due to their well-known cellulosomes systems.[13,14] Cellulosomes are complexes of enzymes which are capable of degrading hemicellulose, cellulose and pectin as well as lignins. Cellulosomes are produced by various anaerobic bacteria such as *Acetivibrio cellulolyticus*, *C. acetobutylicum*, *Bacteroides cellulosolvens*, *C. cellulolyticum*, *C. clariflavum*, *C. cellulovorans*, *C. josui*, *C. thermocellum*, *C. papyrosolvens*, *Ruminococcus flavefaciens* and *R. albus*.[15]

Apart from cellulases, lytic polysaccharide monooxygenases (LPMOs) are key enzymes involved in the degradation of biomass.[16] LPMO are copper dependent enzymes that can cleave cellulose to produce nanofibrils. LPMOs are most abundantly found in saprotrophic fungi with around 30 LPMO-encoding genes.[17] They are secreted when fungi are grown in the presence of cellulose or lignocellulosic material. Recently, LMPOs from fungal source are cloned, engineered, purified and characterized.[18–20]

*C. clariflavum* is a Gram-positive, cellulolytic, thermophilic bacterium, which was first isolated from an anaerobic sewage sludge.[21,22] The genome of *C. clariflavum* contains an abundance of cellulose degrading enzymes. Due to its thermophilic nature and its production of cellulosomal proteins, this bacterium is attracting attention from researchers for biomass degradation.[23] Genome analysis showed the high similarity among *C. clariflavum* and *C. thermocellum*, which exhibits the model machinery for cellulosome production.[24]

To enhance the yield, quality and functionality of industrially important enzymes, genetic engineering has played an important role since the last century. For the efficient production of recombinant proteins, the selection of the right expression vector and its host is very important. *E. coli* is often utilized for expression of exoglucanases gene from different microbes. The optimization of different conditions is another strategy for the maximum production of such a recombinant protein.[25,26] Biochemical characterization of enzymes such as pH and temperature stability of enzyme over a wide range, resistance to various ionic and non-ionic detergents and resistance against various organic solvents contributes to its applications towards industries.[27,28] Cellobiohydrolases have various industrial applications such as in detergent, food, animal feed, textile and bioenergy industries.[29,30]

The present study is concerned with the cloning and expression of a cellobiohydrolase gene of *C. clariflavum* into the *E. coli* BL21(DE3) strain. We also describe the purification and characterization of recombinant cellobiohydrolase enzyme for its use in industrial processes.

# Methodology

## Bacterial strain selection

Genomic DNA from *C. clariflavum* DSM 19732 was obtained from the German Collection of Microorganisms and Cell Culture, DSMZ, Germany (https://www.dsmz.de/). The genomic DNA was used for amplification of the cellobiohydrolase gene, GenBank ID AEV70589.1 (Clocl_4158) (accession number G8LUE1). The pET 21a(+) expression vector was utilized for the expression of cellobiohydrolase gene in *E. coli* BL21(DE3).

## Biomass collection

Three biomass including *Saccharum munja* (soft and hard portions), sugarcane bagasse and sawdust were obtained from different areas of Pakistan. However, hazelnut cob, hazelnut shell and rhododendron plant biomass were provided by our collaborators from Turkey. The biomass was dried and ground in a grinder into very fine particles.[31]

## PCR amplification

Primers were designed using Vector NTI software. The sequence of cellobiohydrolase gene, AEV70589.1, (Clocl_4158) and its protein from *C. clariflavum* is mentioned in ESI file 1.† The restriction sites *Nde*I and *Hind*III were added in the forward and reverse primers. Sequences of the forward and reverse primers are given below:

Forward primer: 5′ GC**CATATG**ATGACAATAACCTATGAC 3′
Reverse primer: 5′ CG**AAGCTT**GAATTAGTTCCGCTATAAG 3′

A PCR mixture was prepared that includes genomic DNA (30 ng μl$^{-1}$), 2 μl forward primer (100 pmol μl$^{-1}$), 2 μl reverse primer (100 pmol μl$^{-1}$), 25 μl master mixture and 19 μl distilled water. The PCR was performed for 5 minutes at 95 °C in 35 amplification cycles where each cycle was incubated at 95 °C for 45 seconds, 57 °C for 45 seconds and 72 °C for 90 seconds.[32] Agarose gel was used for analysis of amplified PCR product, which was purified using a DNA purification kit (QIAquick).

## Cloning of cellobiohydrolase gene in pET-21a(+)

The amplified cellobiohydrolase gene was restricted with *Hind*III and *Nde*I enzymes. After digestion, the cellobiohydrolase gene was purified and ligated into pET-21a(+). *E. coli* BL21(DE3) competent cells were prepared by a standard method and used for transformation of recombinant vector pET-21a(+)/ exoglucanase gene.[33] The screening of positive clones was performed by digesting isolated plasmid pET 21a(+) using and *Nde*I and *Hind*III restriction enzymes.

## Recombinant cellobiohydrolase expression in *E. coli* BL21

In order to determine production of recombinant cellobiohydrolase enzyme, *E. coli* BL21(DE3) cells (transformed) were cultured in Luria–Bertani (LB) medium supplemented with 100 μg ml$^{-1}$ ampicillin. The medium was induced by 0.5 mM IPTG and incubated at 37 °C for 4 h. The medium was then centrifuged at 6000 rpm, at 4 °C for 10 minutes. The supernatant was discarded and the resulting pellet was suspended into Tris–HCl buffer (pH 7, centrifuged at 12 000 rpm and sonicated for 10 min at 4 °C).[34] Expression of the cellobiohydrolase enzyme was determined in extra and intracellular fractions utilizing 12% SDS-PAGE and total proteins were estimated by Bradford method.[35]

## Activity assay

Dinitrosalicylic acid (DNS) method was used to determine enzyme activity[36] by estimating reducing sugars. A 500 µl aliquot of the sample was incubated with 1.5 ml of 100 mM phosphate buffer, pH 6.5, containing 1.0% CMC (Sigma-Aldrich) at 50 °C for 30 min. Then 500 µl DNS reagent (1% DNS; 0.4 M NaOH; 1 M sodium and potassium tartrate) was added, and the resulting mixture was boiled for 5 min. After the addition of 2.5 ml of distilled water the absorbance was measured in a spectrophotometer at 550 nm. Before measuring the absorbance, spectrophotometer was calibrated using a mixture prepared by 2.0 ml of 100 mM phosphate buffer, pH 6.5, with 1.0% carboxymethylcellulose (CMC). Enzyme activity was defined as "one enzyme unit that is required to liberate 1 µmol of reducing sugar per minute at standard conditions of enzyme assay".

## Total protein estimation

The estimation of total protein was carried out using the Bradford method.[35] Bradford reagent (5 ml), 100 µl of cellobiohydrolase enzyme and phosphate buffer (900 µl) were added in a test tube. A control was run parallel in which 5 ml Bradford reagent and 1 ml of phosphate buffer was added. Absorbance was observed in a spectrophotometer at 595 nm. For protein estimation, bovine serum albumin was used as the standard.

## Optimization of cultural conditions

Different parameters were analyzed to determine the optimum conditions for cellobiohydrolase production. The effect of the temperature on enzyme production was optimized by culturing the *C. clariflavum* strain at various temperatures ranging between 16–42 °C. The effect of the initial pH of the culture medium on enzyme production was evaluated by adjusting pH of the medium with sulphuric acid and sodium hydroxide to obtain pH values ranging between 4.0 to 9.0. The effect of temperature of the culture medium on the enzyme production was observed by culturing the bacteria at various temperatures (16 °C, 23 °C, 30 °C, 37 °C and 42 °C). The effect of time of incubation was tested by incubating the bacterial cultures at 37 °C, at 150 rpm for different time durations (1, 2, 3, 4, 5 and 6 h). The impact of the IPTG addition to the culture medium on the cellobiohydrolase enzyme at various concentration of 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 during entire culturing of bacteria was also tested.

