



Biochemical Characterization of Thermostable Carboxymethyl Cellulase and β -Glucosidase from *Aspergillus fumigatus* JCM 10253

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Abstract

Second-generation biofuel production has emerged as a prominent sustainable and alternative energy. The biochemical properties of cellulolytic enzymes are imperative for cellulosic biomass conversion into fermentable sugars. In the present study, thermostable CMCase and β -glucosidase were purified and characterized from *Aspergillus fumigatus* JCM 10253. The enzymes were purified through 80% ammonium sulfate precipitation, followed by dialysis and DEAE-cellulose ion-exchange chromatography. The molecular masses of the purified CMCase and β -glucosidase were estimated to be 125 kDa and 90 kDa, respectively. The CMCase and β -glucosidase demonstrated optimum activities at pH 6.0 and 5.0, respectively. Their respective maximum temperatures were 50 and 60 °C. The cellulase activities were stimulated by 10 mM concentration of Ca^{2+} , Ni^{2+} , Fe^{2+} , Mg^{2+} , Cu^{2+} , Mn^{2+} , Zn^{2+} , and Pb^{2+} ions. The CMCase activity was enhanced by surfactant Triton X-100 but marginally influenced by most inhibitors. The β -glucosidase retained its activity in the presence of organic solvents (30%) isoamyl alcohol, heptane, toluene, and ethyl acetate, while CMCase was retained with acetone during a prolonged incubation of 168 h. The K_m and V_{max} values of the two cellulases were studied. The properties of high thermostability and good tolerance against organic solvents could signify its potential use in biofuel production and other value-added products.

Keywords *Aspergillus fumigatus* · Cellulases · Organic solvents · Surfactant · Thermostable

Introduction

Cellulose is a major polysaccharide component of plant cell walls [1]. Cellulosic waste materials are a renewable carbon source used by various microorganisms and promising feedstock to lower the production cost of cellulosic ethanol and other chemical

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sources [2, 3]. Microbial cellulases are involved in hydrolyzing β -1, 4-glycosidic linkages of cellulose molecules, one of the most stable and prevalent bonds in nature [4, 5]. The cellulases are categorized into three types: endoglucanase or carboxymethyl cellulase (E.C. 3.2.1.4), cellobiohydrolase or exoglucanase (E.C.3.2.1.91), and cellobiase or β -glucosidase (E.C.3.2.1.21) [2, 6, 7]. Endoglucanases randomly cleaves β -1,4-glycosidic bonds in the polymeric structure of cellulose, demonstrating reducing and non-reducing ends. Cellobiohydrolases generate cellobiose and other small oligosaccharides units by acting on reducing and non-reducing ends. Finally, β -glucosidases is a rate-limiting enzyme that cleaves cellobiose to release monomeric sugar molecules [6, 8–11]. Thus, β -glucosidase plays a key role in the final step of cellulose hydrolysis to produce glucose for bioethanol production.

Fungi and bacteria are well-known for their good potential to be used in cellulase production. However, fungal cellulases are preferred over bacterial sources because of easy recovery, high activity, wide thermal and pH stability, and versatile substrate utilization [6, 10, 12]. The low cost and readily available solid substrates like forest wastes, different grasses, cotton stalks, bagasse, rice husk, wheat husk, and other crops residues are reported to provide a nourishing environment for filamentous fungi [6, 13, 14].

Cellulase has become the world's third largest group of enzymes used in the industrial sector [15, 16]. Microbial cellulases have demonstrated their broad applications in biotechnological and other manufacturing industries [17, 18]. The rising concerns of producing cellulase are due to their huge demand in different sectors such as cotton processing, cellulosic-based bioethanol production, detergent formulation, textile, laundry, food, feed, leather, juice extraction, paper, and pulp making industries [2, 19–22]. However, several bacteria and actinomycetes have been reported cellulase yield [22–24]. Species of the genera *Trichoderma*, *Penicillium*, and *Aspergillus* are renowned producers of commercial cellulase [4]. The cellulase accounts for about 20% of the world market as a commercial enzyme [6, 14, 16, 25, 26].

Thermostable cellulases play a significant role in the enzymatic hydrolysis of cellulose. Thermostable enzymes can be used directly after the heating step without pre-cooling, which decreases the processing time and improves fermentation yields [27]. The selective production of more efficient thermostable enzymes has become the objective of much research worldwide. The thermostability of cellulases secreted by fungi for industrial applications has been extensively studied in several model organisms, like *Trichoderma reesei* [28], *Aspergillus fumigatus* [29], and *Phanerochaete chrysosporium* [30]. The cellulases from *Aspergillus* species are broadly studied, such as β -glucosidase by *Aspergillus terreus* [31], the β -glucosidase from *Aspergillus niger* [32], a highly glucose-tolerant β -glucosidase from *Aspergillus oryzae* [33], thermostable β -glucosidase from *A. fumigatus* Z5 [34], and thermostable CMCase from *Aspergillus fumigatus* N2 [29]. However, the high titer of active enzyme production that cleaves β -1,4-glycosidic bonds is still challenging and a bottleneck for biomass conversion.

The present investigation was focused on the extraction and purification of extracellular cellulolytic enzymes by ammonium sulfate precipitation followed by dialysis and DEAE-cellulose ion-exchange chromatography. To the best of our knowledge, this is the first study on the biochemical characterization of purified carboxymethyl cellulase and β -glucosidase from *Aspergillus fumigatus* JCM 10253 with the supplement of agro-industrial waste ragi (*Eleusine coracana*) husk in the fermentation under statistically optimized parameters. In this context, the major goal of this work was to purify cellulases and determine their biocatalytic efficiency under different conditions to assess their potential applications in bioprocessing industries.

Materials and Methods

Fungal Strain and Culture Conditions

A cellulolytic *Aspergillus fumigatus* JCM 10253 was isolated from a soil sample from Warangal district, Telangana, India. The fungus was identified by 18S rRNA sequencing [35]. The fungal strain JCM 10253 was selected based on the zone of clearance after Congo red staining demonstrated by the plate assay method [35]. For the shake flask fermentation, the Mandels and Weber [36] production medium was added with 1% (w/v) carboxymethyl cellulose (CMC) as a carbon source. The composed medium was sterilized at 121 °C, 15 psi pressure. A loopful of *Aspergillus fumigatus* JCM 10253 spores was inoculated into a sterilized medium. The above-prepared medium was supplemented with 1.6% (w/v) ragi husk as a substrate. The medium was adjusted to pH 2.0 and incubated at 48.6 °C, 160 rpm for 8.5 days for the production of CMCase. Simultaneously, the second production medium consisted of 1.17% (w/v) ragi husk. The medium was maintained at pH 3.8, 48.8 °C, and agitation at 160 rpm for 7.6 days for the production of β -glucosidase under previously statistically optimized conditions [37].

Purification of Cellulase

The crude culture from *Aspergillus fumigatus* JCM 10253 was subjected to the purification process at 4 °C. The cells and residual medium were separated from the crude culture by centrifugation at 10,000 rpm for 30 min. The supernatant was exposed to ammonium sulfate precipitation (60–90% saturation) for overnight. After precipitation, a pellet was recovered from the supernatant by centrifugation at 10,000 rpm for 20 min. The pellet was resuspended in 10 mL of 10 mM sodium phosphate buffer (pH 7.0) and dialysis tubing with a molecular weight cut off 10 kDa (HiMedia, Mumbai, India) against the same buffer. The dialyzed sample was equilibrated with freshly prepared sodium phosphate buffer and loaded onto the DEAE-cellulose column (2.5 × 20 cm, Bio-Rad Laboratories, USA). The enzyme fraction was allowed to bind with the matrix, and the unbound fraction was collected to estimate the enzyme activity and protein content. The elution of the bound fraction was done by a linear gradient of NaCl (0.1–1.0 M) at a flow rate of 1.0 mL/min. The active fractions were collected separately and dialyzed against the same buffer. The standard assays were used to measure the enzyme activity and protein content in the dialyzed samples.

Enzyme Assays and Protein Quantification

Carboxymethyl cellulase (CMCase) was assayed according to the method described by Ghose [38]. The CMCase activity was determined by reaction mixture containing 0.25 mL of the purified enzyme and 0.25 mL of 2% substrate (CMC dissolved in 50 mM sodium citrate buffer (pH 4.8)) incubated at 50 °C for 30 min. After incubation, 1.5 mL of 3,5-dinitrosalicylic acid (DNS) reagent was added to the reaction mixture and kept in a boiling water bath for 5 min [39]. The reducing sugar was quantified at 540 nm by UV–visible spectrophotometer (Jasco-V-630). β -Glucosidase was assayed according to the method demonstrated by Grover et al. [40] using the *p*-nitrophenyl- β -D-glucopyranoside (*p*NPG) as a substrate [41]. The reaction mixture contains 0.5 mL of sodium acetate buffer (0.1 M; pH 5.0), 0.25 mL of the purified enzyme, and 0.25 mL of 0.02 M *p*NPG incubated at 37 °C

for 15 min. After incubation, 1.0 mL sodium carbonate (0.2 M) was added to the reaction mixture and measured at 400 nm. One unit (U) of enzyme activity is defined as the amount of enzyme releasing 1 μmol of glucose or *p*-nitrophenol per min from their respective substrates under the standard assay conditions. The protein content of each fraction was monitored at 280 nm using UV spectrophotometer after every step of chromatography. The protein was quantified by the Bradford method [42] using the standard as bovine serum albumin (BSA).

Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed according to the method described by Laemmli [43]. The molecular weight was determined using 3 mm slab gel with 12% polyacrylamide separating gels. The gel was prepared by mixing 1.68 mL of MilliQ water, 2.0 mL of acrylamide mixture (30%), 1.25 mL of Tris–HCl (1.5 M, pH 8.8), 50 μL of sodium dodecyl sulfate (SDS) (10%), 25 μL of ammonium persulfate (10%), and 2.5 μL of tetramethylethylenediamine (TEMED). The stacking gel (4%) was prepared of 3.05 mL of MilliQ water, 665 μL of acrylamide (30%), 1.25 μL of Tris–HCl (0.5 M, pH 6.8), 50 μL of SDS (10%), 25 μL of ammonium persulfate (10%), and 5 μL of TEMED. The staining solution was used (0.1% Coomassie Brilliant Blue R-250, 50% methanol, and 10% glacial acetic acid) for 4 h, and destaining (40% methanol and 10% glacial acetic acid) was performed overnight on a gel rocker. The unstained protein markers (10–250 kDa, Thermo Fisher Scientific, USA) were used to determine the molecular weight of the purified enzyme.

Screening of Purified CMCase and β -Glucosidase Activities

CMCase Activity The activity staining was performed by adding 1% (w/v) of CMC and agarose in 25 mL of 10 mM sodium phosphate buffer (pH 7.0) and vigorously mixed while heating and poured onto the sterile Petri plates. Wells were punched into the gel, and 0.05 mL of the purified enzyme was pipetted into wells. Then, plates were incubated at 30 °C for 24 h. After the incubation period, the staining of plates with 1% Congo red solution was performed, a clear zone marked as cellulose degradation.

β -Glucosidase Activity Esculin gel diffusion assay was performed to screen the β -glucosidase activity [44]. Approximately 4% of agar mixed in 0.2 M sodium acetate buffer (pH 5) was autoclaved and incubated at 50 °C to avoid agar solidification. Esculin of 0.2% was mixed in 6 mL of 1% FeCl_3 and incubated at 50 °C in a boiling water bath. These two solutions were mixed gently and poured into sterile Petri dishes. Esculin gel was allowed to solidify, and wells were punched. A total of 18 μL of the purified enzyme was pipetted into wells and incubated at 37 °C for 24 h. Plates were perceived for the appearance of a brown-black zone, demonstrating esculin hydrolysis.

Biochemical Characterization of Purified Cellulase

Effect of Temperature on Enzyme Activity and Stability

The impact of temperature on cellulase activity was studied by incubating purified enzyme at different temperatures (40, 45, 50, 55, 60, 65, and 70 °C) for 30 min. The thermal

stability was measured by incubating purified enzyme at different temperatures (40, 45, 50, 55, 60, 65, and 70 °C) for 1 h. The CMCCase activity was determined by conducting a reaction at pH 6.0 and 50 °C. Similarly, the β -glucosidase activity was measured at pH 5.0 and 60 °C. The relative activity without incubation (unheated) was defined as 100% under the standard assay conditions.