## Enzyme purification

**Partial purification by heat treatment.** The cells obtained from overnight culture were sonicated as mentioned above. The cell contents were heated at 70 °C for 30 min to denature host proteins.[37] Host cell proteins were separated by centrifugation at 8000 rpm for 10 min.

**Immobilized metal ion affinity chromatography.** Recombinant cellobiohydrolase was purified by Immobilized Metal Ion Affinity Chromatography (IMAC) using Protino® Ni-TED kit. This kit facilitates the purification of proteins by adding 6 histidine residues at N-terminal of proteins. The heated contents (1 ml) of the cell extract that include the maximum amount of recombinant cellobiohydrolase enzyme was passed through the column. Elution buffer (50 µl) was added in the column and the contents were allowed to leave the column by gravity. After regular intervals, elution fractions were collected SDS-PAGE analyses to determine the purity of the enzyme.[38]

**Molecular mass determination.** The molecular weight of purified cellobiohydrolase enzyme was determined by SDS-PAGE by the Laemmli method[39] using pre-stained protein ladder (10–250 kDa). Bands on the gel were visualized by using Coomassie Brilliant Blue dye.

## Characterization

**Optimization of enzyme activity.** Optimal pH and temperature for activity of recombinant cellobiohydrolase enzyme were determined in order to obtain the maximum enzyme activity. Different buffers with a range of pH (5.0–8.0) were utilized to prepare the 1% CMC solution and then enzyme activity was determined under standard assay condition. Similarly, to determine the optimal temperature for maximum cellobiohydrolase activity, enzyme substrate complex was incubated at various temperatures (30–70 °C) for 30 minutes and afterward enzymes activity was calculated.

**pH and temperature stability.** The pH stability of cellobiohydrolase was determined by maintaining the enzyme in pH range 4.0–9.0 for 1 to 5 h at room temperature. The temperature stability of the purified enzyme was determined by incubating it at different temperatures ranging between 55–80 °C at varying time intervals (1–5 h). After all treatments, the enzyme activity of cellobiohydrolase was determined using enzyme assay.

**Surfactant.** The effect of different inhibitors like Tween-20, Tween-80, sodium dodecyl sulfate and urea was studied on purified recombinant cellobiohydrolase by pre-incubating it with 1–3% of these inhibitors at room temperature for 1 h. The activity of the cellobiohydrolase was calculated from enzyme assays.

**Organic solvents.** The activity of the purified cellobiohydrolase was determined for 10–30% organic solvents *i.e.*, acetone, *n*-butanol, methanol, absolute ethanol, and isopropanol at room temperature for 1 h. The residual activity of cellobiohydrolase was also determined using enzyme assay.

**Metal ions.** The effect of various metal ions ($Mg^{2+}$, $Ca^{2+}$, $Mn^{2+}$, $Zn^{2+}$, $Ni^{2+}$, $Fe^{2+}$, $Hg^{2+}$ and $Co^{2+}$) on enzyme stability was determined. The purified enzyme was incubated with various concentrations (1 mM to 10 mM) of metal ions for 1 h at room temperature. The enzyme activity was determined using enzyme assay.

**Pre-treatment of biomass.** The pretreatment of plant biomass was carried out by following the method given by Hoşgün *et al.*[40] Pretreatments of 6 plant biomass (*Saccharum munja*, sugarcane bagasse, sawdust, hazelnut cob, hazelnut shell and rhododendron) were performed by taking into account various parameters (time, NaOH concentration and temperature). The resulting optimum parameters were used for pre-treatment of biomass in this study. All biomasses were pretreated with 10% (w/v) NaOH at 30 °C for 72 h. After completion
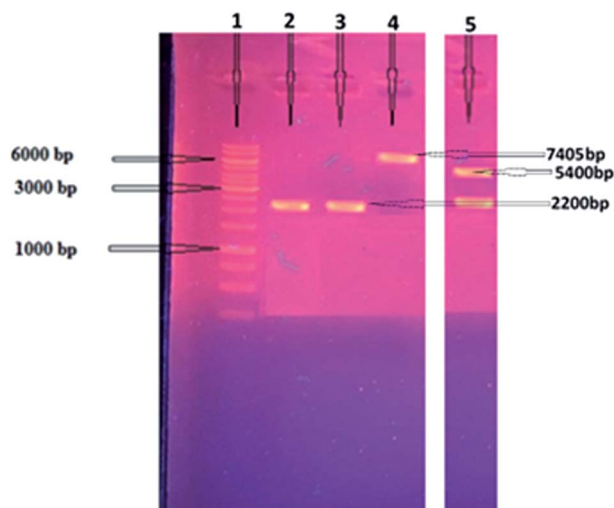
**Paper**

Fig. 1 Amplification of cellobiohydrolase gene and restriction analysis of ligated cellobiohydrolase gene with pET-21-a(+) plasmid on agarose gel. DNA marker is shown in lane 1. Amplified cellobiohydrolase gene (2200 bp) is present in lanes 2 and 3, single restricted recombinant pET-21a(+) ligated with exoglucanase gene (7405 bp) is shown in lane 4, double restricted recombinant pET-21a(+) plasmid ligated with exoglucanase gene showing two separate bands of pET 21a(+) (5400 bp) and cellobiohydrolase gene (2200 bp) as shown in lane 5.

of procedure, the pre-treated biomass was filtered and solid obtained was air dried and stored at 4 °C for enzymatic processing (saccharification).

**Saccharification activity.** Purified recombinant cellobiohydrolase enzyme was checked for its saccharification potential against plant biomass for use in the biofuel industry. Various types of plant biomass (*Saccharum munja* (soft and hard portions), sugarcane bagasse, sawdust, hazelnut cob, hazelnut shell and rhododendron) were used to investigate the saccharification capability of cellobiohydrolase enzyme.

Pretreatment analysis was performed by scanning electron microscopy. For saccharification experiment, 1 g of pretreated biomass was incubated with 50 ml (140 U) (28 mg) enzyme at 50 °C for 24 h in a shaking incubator at 100 rpm. To prevent the contamination by different microorganisms, tetracycline (10 μg ml$^{-1}$) was added along with cellobiohydrolase enzyme. Various conditions (incubation temperature, pH, incubation period, enzyme units, substrate concentration) were optimized to attain the maximum saccharification of plant biomass. A range of temperature (30–70 °C), pH (5–9), incubation period (6–30 h), substrate concentration (0.5–2.5%), enzyme units (50–250) and agitation speed (50–250 rpm) was used obtain the optimized conditions of plant biomass saccharification. The hydrolysis of

plant biomass was checked by measuring the liberated reducing sugars using the protocol given by Miller.[36] The percentage saccharification was calculated from the following formula:

$$\text{Saccharification } (\%) =$$

$$\frac{\text{cellobiose } (\text{mg ml}^{-1}) \times 0.9 \times \text{total reaction volume} \times 100}{\text{biomass contents } (\text{g})}$$

## Results

### Cloning and expression of cellobiohydrolase gene into pET-21a(+)

The cellobiohydrolase gene, GenBank ID AEV70589.1, (Clocl_4158) of *C. clariflavum* was amplified (2200 bp) and then restricted with specific enzymes as mentioned in methodology. The restricted gene was ligated with pET-21a(+) expression vector and then transferred into competent cells of *E. coli* BL21(DE3). Isolated recombinant plasmids were restricted (7643 bp) to confirm the cloning process (Fig. 1). Double restriction utilizing *Hind*III and *Nde*I showed two bands, 5400 bp and 2200 bp, depicting the successful ligation into expression vector. Expression of the cellobiohydrolase was investigated utilizing SDS-PAGE and its activity in extracellular fractions was calculated to be 2.14 U mg$^{-1}$ by using DNS method.[36]

### Purification of recombinant protein

The heat treated denatured host proteins were separated from crude cell lysate by centrifugation and subjected to Protino® Ni-TED columns for further purification. Six His-tagged residues were added at the amino end of the cellobiohydrolase enzyme due to presence of 6 histidine residues located in the pET-21a(+) plasmid at position 140–157. After SDS-PAGE analysis, the purification fold and specific activity of the cellobiohydrolase enzyme was determined to be 3.90 fold and 57.4 U mg$^{-1}$ respectively (Table 1).

### Determination of molecular weight

The molecular weight of recombinant cellobiohydrolase enzyme was determined by SDS-PAGE and observed to be 78 kDa. Cell lysates of wild *E. coli* BL21, non-induced recombinant wild *E. coli* BL21, induced recombinant wild *E. coli* BL21 and heat treated partially purified cellobiohydrolase enzyme fraction were also run in parallel on SDS-PAGE (Fig. 2).

Table 1 Purification summary for the recombinant exoglucanase enzyme

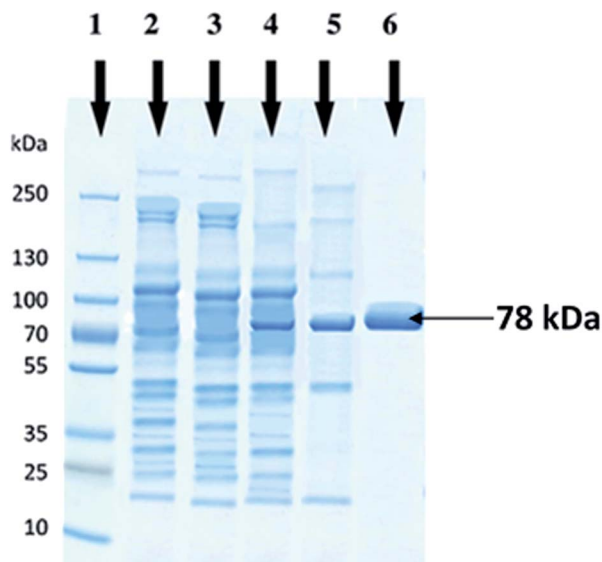| Purification steps | Exoglucanase unit (U) | Total protein (mg) | Specific activity (U mg$^{-1}$) | Purification fold | Recovery (%) |
|---|---|---|---|---|---|
| Crude exoglucanase | 2780 | 189 | 14.7 | 1 | 100 |
| Heat-treated | 1590 | 55 | 28.90 | 1.96 | 57.19 |
| IMAC | 978 | 17 | 57.4 | 3.90 | 35.17 |

**Fig. 2** SDS-PAGE analysis for the expression of purified recombinant cellobiohydrolase enzyme from *Clostridium clariflavum*. In lane 1, prestained protein marker (10–250 kDa) is loaded, lane 2 represent cell lysate of wild *E. coli* BL21(DE3), lane 3 represent cell lysate of non-induced recombinant *E. coli* BL21(DE3), lane 4 depicts cell lysate of induced recombinant *E. coli* BL21(DE3), in lane 5 heat-treated recombinant exoglucanase enzyme was loaded and in lane 6 purified recombinant cellobiohydrolase enzyme sample is present.

## Optimization of recombinant cellobiohydrolase gene expression

Utilization of various IPTG concentrations (0.1–0.6 mM) revealed that maximum cellobiohydrolase expression ($2.16 \pm 0.045$ U ml$^{-1}$) was achieved when recombinant *E. coli* BL21(DE3) bacterial cells were induced with 0.5 mM IPTG. However, reduction in cellobiohydrolase expression was observed at lower concentrations (Fig. 3A). Effect of time of induction was also monitored to obtain maximum production of cellobiohydrolase enzyme. Various time scale (1 to 6 h) was used for incubation of recombinant culture. The maximum production ($2.35 \pm 0.085$ U ml$^{-1}$) of the cellobiohydrolase gene with $1.04 \pm 0.012$ mg ml$^{-1}$ total protein contents was determined after 4 h of incubation at 37 °C and 0.5 mM IPTG induction (Fig. 3B). Reduced production of enzyme ($1.38 \pm 0.036$ U ml$^{-1}$, $1.72 \pm 0.104$ U ml$^{-1}$ and $1.76 \pm 0.124$ U ml$^{-1}$) was obtained after 2 h, 3 h and 5 h incubation, respectively at 37 °C. Effect of temperature was also investigated to get the most favorable temperature for high production of cellobiohydrolase. Recombinant *E. coli* cells were cultured at different temperatures (16 °C to 42 °C) after induction with 0.5 mM IPTG for 4 h. Highest expression ($2.57 \pm 0.041$ U ml$^{-1}$) with $1.56 \pm 0.014$ mg ml$^{-1}$ total protein contents was obtained at 37 °C for 4 h. At elevated temperatures, enzyme activity was decreased and dropped to $1.81 \pm 0.121$ U ml$^{-1}$ at 42 °C (Fig. 3C). pH of the medium has obvious effect on the growth of microorganisms. Maximum production ($2.78 \pm 0.145$ U ml$^{-1}$) of cellobiohydrolase enzyme was observed at pH 6.0. At pH 4, 5, 7, 8 and 9 less enzyme activity ($1.25 \pm 0.987$ U ml$^{-1}$, $1.68 \pm 0.72$ U ml$^{-1}$, $2.11 \pm 0.921$ U ml$^{-1}$, $1.56 \pm 0.998$ U ml$^{-1}$ and $0.98 \pm 0.958$ U ml$^{-1}$) was obtained (Fig. 3D). The best culture conditions

for cellobiohydrolase production was obtained at 37 °C, at initial pH 6.0, with addition of 0.5 mM IPTG in the medium for 4 h incubation.

## Characterization of recombinant cellobiohydrolase

Purified recombinant cellobiohydrolase enzyme was characterized for the various parameters below:

**Optimization of pH and temperature.** To determine the optimal pH for maximum enzyme activity enzyme was incubated with buffers having different pH values (5.0 to 8.0) along with its substrate (1% CMC). Maximum enzyme activity (2.69 U ml$^{-1}$) was found at pH 6.5. While at other pH values like 5.0, 5.5, 6.0, 7.0, 7.5 and 8.0, the reduced enzyme activity (0.74 U ml$^{-1}$, 1.3 U ml$^{-1}$, 1.95 U ml$^{-1}$, 1.99 U ml$^{-1}$, 1.28 U ml$^{-1}$ and 0.82 U ml$^{-1}$, respectively) as shown in Fig. 4A. Optimal temperature for maximum cellobiohydrolase activity (2.58 U ml$^{-1}$) was observed as 50 °C. At other temperature such as 30 °C, 40 °C, 60 °C and 70 °C, the calculated enzyme activity was 0.43 U ml$^{-1}$, 1.2 U ml$^{-1}$, 1.43 U ml$^{-1}$ and 0.87 U ml$^{-1}$, respectively as shown in Fig. 4B.

**Thermostability.** To determine the thermostability of purified recombinant cellobiohydrolase enzyme, incubations were performed at different temperatures (50 °C to 90 °C) for 1–4 h. A high residual activity (61%) of cellobiohydrolase was observed after 2 h incubation at 70 °C, which diminished to 40% activity after 4 h at 70 °C. After 4 h incubation at 60 °C, the enzyme showed 51% residual activity. At high temperatures of 80 °C and 90 °C, the residual activity of the purified enzyme was determined as 63% and 40%, respectively, after 1 h (Fig. 5A). The results indicate that the cellobiohydrolase enzyme was comparatively stable at high temperatures.