Effect of pH on Enzyme Activity and Stability

The impact of pH on the cellulase activity was determined from 3.0 to 10.0 pH range using suitable buffers at 10 mM concentration (3–5.5 sodium citrate buffer, 4.5–6 sodium acetate buffer, 6–8 sodium phosphate buffer, 8.5–9 Tris–HCl buffer, 9.5–10 glycine–NaOH buffer) under the standard assay conditions. The pH stabilities of CMCCase and β -glucosidase were measured by incubating 100 μ L of the purified enzyme mixed with 100 μ L of the buffer solutions of different pH (3.0–10.0) for 1 h at 50 and 60 °C, respectively. The aliquots of the reaction mixture were used to evaluate the relative activities under the standard assay conditions.

Effect of Metal Ions on Enzyme Activity

The impact of metal ions (Ca^{2+} , Ni^{2+} , Fe^{2+} , Mg^{2+} , Cu^{2+} , Hg^{2+} , Mn^{2+} , K^+ , Na^+ , Zn^{2+} , Ba^{2+} , Co^{2+} , Cd^{2+} , and Pb^{2+}) were determined (at concentrations of 2, 5, and 10 mM of metal chlorides) on the cellulase activity. The incubation of purified CMCCase and β -glucosidase was carried out using different metal ions at 50 and 60 °C for 1 h, respectively, and assayed under standard conditions. The relative activity in the absence of metal ions was defined as 100% under standard assay conditions.

Effect of Inhibitors on Enzyme Activity

The effect of inhibitors (urea, dithiothreitol (DTT), β -mercaptoethanol (v/v), sodium dodecyl sulfate (SDS), sodium sulfite, ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), and sodium azide) on the cellulase activity was studied at 5 and 10 mM concentrations. The incubation of purified CMCCase and β -glucosidase was carried out using the above reagents at 50 and 60 °C for 1 h, respectively. The relative activity without inhibitors was defined as 100% under standard assay conditions.

Effect of Organic Solvents on Enzyme Stability

The purified cellulase was mixed with 30% (v/v) concentration of different organic solvents, viz., 2-propanol, propan-1-ol, isoamyl alcohol, 1-butanol, *n*-pentane, heptane, ethanol, methanol, acetone, xylene, benzene, toluene, ethylene glycol, chloroform, and ethyl acetate, and incubated for 7 days at 120 rpm in the tight screw cap tubes. The incubation of purified CMCCase and β -glucosidase was carried out using 30% organic solvents at 50 and 60 °C, respectively, and assayed under standard conditions. The relative activity without the solvents was defined as 100% under standard assay conditions.

Effect of Surfactants on Enzyme Activity

The impact of surfactants on cellulase activity was determined by incubating a reaction mixture of a purified enzyme with surfactants Triton-X-100, Tween-20, Tween-80, and dimethyl sulfoxide (DMSO) at concentrations of 1% and 5% (v/v). The incubation of purified CMCase and β -glucosidase was carried out using different surfactants at 50 and 60 °C for 1 h, respectively, and assayed under standard conditions. The relative activity without surfactants was defined as 100% under the standard assay conditions.

Determination of Substrate Specificity

The purified cellulase activity was measured against different substrates. The substrates were carboxymethyl cellulose (CMC), *p*-Nitrophenyl- β -D-glucopyranoside (*p*NPG), cellobiose, Avicel, filter paper (strip), and beechwood xylan. The reaction mixtures of 1% (w/v) substrates for CMCase and β -glucosidase were prepared in 10 mM sodium phosphate buffer (pH 6.0) and sodium acetate buffer (pH 5.0) and assayed at 50 and 60 °C under standard assay conditions, respectively.

Kinetic Parameters

To determine the kinetic parameters (K_m and V_{max}), the purified CMCase and β -glucosidase were assayed at 50 and 60 °C using substrates CMC and *p*NPG concentrations ranging from 1.0 to 40 mg/mL in 10 mM sodium phosphate buffer of pH 6.0 and sodium acetate buffer of pH 5.0 under standard assay conditions, respectively. Kinetic parameters were determined by the Michaelis–Menten constant (K_m , mg/mL) and maximum reaction velocity (V_{max} , $\mu\text{mol}/\text{mg}/\text{min}$) through the non-linear regression of the Michaelis–Menten equation.

Results and Discussion

Screening Plates of CMCase and β -Glucosidase Activities

Cellulases are well known for breaking down cellulose polymer into glucose subunits. The plate assays were employed to screen the activities of purified enzymes from *Aspergillus fumigatus* JCM 10253. The purified enzymes were transferred to screening plates to assess CMCase and β -glucosidase activities. After 24 h of incubation, the plates were stained with Congo red dye that produced a clear zone surrounding the inoculated purified CMCase, as depicted in Fig. 1B. The area covered by a clear zone demonstrates the ability of the enzyme to hydrolyze cellulose [45]. The enzyme breaks down the polysaccharide into smaller monosaccharides. Thus, the Congo red dye cannot bind strongly with monosaccharides resulting in a clear zone [46]. In the gel diffusion assay, esculin (6,7-dihydroxycoumarin-6- β -D-glucopyranoside) was used as a substrate

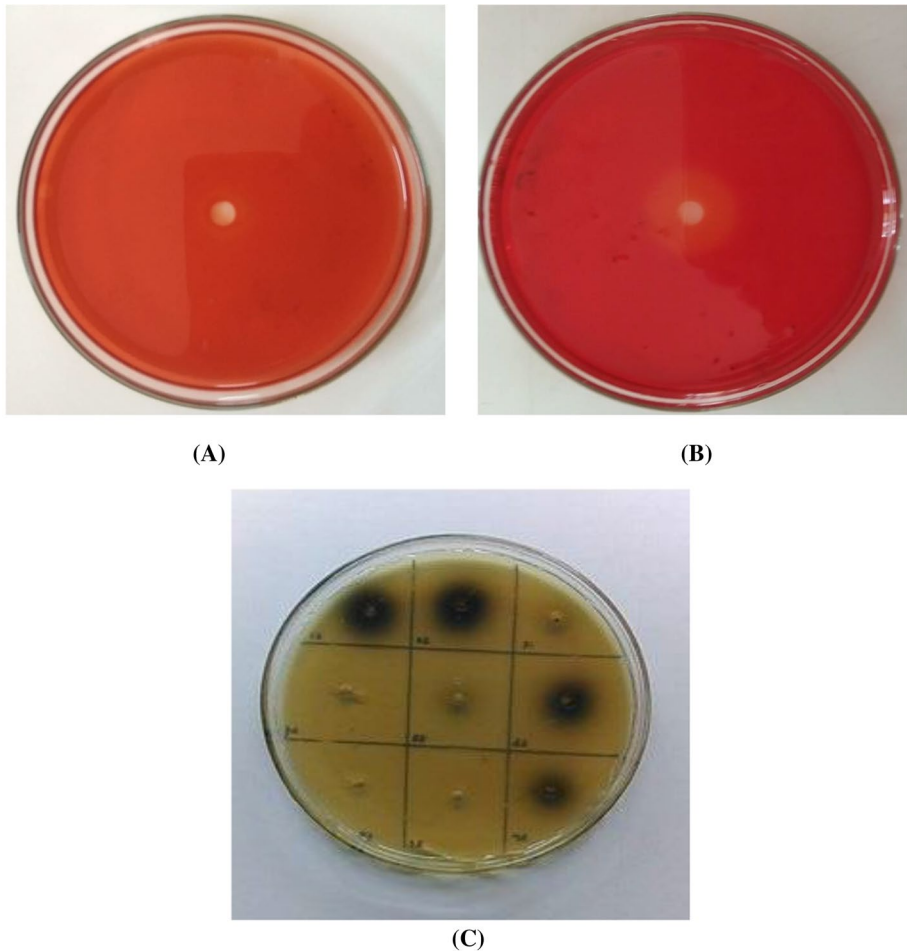


Fig. 1 Screening of purified CMCCase and β -glucosidase activities from *Aspergillus fumigatus* JCM 10253 by plate assays. Plate assay show the zone of clearance after 24 h of Congo red staining by purified CMCCase (B), and plate without enzyme considered as control (A); C Esculin gel diffusion assay of purified β -glucosidase activity showing brown-black zone after 24 h of incubation, and wells without enzyme considered as control (no dark regions)

for β -glucosidase activity, which is expressed by the production of a brown-black zone on esculin gel (Fig. 1C).

Polyacrylamide Gel Electrophoresis for Molecular Weight Determination

The cellulase from *Aspergillus fumigatus* JCM 10253 was purified from crude enzyme extract by fractional ammonium sulfate precipitation, followed by dialysis and DEAE-cellulose ion-exchange chromatography. The results of purification steps are summarized in Tables 1 and 2. The optimum activity was obtained in the fraction by the extension of 80% ammonium sulfate. The active fraction collected from DEAE-cellulose column

Table 1 Purification of CMCase from *Aspergillus fumigatus* JCM 10253

Purification steps	Enzyme activity (U/mL)	Total protein (mg/mL)	Specific activity (U/mg)	Yield (%)	Purification fold
Culture filtrate	94.9	2.4	39.5	100	1
Dialysis (10 kDa cut off)	48.5	1.05	46.2	51.1	1.16
DEAE—cellulose	19.5	0.34	57.3	20.5	1.45

Table 2 Purification of β -glucosidase from *Aspergillus fumigatus* JCM 10253

Purification steps	Enzyme activity (U/mL)	Total protein (mg/mL)	Specific activity (U/mg)	Yield (%)	Purification fold
Culture filtrate	0.18	0.92	0.19	100	1
Dialysis (10 kDa cut off)	0.15	0.5	0.3	83.3	1.5
DEAE—cellulose	0.083	0.16	0.51	46.2	2.73

showed CMCase activity of 19.5 U/mL with a protein content of 0.34 mg/mL. The purified CMCase had a specific activity of 57.3 U/mg of protein, demonstrating a 1.45 fold increase in activity with a yield of 20.5% (Table 1). Besides, the active fraction released from DEAE-cellulose column showed a β -glucosidase activity of 0.083 U/mL with a protein content of 0.16 mg/mL. The purified β -glucosidase exhibited a 2.73 fold increase in activity with a recovery of 46.2%. The specific activity was 0.51 U/mg of protein, as described in Table 2.

The dialyzed sample obtained after the ammonium sulfate precipitation method was employed in the next step of the purification process by applying ion-exchange chromatography. The dialyzed sample (2 mL) was equilibrated with sodium phosphate buffer (10 mM, pH 7.0) before being loaded into the DEAE-cellulose column. The unbound fraction was collected, passing through the column, and simultaneously determined its total protein content and enzyme activity. The no activity in the unbound fraction indicates that total cellulase is bound to a matrix. The bound enzyme with matrix was eluted using sodium phosphate buffer of pH 7 having NaCl with increasing concentration at gradient of 0.1 M. The maximum activities of CMCase and β -glucosidase were detected in the fractions released from 0.2 M and 0.3 M NaCl using DEAE-cellulose column, respectively. The active fractions were pooled together, followed by dialysis to remove excess salt. Thereafter, the molecular masses of purified enzymes were determined. The SDS-PAGE showed that the molecular masses of CMCase and β -glucosidase were approximately 125 and 90 kDa, respectively (Fig. 2). Hence, this demonstrated the purity of elution fractions by a single band on SDS-PAGE. Two β -glucosidases purified from strains T1 and T2 of *Aspergillus tubingensis* showed molecular masses of 111 kDa and 102 kDa, respectively [47]. The molecular weight of purified cellulase from *Aspergillus ochraceus* MTCC 1810 was ~78 kDa described by Asha et al. [48]. Oh et al. [49] reported two β -glucosidases, BGL1 and BGL2, from cellulolytic fungi *Aspergillus* sp. YDJ216 with molecular masses of 97 kDa and 45 kDa, respectively. Narasimha et al. [50] described the molecular mass of β -glucosidase from *Aspergillus niger* as estimated to be 95 kDa. The β -glucosidase from *Penicillium piceum* showed a molecular weight of 92 kDa [51]. The molecular mass