**pH stability.** The stability of cellobiohydrolase enzyme was studied by incubating purified enzyme in several buffers (pH 4.0–9.0) for 1 to 3 h. It was observed that residual activity of the enzyme was 78% and 69% when pre-incubated at pH 6.0 and pH 7.0 respectively for 3 h. At pH 8.0 and pH 9.0, the enzyme activity declined to 38% and 19%, respectively after 3 h incubation. However, the residual activity dropped to 16% at pH 4.0, after 3 h but at pH 5.0 considerable stability (46%) was maintained by the enzyme after 3 h (Fig. 5B).

**Effect of metal ions.** The effect of various metal ions (Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, Ni$^{2+}$, Fe$^{2+}$, Hg$^{2+}$ and Co$^{2+}$) on purified enzyme stability was determined. Purified enzyme was incubated with various concentrations (1 mM to 10 mM) of metal ions for 1 h at room temperature. The results indicated the increased residual activity of the enzyme (115%) in the presence of 1 mM Ca$^{2+}$. Mg$^{2+}$ ions did not significantly affect the enzyme activity at lower concentrations (1 mM and 5 mM), however, higher concentration (10 mM) deceased the enzyme activity to 72% (Fig. 5C). Metal ions such as Mn$^{2+}$ and Zn$^{2+}$ had a negligible effect on residual activity of the enzyme, while in the presence of 1% Co$^{2+}$ ions, the enzyme showed an enhanced residual activity of 112% and ones of 105% and 95% at 5 mM and 10 mM respectively. On the other hand, higher concentrations of Ni$^{2+}$ (10 mM) did not significantly affect the activity and stability of the enzyme that retained 46% residual activity, however high
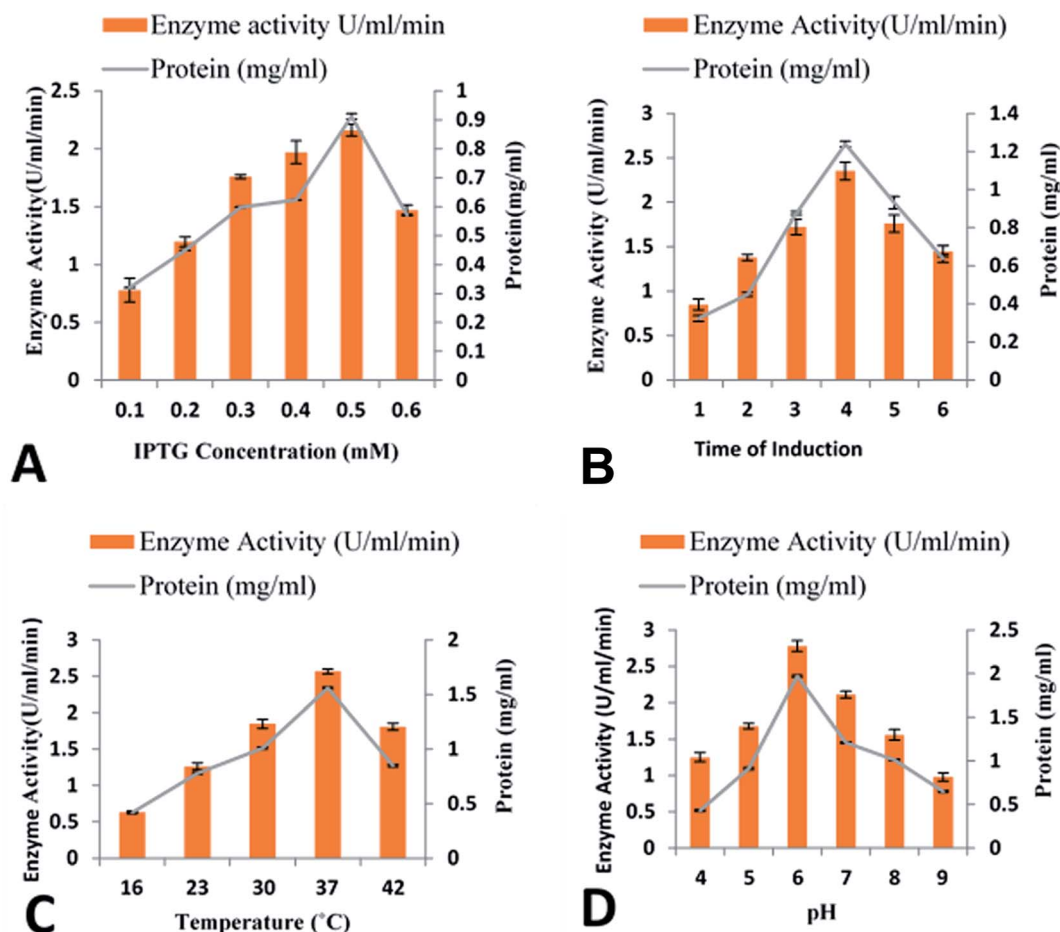
**Fig. 3** Optimization of cloned cellobiohydrolase gene expression: (A) effect of IPTG concentration (B) effect of time of induction (C) effect of incubation temperature (D) effect of pH.
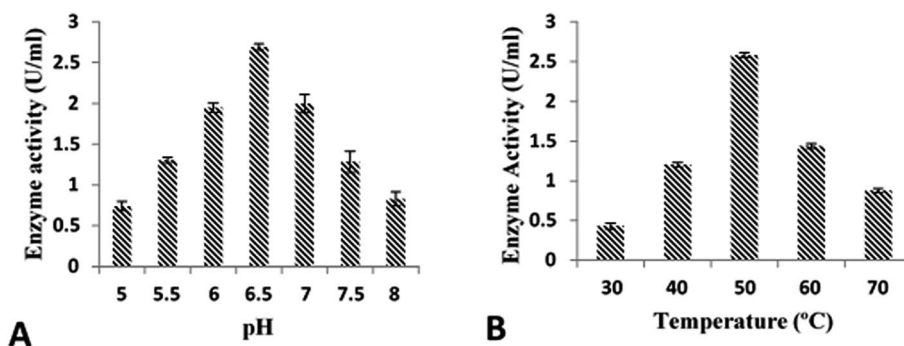


**Fig. 4** Optimization of cloned cellobiohydrolase enzyme activity: (A) effect of pH (B) effect of incubation temperature.

concentration of $Hg^{2+}$ significantly reduced the catalytic activity of enzyme (Fig. 5C).

**Effect of surfactants.** The effect of surfactants on the activity and stability of purified enzyme was also analyzed. The enzyme was incubated with various surfactants including Tween 80, Tween 20, SDS and urea in various concentration (1–3%) at room temperature for 1 h. Purified cellobiohydrolase showed considerable enzyme activity (46%) even in higher concentration (3%) of Tween 80. The enzyme seems to be resistant (52%

and 44%) in the presence of 1% Tween 20 and 1% urea but higher concentrations of these surfactants showed decreased enzyme activity (Fig. 5D). SDS greatly influenced the activity and stability of cellobiohydrolase enzyme and 12% residual activity was found in the presence of 3% SDS (Fig. 5D).

**Effect of organic solvents.** The stability of the cellobiohydrolase enzyme was also studied by the pre-incubation of purified enzyme with various concentrations (10–30%) of organic solvents (acetone, methanol, absolute ethanol, *n*-
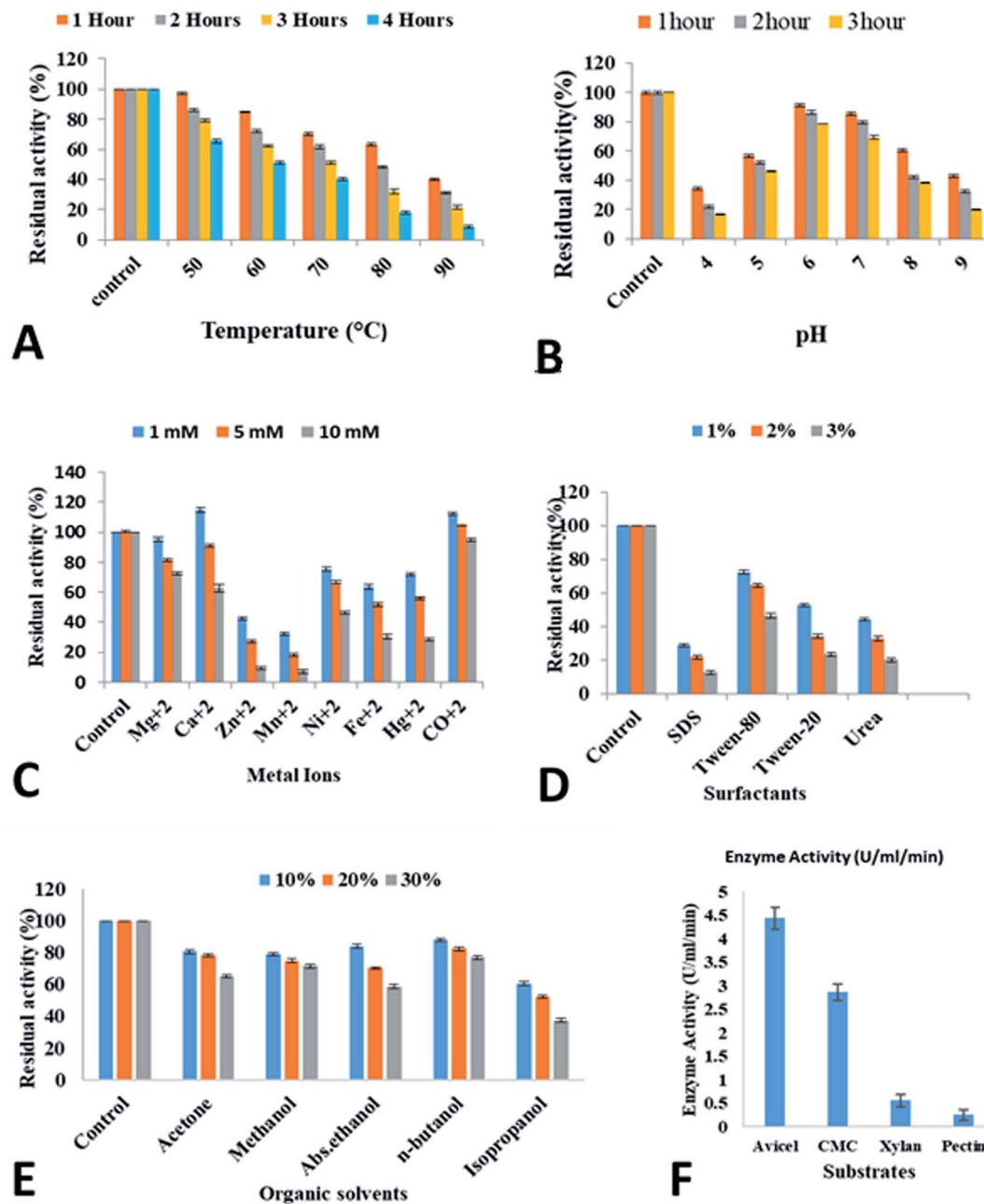
**Fig. 5** Characterization of recombinant purified cellobiohydrolase enzyme. (A) Thermostability (B) pH stability (C) effect of metal ions (D) effect of surfactants (E) effect of organic solvents (F) substrate specificity.

butanol, isopropanol) for 1 h at room temperature. Purified enzyme showed higher stability in the presence of organic solvents even at higher concentration (30%) of organic acids. In the presence of 30% methanol, acetone, absolute ethanol, isopropanol and $n$-butanol, the residual activity was calculated as 71%, 65%, 58%, 37% and 77% respectively (Fig. 5E).

**Substrate specificity.** The specificity of the purified enzyme for substrate was determined using 2% various substrates (Avicel, CMC, xylan, pectin). The enzyme assays were performed under specific conditions. The results showed that the maximum enzyme activity ($4.43 \pm 1.45$ U ml$^{-1}$) was obtained in

the presence of 2% Avicel (Fig. 5F). With 2% CMC, xylan and pectin, enzyme activity was calculated as $2.86 \pm 1.21$ U ml$^{-1}$, $0.56 \pm 1.114$ U ml$^{-1}$ and $0.25 \pm 1.014$ U ml$^{-1}$, respectively.

**SEM analysis of pretreated biomass.** Scanning electron microscopy was used to characterize the structures of biomass after pretreatment with enzyme. The electron micrographs of native sample showed the compact, regular and smooth surface, indicating the highly well-arranged surface structure, whereas the pretreated samples exhibited an uneven and irregular structures and showed loosened inner zones of cellulose and hemicellulose. Pretreatment methods also enhanced

the surface areas of biomasses in order to provide better accessibility to enzymatic hydrolysis. The images of different biomasses are shown in Fig. 6.

**Plant biomass saccharification.** The saccharification of the pretreated biomass (*Saccharum munja* (soft and hard portions), sugarcane bagasse, sawdust, hazelnut cob, hazelnut shell and

rhododendron) utilizing 140 units of recombinant cellobiohydrolase resulted in release of high cellobiose levels. The reducing sugars yield achieved was 4.79 mg ml$^{-1}$ from pretreated sugarcane bagasse. While for other substrates like *Saccharum munja* hard, *Saccharum munja* soft, sawdust, hazelnut cob, hazelnut shell and rhododendron the calculated reducing
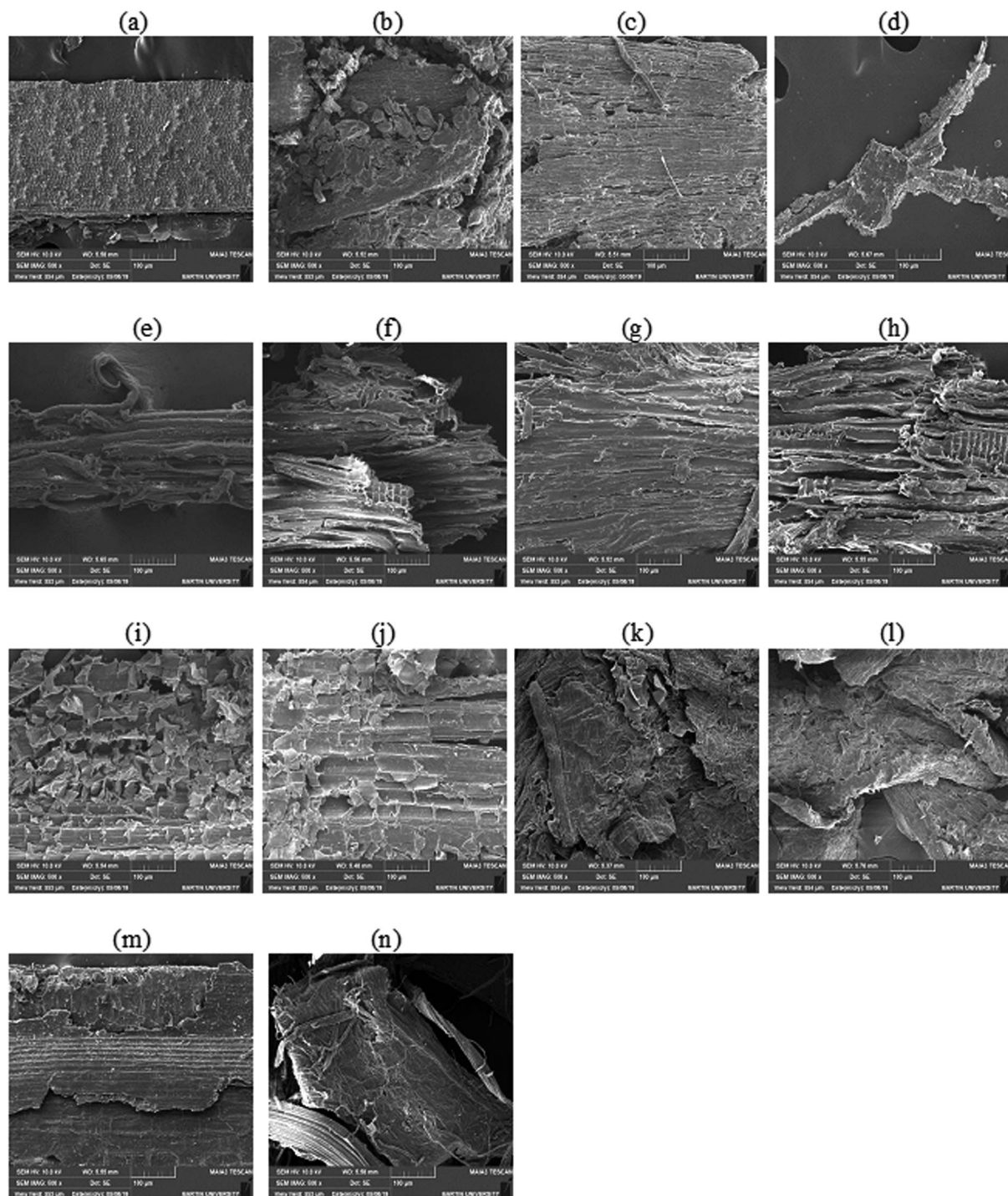