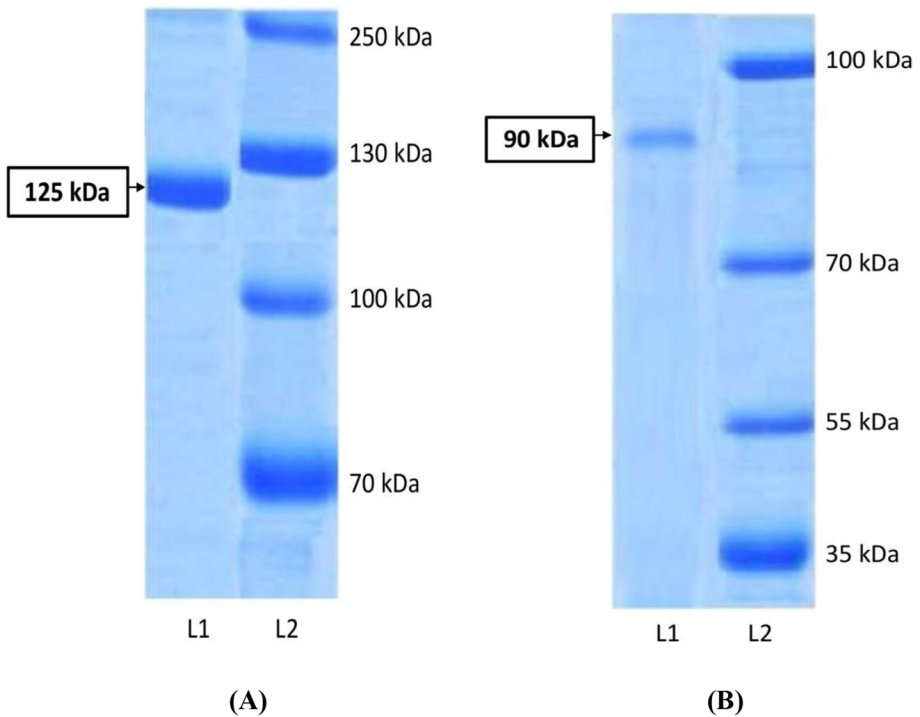


Fig. 2 SDS-PAGE of purified CMCase (A) and β -glucosidase (B), produced from *Aspergillus fumigatus* JCM 10253. L1, the active fraction of purified enzyme after DEAE-cellulose ion-exchange column chromatography; L2, molecular weight protein markers (kDa)

of the purified cellulase from *Rhizopus oryzae* SN5 was determined as ~ 80 kDa [52]. In another study, *Penicillium citrinum* and *Aspergillus clavatus* were demonstrated three isoforms of cellulase with a molecular weight of 130 kDa [53].

Biochemical Characterization of Purified Cellulase

Effect of Temperature on Enzyme Activity and Stability

Temperature is one of the significant factors affecting the microorganism's growth and metabolic activities [54, 55]. As the incubation temperature increases, the collision rate increases along with the reaction rate, as seen in the several chemical reactions. The enzyme stability decreases due to thermal degradation, and holding the enzyme at a high temperature may denature the enzyme. In the present study, the temperature effect on purified enzyme was measured across a wide range from 40 to 70 °C. The optimum CMCase and β -glucosidase activities were obtained at 50 and 60 °C, respectively. Thus, further increasing temperature showed a decline in enzyme activities (Fig. 3a and b). In fungi, cellulase activity reached maximum temperatures, usually between 50 to 60 °C and stable until 50 to 55 °C [56, 57]. The purified cellulase from *Aspergillus terreus* showed optimum activity in the range of 46–62 °C [58]. The purified CMCase from halophilic fungi *Aspergillus flavus* TISTR 3637 exhibited maximal activity at 60 °C [59]. The report

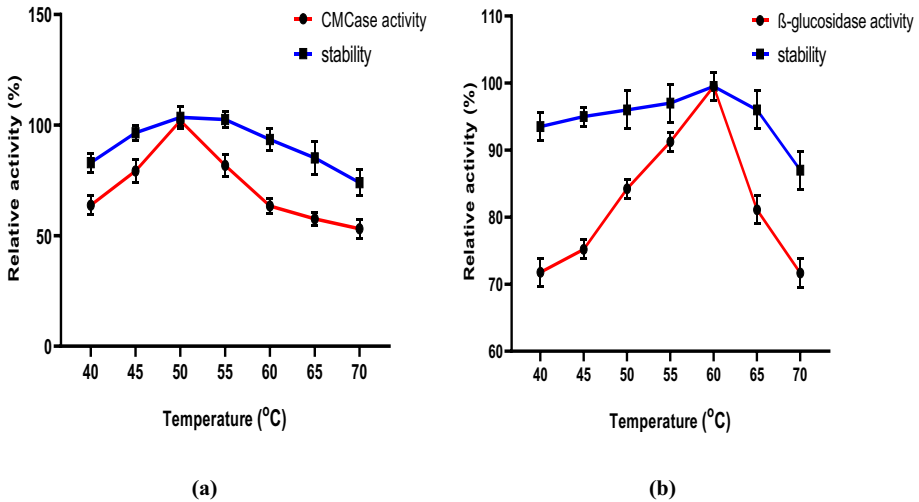


Fig. 3 Effect of temperature on cellulase activity and stability. The optimum temperature was determined by incubating the enzyme at different temperatures (40–70 °C). The stability of the enzyme was measured by pre-incubating at respective temperatures for 1 h and assayed under standard conditions. Results are the mean of triplicate experiments with standard deviation represented by error bars

on purified thermostable CMCase and β -glucosidase from *Penicillium* sp. LMI01 showed optimum activities at 60 °C [60]. In addition, β -glucosidases usually demonstrate maximum temperatures in the range of 40 to 60 °C [61, 62]. The report revealed that the purified β -glucosidases (BGL1 and BGL2) from *Aspergillus* sp. YDJ216 were optimally active at 60 °C [49]. Similarly, purified endo- β -1,4-glucanase and β -glucosidase from *Penicillium chrysogenum* were exhibited optimum activities at 50 and 60 °C, respectively [63].

The thermophilic filamentous fungi are an excellent source of cellulases due to their high thermostability, which is one of the most desirable characteristics for industrial applications [64]. The purified CMCase and β -glucosidase from *Aspergillus fumigatus* JCM 10253 showed stabilities in the temperature range of 40–65 °C. At temperatures 50 and 60 °C, CMCase and β -glucosidase activities retained 100% after 1 h of incubation, respectively. At 65 °C, the CMCase and β -glucosidase were retained 85% and 96% after 1 h, respectively, demonstrating a significant decrease in activities (Fig. 3 a and b). The CMCase from *Penicillium* sp. LMI01 showed stability between 50 and 60 °C, about 95.7% of activity was retained after 1 h [60]. Martins et al. [65] studied β -glucosidase thermostability revealed 100% and 48.6% when exposed at 60 and 65 °C, respectively. The cellulase from *Penicillium pinophilum* MS 20 exhibited stability up to 2 h at 50 °C and retained 100% of the activity [66]. The report described that β -glucosidase from *Fusarium chlamydosporum* HML278 was stable below 70 °C and had 75% of the enzyme activity after 1 h [67]. The enzymes that are stable at 60 °C and above are known to be heat-resistant enzymes. These enzymes play a significant role in the enzymatic saccharification and fermentation of lignocellulosic biomass [68].