Fig. 6 Scanning electron micrographs (500×) of (a) control hazelnut shell (b) treated hazelnut shell (c) control rhododendron root (d) treated rhododendron root (e) control hazel nut cob (f) treated hazel nut cob (g) control saw dust (h) treated saw dust (i) control *Saccharum munja* (soft) (j) treated *Saccharum munja* (soft) (k) control *Saccharum munja* (hard) (l) treated *Saccharum munja* (hard) (m) control sugarcane bagasse (n) treated sugarcane bagasse.

Table 2 Percentage saccharification of pretreated plant biomass

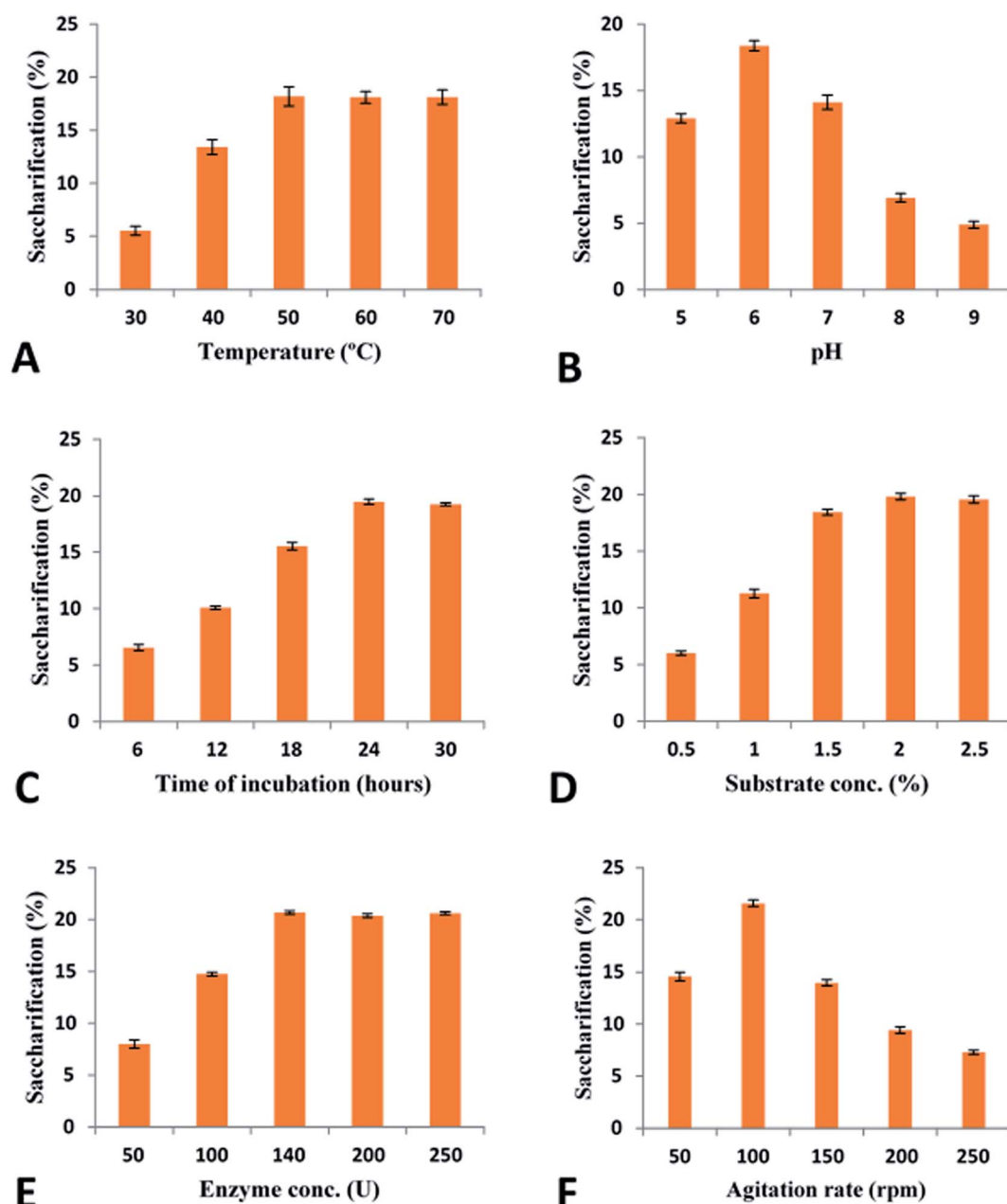| Pretreated biomass type | Reducing sugars released (mg ml$^{-1}$) | Saccharification (%) | Cellulose contents in biomass (%) |
| --- | --- | --- | --- |
| Sugarcane bagasse | 4.79 | 21.57 | 62 (Guilherme et al., 2015)[107] |
| Saccharum munja (hard) | 1.77 | 7.96 | 35 (Kumar et al., 2019)[108] |
| Saccharum munja (soft) | 3.86 | 17.4 | 70 (Singh et al., 2017)[109] |
| Sawdust | 3.11 | 13.9 | 45 (Klash et al., 2010)[110] |
| Hazelnut cob | 3.5 | 15.75 | 52 (Demirbraş, 2005)[111] |
| Hazelnut shell | 2.5 | 11.5 | 42 (Demirbraş, 1999)[112] |
| Rhododendron | 2.88 | 12.96 | 47 (Shrestha & Budhathoki, 2012)[113] |



Fig. 7 Optimization of saccharification conditions: (A) effect of temperature (B) effect of pH (C) effect of incubation time (D) effect of substrate concentration (E) effect of enzyme concentration (F) effect of agitation rate.

sugars were 1.77 mg ml⁻¹, 3.86 mg ml⁻¹, 3.11 mg ml⁻¹, 3.5 mg ml⁻¹, 2.5 mg ml⁻¹, and 2.88 mg ml⁻¹, respectively. This reflected the higher performance of the minimal enzyme system developed based on this tailor-made strategy. The saccharification percentage obtained from all other tested plant biomass (sawdust, hazelnut cob, hazelnut shell, rhododendron, *Saccharum munja* hard, *Saccharum munja* soft) under optimized conditions (pH 6, at 50 °C, 24 h of incubation, 2% substrate concentration, 140 units of enzyme, 100 rpm) were 13.9%, 15.75%, 11.5%, 12.96%, 7.96% and 17.4%, respectively as shown in Table 2. Among all tested pretreated plant biomass, sugarcane bagasse resulted comparatively better results and higher percentage of saccharification (21.57%). The results obtained are represented in Table 2.