Effect of pH on Enzyme Activity and Stability

The pH is a critical factor that affects enzyme activity. The cellulase activity was active over a pH range from 3.0 to 9.0. The optimum activities were obtained at pH 6.0 and 5.0

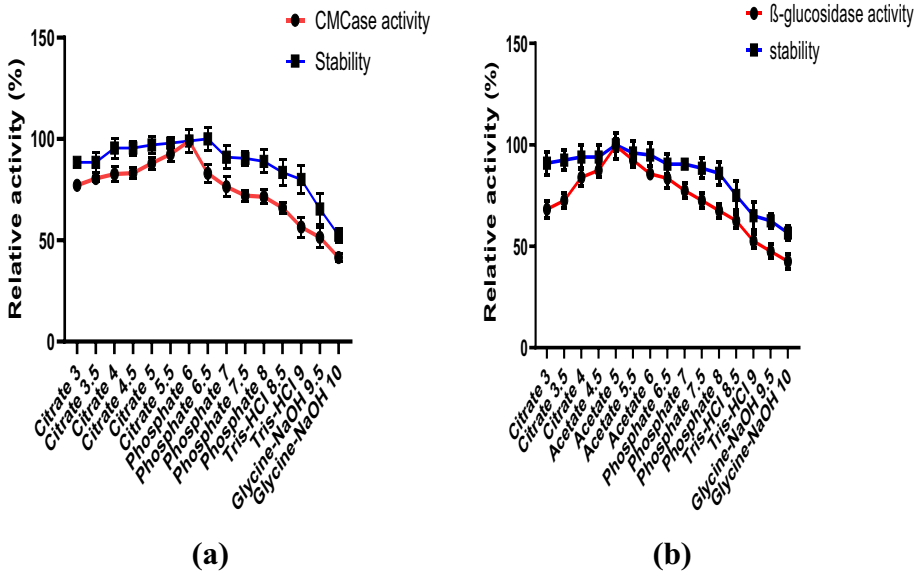


Fig. 4 Effect of pH on cellulase activity and stability. The optimum pH was measured by incubating at different pH (pH 3–10) using various buffers for 30 min at 50 and 60 °C for CMCCase (a) and β -glucosidase (b), respectively. The stability of the enzyme was measured by pre-incubation at respective temperatures for 1 h and assayed under standard conditions. Results are the mean of triplicate experiments with standard deviation represented by error bars

for CMCCase and β -glucosidase, respectively (Fig. 4a and b). The β -glucosidases of different fungal species reported the highest activity in the acidic medium between pH 4.0 and 6.0 [61, 69, 70]. The high levels of these enzymes are an important factor for biotechnological processes.

Microorganisms are susceptible to hydrogen ion concentration present in the fermentation medium. The pH factor is important in inducing microbial morphology and enzyme production [54, 71]. Gupta et al. [72] described that pH is crucial machinery that affects the production of enzymes, and changes in pH influence the medium's stability. The reports on purified β -glucosidases from *Penicillium chrysogenum* and *Penicillium citrinum* UFV1 showed maximal activities at pH 5.0 [63, 64]. Narasimha et al. [50] determined the optimum activity of β -glucosidase from *Aspergillus niger* at pH 5.0. The purified cellulase from *Aspergillus terreus* demonstrated maximal activity from a pH range of 5.0 to 6.0 [58]. In another studies, cellulases from *Penicillium pinophilum* MS 20 and *Aspergillus awamori* showed optimum activities at pH 5.0 [66, 73].

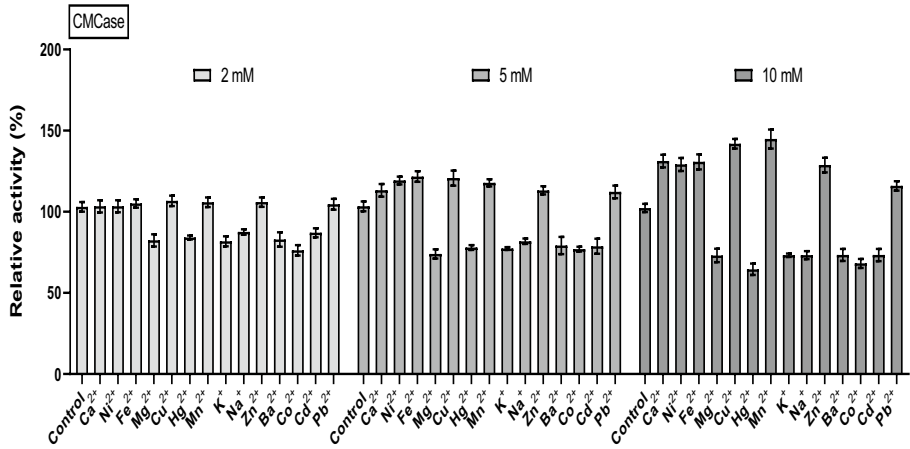
In the present study, the purified CMCCase and β -glucosidase of *A. fumigatus* JCM 10253 were stable over pH 4.0 to 9.0 (Fig. 4a and b). The pH stabilities of CMCCase and β -glucosidase were retained 100% after 1 h of incubation at pH 6.5 and 5.0, respectively. The purified CMCCase and β -glucosidase show more than 90% of the activities within a pH range from 4.0 to 7.0. The report on cellulase from *Trichoderma longibrachiatum* showed stability of pH range from 3 to 6 [46]. The cellulase from *P. pinophilum* MS 20 showed wide pH (4–7) stability and retained 90% of the activity [66]. The β -glucosidase from the thermophilic fungus *Malbranchea pulchella* (MpBg3) showed stability between the pH range of 4.0 to 6.0 [61]. Qin et al. [67] described the β -glucosidase stability of *F. chlamydosporum* HML278 from pH 4.0 to 10.0 and optimum activity at pH 5.0. Da Costa

et al. [64] described the β -glucosidase of *Penicillium citrinum* UFV1 showed stability in a pH range from 5 to 8. The β -glucosidase of *Penicillium verruculosum* determined maximum activity at pH 4.5 and maintained 90% of the activity at pH 4.0–5.0 [74]. Similarly, β -glucosidase (*bgl* T2) of *Aspergillus fresenii* revealed optimum stability in the range of pH 3.0–8.0 [75].