**Optimization of saccharification of plant biomass.** Maximum saccharification of plant biomass was obtained when treated with purified recombinant cellobiohydrolase and incubated at 50 °C, pH 6.0, for 24 h of incubation at 100 rpm agitation. In saccharification process, 2% substrate and 140 units of enzyme was used (Fig. 7). Elevated temperatures did not considerably affect the saccharification rate while temperature below 50 °C resulted in less saccharification percentage (Fig. 7A). Similarly, optimized pH for the saccharification of plant biomass was estimated to be 6.0 while less hydrolysis of plant biomass was observed with other pH values (12% at pH 5.0, 14% at pH 7.0, 6% at pH 8.0, 4.8% at pH 9.0) (Fig. 7B). Incubation period of enzyme along with plant biomass is optimized after 24 h (19.48%) but prolonged incubation did not seem to affect the plant biomass hydrolysis. On the other hand, less incubation time rendered less saccharification of plant biomass (15.55% after 18 h, 10% after 12 h and 6.5% after 6 h) was observed (Fig. 7C). Maximum saccharification of plant biomass with cellobiohydrolase was acquired by using 2% pretreated plant biomass with 140 units of enzymes as shown in Fig. 7D and E respectively. Higher enzyme concentrations as well as substrate concentrations did not resulted in high saccharification percentage. With 50, 100 and 200 units of enzyme, saccharification percentage was calculated to be 8%, 14.7% and 20.36% respectively. A range of agitation speed (50–250 rpm) was tested to optimize saccharification process. Agitation rate of 100 rpm was calculated to be optimum producing maximum saccharification (21.57%) and higher agitation rates resulted in reduced saccharification (Fig. 7F).

## Discussion

Biofuel production from lignocellulosic plant biomass is attracting the intense attention of researchers in recent decades in response to the search for alternative fuels. The major portion of lignocellulosic plant biomass is comprised of complex cellulose polysaccharides and the degradation of this complex cellulose requires a complete set of cellulases. Exoglucanase is one of the most important cellulases which acts upon the ends of cellulose chains and results in the production of cellobiose and glucose units. In this study, the cellobiohydrolase gene from *C. clariflavum* was cloned into *E. coli* BL21(DE3) using the expression vector pET 21a(+). The

recombinant cellobiohydrolase enzyme activity (2.78 ± 0.145 U ml⁻¹) was obtained with 4 h of IPTG (0.5 mM) induction at 37 °C. Higher concentrations of IPTG resulted in reduced cellobiohydrolase enzyme production due to the toxicity of IPTG that reduced the rate of ribosomal RNA synthesis.

The present study showed much higher purifications when compared to many recent reports. The specific activity of the purified enzyme reached 57.4 U mg⁻¹ after IMAC processing. Similarly, the percentage recovery was 35.17% and a purification fold of 3.90 was attained. In comparison to this current study, much less purification fold of cellobiohydrolases has been recently reported. There, a purification fold of 1.07 and 1.11 was reported for glucanases from *Trichoderma viride* and *C. thermocellum* respectively.[41,42] Similarly, reduced recovery yields of 10.26% and 1.06% was achieved after the final purification of cellobiohydrolases from *T. harzianum* and *Bacillus stearothermophilus*.[43,44] In another study, a similar low recovery of cellobiohydrolase from *Aspergillus oryzae* was reported after its purification which was in-compatible with recovery obtained in present study.[45]

In this study, the enzyme retained maximum activity when incubated at acidic pH (6.0). Many studies involving cellobiohydrolase (cellobiohydrolase) from thermophiles showed similar results. Cellobiohydrolase from *C. thermocellum* showed maximum activity at pH 5.7.[46] Similarly, the cellobiohydrolases from *Thermoascus aurantiacus*[47] and *Actinomyces* sp.[48] remained stable at pH 6.0 and the cellobiohydrolase from *Thermotoga petrophila* retained 79% activity at pH 4.[49] On the other hand, *T. maritima* showed stability at varied pH range between 6.0–7.5.[50] However, various cellulases from *T. reesei* and *Rasamsonia emersonii* showed stability between pH values 4–6.[51]

One study makes use of the presence of all nine tryptophan residues in the catalytic domain of cellobiohydrolase from mutant *T. reesei* to demonstrate the effect of pH on the enzyme activity utilizing fluorescence measurement.[105] It was found that drastic changes occur in the catalytic site of the enzyme at lower and higher pH values that resulted in less binding with substrate and showed less fluorescence intensity due to change in polarity of some of all tryptophans in the active site tunnel of Cel7A when substrate is bound. Similar results indicated high alkaline pH value caused radical changes in secondary structure of cellobiohydrolase from *T. harzianum* and resulted in drastic decrease in enzyme activity.[106]

An important feature of cellobiohydrolase is its stability at different ranges of temperatures from 50–90 °C for 1–4 h. Specifically, the cellobiohydrolase retained 63% activity at 80 °C for 1 h. Jeong *et al.*[52] demonstrated that cellobiohydrolase from *Fervidobacterium islandicum* was stable at 65 °C for 1 h. In the present study, cellobiohydrolase retained reasonable stability of 52% for 4 h at 60 °C. In one study, cellobiohydrolase from *T. petrophila* showed 81% residual activity at 70 °C for 4 h.[49] Recently, around 50% cellobiohydrolase activity from *Lentinula edodes* was demonstrated at 60 °C.[53] Several reports have reported less residual activities of thermostable cellobiohydrolases at pre-incubation at high temperature. Cellobiohydrolase obtained from *C. thermocellum*,[42] *T. maritima*,[50] hot spring isolate,[54] *Thermoanaerobacterium thermosaccharolyticum*,[55] *Thermoascus*

*aurantiacus*,[47] *Fervidobacterium islandicum*[52] all showed highly reduced enzyme activity when incubated between 70 and 90 °C. However, two reports demonstrated that cellobiohydrolase from *T. maritima*[50] and *T. neapolitana*[56] maintained their catalytic activity during pre-incubation at 90 °C.

It has been proposed that the presence of rigid protein structures at high temperatures establish fluidity and flexibility in their structural characteristics.[57,58] Helix stabilization is one of the key factors that is governed by the low level presence of C-β-branched amino acids like valine, isoleucine and threonine.[59] Presence of specific amino acids like proline at helical ends also contribute in helix stabilization and leads to enzyme stability.[60] Similarly, there are many interactions between domain/subunits that can stabilize the structure of thermostable enzyme. These interactions include hydrophobic interactions, hydrogen bonds, disulphide bridges aromatic interactions, docking loose ends and ion-pair networks.[61–65] Sometimes oligomer formation helps in the prolonged stability of enzyme at high temperature.[60,63,66] Apart from this, dense packing in enzyme structures due to increased core hydrophobicity[63] and the presence of stable surface-exposed amino acids like glutamine that cause deamidation[60] or oxidative degradation due to presence of cysteine and methionine[67] contribute significantly to enzyme stability.

It is well established that thermostable enzymes possess rigid structure that could be converted into flexible and fluidity structures at high temperature.[42,56] In another interesting study, the enzymatic activity of thermophilic α-amylase obtained from *Bacillus licheniformis* has been reported at high temperature, however, loss of enzyme activity has been described at high temperature in mesophilic *B. amyloliquefaciens*.[68] On the other hand, the main contributors that sustain the molecular integrity of enzymes from psychrophilic microorganisms near zero celsius are decreased rigidity in enzyme core and reduced inter domain interactions but their enzyme structures resemble with mesophilic and thermophilic enzymes.[69] Decreased rigidity in protein core and decreased inter domain interactions contribute in the molecular structure of psychrophilic microorganisms at sub-zero temperature.[70] Further, it is also generally assumed that the increase in stability from psychrophilic to thermophilic proteins arises from a decrease in conformational flexibility.[71,72]

In industrial applications, generally thermostable enzymes are preferred that can survive high temperature (>55 °C) needed in industrial processes. Additional advantages of thermostable enzymes usage include high tolerance for organic solvents,[73] reduced risk of contamination and increased stability specially when used in raw material pre-treatments. The stability of cellobiohydrolase from *C. clariflavum* make it the enzyme of choice for industrial use especially in saccharification of biomass. The wide range of industrial uses of thermostable cellobiohydrolase including paper and pulp industry, food industry, textile industry as well as in biofuel industry has been reported.[74]

Cellobiohydrolase from *C. clariflavum* was studied in the presence of various metal ions. The cellobiohydrolase showed tolerance and increased enzymatic activity in the presence of $Ca^{2+}$, $Ni^{2+}$, $Co^{2+}$ and $Mg^{2+}$. However, $Mn^{2+}$, $Fe^{2+}$ and $Zn^{2+}$ and $Hg^{2+}$ showed adverse effect on the activity of the enzyme. Many

previous reports on cellobiohydrolase have shown similar results. It has been shown that *Rhizopus oryzae* cellobiohydrolase demonstrated low activity in the presence of $Zn^{2+}$ and $Hg^{2+}$.[75] Recently, it has been illustrated that cellobiohydrolase from *Fervidobacterium islandicum* remained stable in the presence of $Co^{2+}$, $Na^{2+}$, $Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ ions while showed reduced enzymatic activity in the presence of $Zn^{2+}$, $Fe^{2+}$ and $Cd^{2+}$ metal ions.[52] Other studies by Ghori *et al.*[76] and Nasir *et al.*[41] reported that cellobiohydrolase from *A. niger* and *T. viride* respectively were inhibited in the presence of $Hg^{2+}$ and $Fe^{2+}$ while enzymes retained high residual activity in the presence of other metal ions. Most of the glycoside hydrolases are inhibited by $Hg^{2+}$ that oxidizes indole rings of tryptophan residues of the enzyme.[77] This phenomenon has been observed in cellulases from *Bacillus* sp. BG-CS10 and *B. sphaericus*.[78,79] On the other hand, damage of active site due to disturbance in thiol groups in the cellobiohydrolase enzyme could also be the main cause of the decreased activity of cellobiohydrolase.[80] Some studies have demonstrated the increased residual activity of cellobiohydrolase in the presence of $Ca^{2+}$.[81,82] The possible effect of metal ions on cellobiohydrolase has not been clearly explained in the literature. However, it is assumed that it could probably occur due to redox effect on amino acids, thus, enhancing or reducing the enzyme activities. Binding of metal ions with sulfhydryl (–SH) groups of cysteine result in denaturing of thiol group and consequently change in conformational structure of enzyme disallow binding with the substrate.[83,84]

The effect of divalent ions on cellobiohydrolase is not clearly understood and possibly occurs by redox effects on the amino acids, increasing or decreasing their activities. The reduced activity of the enzyme is probably due to the damage of the active site by denaturation of the existing thiol groups.[83] It is assumed that metal ions generally bind to sulfhydryl (–SH) groups of the cysteine present in an enzyme and by denaturing of the thiol group, thus resulting in the conformational change of an enzyme which prohibits binding with the substrate.[82,84] Previously, stereochemical aspect of α-amylase in hyperthermophilic archaebacterium *Pyrococcus furious* showed that higher activation energies are required for optimum function of enzyme at high temperature.[71]

One of the significant feature of cellobiohydrolase from *C. clariflavum* is its tolerance against various organic compounds and surfactants. Azadian *et al.*[85] has shown that catalytic activity of cellobiohydrolase from *Bacillus* was increased to 161% and 152% in the presence of methanol and chloroform respectively while it was inhibited to 61% by DMF. Thomas *et al.*[86] has explained the organic solvent, *n*-hexane and isooctane, tolerant cellobiohydrolase enzyme from *Promicromonospora* sp. Gaur and Tiwari[87] showed increased residual activity of cellobiohydrolase from *B. vallismortis* to 242.5, 178.7, 117, 170.3, 180.2, 143, 160.3, 115.9, 131.9 and 133% after incubation for 24 h to 48 h with 30%, *n*-decane, *n*-dodecane, iso-octane, toluene, xylene, *n*-hexane, acetone, methanol, *n*-butanol and cyclohexane. Similarly, other studies have reported that cellobiohydrolase from hot spring isolate and *Hermetia illucens* remained stable in the presence of isopropanol, propanol and methanol.[54,88] However, some studies reported decreased residual

activity of cellobiohydrolase enzyme from soil metagenome library.[89] It has been shown previously that β-glucosidases from *T. aurantiacus*, *A. oryzae* and *Fusarium oxysporum* were activated by short chain alcohols.[90–92] It has been proposed that alcohols alter the polarity of the medium that help to stabilize the enzyme conformation.[93] Less cellobiohydrolase activity has been demonstrated in the presence of 1% various surfactants like Tween 80, Tween 20, Triton X-100, SDS and $H_2O_2$. Generally, it is suggested that stability of cellulases in presence of Tween 80 corresponds to the hydrophobicity and secondary structure propensity of these proteins.[94]

Some groups have studied the cellobiohydrolase obtained from commercial company, microbes isolated from agricultural wastes and bacteria isolated from hot spring.[41,54] It is assumed that a several complex mechanisms confers adaptations and allow bacteria to survive against organic solvents.[95] The development of reflux pumps in bacteria due to periplasmic space helps them tolerate against the organic solvents.[96] Structurally and functionally unrelated compounds are removed from the cytoplasm of the bacteria due to these efflux pumps.[97] On the other hand, organic solvents can penetrate the active sites of the enzyme thus making these enzymes ineffective.[98,99] Water molecules interactions with enzymes help retain the three dimensional structure and its activity.[100]

For the complete degradation of cellulose to simple sugars, cellobiohydrolases play a very important role. These enzymes hydrolyze the cellulose at the polysaccharide chain ends and results in production of cellobiose as well as glucose units.[101] In this study recombinant purified cellobiohydrolase from *C. clariflavum* was found to be very active for the hydrolysis of pretreated plant biomass. Maximum saccharification (21.57%) was observed against pretreated sugarcane bagasse while all other tested biomass (sawdust, hazelnut cob, hazelnut shell, rhododendron, *Saccharum munja* hard, *Saccharum munja* soft) were also significantly hydrolyzed by the action of recombinant enzyme (Table 2). Very few literatures on the exclusive use of cellobiohydrolase for the saccharification of plant biomass has been previously reported although the saccharification of plant biomass by the combined effect of cellulases is reported in previous reports. In a previous study, 9.03% saccharification was obtained against sugarcane bagasse and 5.92% saccharification of sawdust utilizing the cellulases of *A. oryzae*[102] while 5.12% saccharification of wheat straw and 7.31% saccharification of sugarcane bagasse was reported for the endoglucanase of *C. thermocellum* in another study.[103] A 24% saccharification of sugarcane bagasse was observed by the action of endoglucanase from *B. licheniformis*.[104]

## Conclusion

The present study describes the cloning of cellobiohydrolase gene from a thermophilic bacterium *C. clariflavum* in *E. coli* BL21(DE3) and its expression in pET21a(+) expression system. This study presented the optimized conditions for the maximum production of cellobiohydrolase enzyme in *E. coli* BL21(DE3) using pET21a(+) expression system. This study is innovative for the industrial scale production of recombinant cellobiohydrolase enzyme.

## Conflicts of interest

We have no competing interests.

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