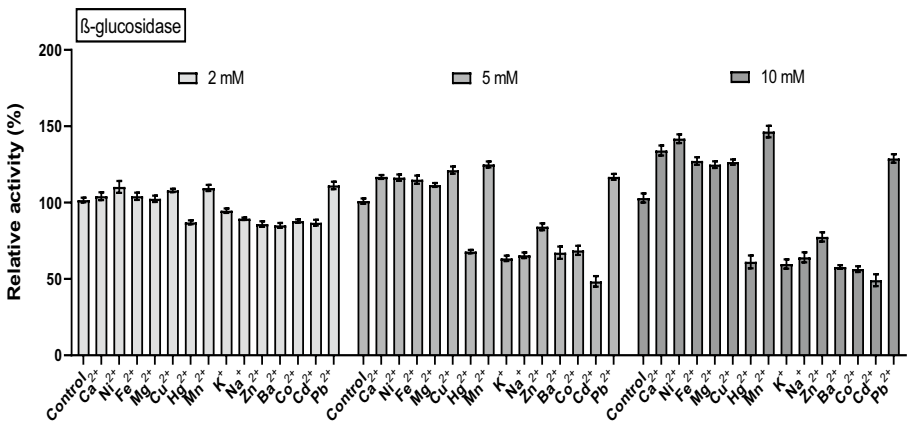
Effect of Metal Ions on Enzyme Activity

Metal ions are known to influence enzyme activity. However, the interference mechanisms are not well understood due to a lack of supporting data to confirm that activation or inhibition occurs via allosteric or non-allosteric mechanisms [76]. In this study, the effects of common metal ions on CMCase and β -glucosidase activities were measured. The metal ions positively modulated the CMCase activity at 10 mM concentration of Ca^{2+} (131.5%), Ni^{2+} (129.3%), Fe^{2+} (130.7%), Cu^{2+} (141.9%), Mn^{2+} (145%), Zn^{2+} (128.6%), and Pb^{2+} (115.9%), as shown in Fig. 5A. However, metal ions such as Mg^{2+} , Hg^{2+} , K^+ , Na^+ , Ba^{2+} , Co^{2+} , and Cd^{2+} inhibited the CMCase activity (negative modulation). On the other hand, metal ions positively modulated the β -glucosidase activity by Ca^{2+} (134%), Ni^{2+} (139.8%), Fe^{2+} (127.8%), Mg^{2+} (124.9%), Cu^{2+} (126.5%), Mn^{2+} (146.5%), and Pb^{2+} (128%) at concentration of 10 mM (Fig. 5B), whereas Hg^{2+} , K^+ , Na^+ , Zn^{2+} , Ba^{2+} , Co^{2+} , and Cd^{2+} showed negative modulation on the β -glucosidase activity. In addition, metal ions like Hg^{2+} , K^+ , Na^+ , Zn^{2+} , Ba^{2+} , Co^{2+} , and Cd^{2+} at concentrations of 2 and 5 mM moderately declined the cellulase activities. The CMCase and β -glucosidase activities were enhanced with Mn^{2+} by 145% and 146.5%, respectively. The result suggests that Mn^{2+} might be acting as a co-factor to enhance cellulase activity.

In several studies, Ca^{2+} has been reported as cellulase activators [63, 77]. Feng et al. [78] showed that the Ca^{2+} enhanced β -glucosidase CfGlu1C activity by 39.4%. Similarly, the Ca^{2+} activated the cellulase from *Aspergillus awamori* by 53.4% [73]. The activation and inhibition of cellulases occur through multiple interactions among ions and enzymes. Metal ions such as Na^+ , K^+ , Co^{2+} , and Hg^{2+} are involved in reducing cellulase activity. In contrast, divalent cations like Ca^{2+} , Fe^{2+} , Zn^{2+} , and Pb^{2+} had enhanced the cellulase activity [63, 66, 73]. The reports on bivalent ions Hg^{2+} and Co^{2+} inhibited enzyme activities. The authors revealed that Hg^{2+} could interact with cysteine residues in sulfhydryl groups, particularly -SH group, which can modify the tertiary structure of the protein [67, 79–81]. Bai et al. [82] described that the β -glucosidase activity from *Penicillium simplicissimum* H-11 was enhanced by Mn^{2+} while inhibited by Co^{2+} . Similarly, the β -glucosidase activity of *Aspergillus niger* was enhanced by Fe^{2+} and Mn^{2+} [83]. Ba^{2+} inhibited two β -glucosidases (BGL1 and BGL2) activities from cellulolytic fungus *Aspergillus* sp. YDJ216 [49]. The report described that the β -glucosidase activity of *Aspergillus niger* was stimulated by Mn^{2+} up to 20% and inhibited by Hg^{2+} [50]. The study demonstrated that the Co^{2+} , Zn^{2+} , and Hg^{2+} inhibited the β -glucosidase activity of *F. chlamyosporum* HML278 and was significantly activated by Mn^{2+} , Ca^{2+} , Mg^{2+} , and Fe^{3+} [67]. Pachauri et al. [46] studied that cellulase from *Trichoderma longibrachiatum* was activated by Ca^{2+} , Mg^{2+} , Zn^{2+} , and Fe^{2+} . Bansal et al. [84] described that the purified cellulase activity of *A. niger* NS-2 was enhanced by Ca^{2+} and Mn^{2+} while inhibited by Hg^{4+} . Similarly, Fe^{2+} and Mn^{2+} ions had enhanced cellulase activity of *Cochliobolus sativus* up to fivefold [85]. Prajapati et al. [86] described that cellulase activity from *Aspergillus tubingensis* NKBP-55 had significantly stimulated through



(A)



(B)

Fig. 5 **A** Effect of metal ions on CMCase activity. The CMCase activity was determined in the presence of different metal ions by pre-incubation with a purified enzyme in the reaction mixture at 50 °C for 1 h and assayed under standard conditions. The enzyme activity without incubation with metal ions was defined as 100%. Results are the mean of triplicate experiments with standard deviation represented by error bars. **B** Effect of metal ions on beta-glucosidase activity. The beta-glucosidase activity was determined in the presence of different metal ions by pre-incubation with a purified enzyme in the reaction mixture at 60 °C for 1 h and assayed under standard conditions. The enzyme activity without incubation with metal ions was defined as 100%. Results are the mean of triplicate experiments with standard deviation represented by error bars

Cu²⁺ and Mn²⁺, whereas Hg²⁺ inhibited. The activating effect in cellulase by Mn²⁺ ions is due to changes in electrostatic bonding, which stabilize the enzymes' tertiary structure [86]. Subsequently, the authors suggest that this activation is due to ions' effect on the enzyme structural conformation or may be due to the reaction between cations and amino acid residues associated with the catalytic site. The enzymes act differently with

these metal ions shows differences in their catalytic domains. Therefore, this kind of interaction may be either positive or negative modulation [76, 80, 87, 88].

Effect of Inhibitors on Enzyme Activity

The impact on cellulase activity by urea, DTT, β -mercaptoethanol, SDS, sodium sulfite, EDTA, PMSF, and sodium azide was taken into account at 5 and 10 mM concentrations. The effect of inhibitors on cellulase activities is summarized in Table 3. The results found no significant inhibitory effect on cellulase activities. Approximately 10–20% of the reduction was determined in the CMCase activity. The minimal reduction of CMCase activity was determined by urea, β -mercaptoethanol, and SDS. The report described that the chemical reagents EDTA, SDS, PMSF, β -mercaptoethanol, DTT, and sodium azide had no significant inhibitory effect on cellulase activity from *T. longibrachiatum* [46]. The cellulase activity of *Aspergillus awamori* exhibited a minor inhibitory effect by PMSF, DTT, and sodium azide, while SDS had a major effect [73]. The report described that the cellulase activity of *Aspergillus niger* was inhibited by EDTA [89]. The inhibitory effect by metal chelating agents makes an inactive complex with inorganic groups of the enzyme [73, 89].

The β -glucosidase showed approximately 35–45% loss of activity. The SDS showed a maximal reduction of β -glucosidase activity to 53.2% at 10 mM and 59.7% at 5 mM. Approximately 13–19% loss of activity was determined by PMSF, a minimal reduction

Table 3 Effect of different inhibitors on cellulase activity

Reagents	Concentration	Relative activity (%)	
		CMCase activity	β -glucosidase activity
Control		100	100
Urea	5 mM	94.5	66.5
	10 mM	91.2	60.2
DTT	5 mM	89.5	66.3
	10 mM	77.3	59.8
β -mercaptoethanol	0.1 (%)	98.8	68.5
	1.0 (%)	92.4	63.2
SDS	5 mM	93.5	59.7
	10 mM	90.9	53.2
Sodium sulfite	5 mM	87.7	71.5
	10 mM	80.4	68.8
EDTA	5 mM	86.7	67.9
	10 mM	79.4	60.3
PMSF	5 mM	84.9	87.4
	10 mM	79.2	81.3
Sodium azide	5 mM	89.6	69.3
	10 mM	84.4	60.8

CMCase and β -glucosidase were pre-incubated with different inhibitors at 50 and 60 °C for 1 h, respectively, and assayed under standard conditions. The enzyme activity incubated without inhibitors was defined as 100%. Results are expressed as mean of triplicates \pm standard deviation for all the values that are $< \pm 5.0\%$

among inhibitors. The β -glucosidase activity was inhibited up to 40% by urea, DTT, EDTA, and sodium azide at 10 mM concentration. The β -glucosidase activity from *Lycoperdon pyriforme* was not affected by EDTA, DTT, and PMSF [90]. In contrast, SDS completely inhibited the activity, which revealed that the integrity of its three-dimensional conformation is crucial for catalytic activity [90]. SDS and DMSO had no significant effect on the β -glucosidase activity from *Aspergillus niger*, while EDTA declined the activity to 27% [83]. The glucose-tolerant β -glucosidase of *Malbranchea pulchella* (MpBg3) showed a lack of effect by β -mercaptoethanol and EDTA [61]. The β -glucosidase from the thermophilic fungus *Myceliophthora thermophila* M.7.7 had no appreciable impact by SDS, EDTA, and DTT when pNPG was used as substrate [80].

Effect of Organic Solvents on Enzyme Stability

The product inhibition of cellulases by various factors is a basic concern that influences the efficiency of the enzyme. The motive of this study was to measure the stability of cellulase in the presence of a wide range of hydrophilic and hydrophobic organic solvents. These studies are important in the enological applications like ethanol-tolerant enzymes for alcohol fermentation. In addition, tolerance to solvents can also be significant for its potential application in solvent-based industries for high value-added products [91, 92]. In the present study, the purified CMCCase from *Aspergillus fumigatus* JCM 10253 was incubated with different organic solvents (30%, v/v) at 50 °C for a week, and obtained values are summarized in Table 4. The results showed that only acetone retained its activity with purified CMCCase throughout 168 h of incubation

Table 4 Stability of CMCCase in the presence of various organic solvents

Organic		Relative activity (%)							
Solvents (30%)	Log P	1 h	24 h	48 h	72 h	96 h	120 h	144 h	168 h
2-Propanol	0.16	74.6	84.7	95.6	91.8	85.2	78.6	70.5	66.4
Propan-1-ol	0.32	42.2	68.5	76.4	69.5	63.9	60.5	59.7	53.5
Isoamyl alcohol	1.28	68.3	77.9	87.9	82.6	78.6	73.7	66.3	57.5
1-Butanol	0.8	66.2	72	85.7	81.2	75.5	68	59.2	51.3
<i>n</i> -pentane	3.2	87.8	90.2	95.4	92.7	87.3	81.3	76.5	72.9
Heptane	4.0	48.3	62.7	73.8	66.9	63.7	58.8	56.9	50.5
Ethanol	−0.24	46.9	77.4	85.7	81.5	77.3	73.8	70.6	68.3
Methanol	−0.76	43.4	48.9	60.5	66.9	75.3	72.6	68.9	62.3
Acetone	−0.2	106	110.4	115.7	119.3	114.2	107.5	104.3	101.8
Xylene	3.1	67.9	72.6	84.5	79.2	67.2	62.5	53.7	49.6
Benzene	2.13	42.6	51.7	67.8	62.5	54.6	50.6	47.3	43.5
Toluene	2.5	48.4	66.9	80.5	78.3	75.4	67.3	60	55.0
Ethylene glycol	−1.6	45.6	63.3	71	77.2	61.8	59.4	54.7	49.4
Chloroform	1.8	68.5	76.3	88.5	81.5	77.5	73.9	71.3	69.5
Ethyl acetate	0.7	39.6	50.9	67.4	74.8	72.6	70.5	66.8	60.2

CMCase was pre-incubated with different organic solvents at a concentration of 30% (v/v) at 50 °C for 168 h and assayed under standard conditions. The enzyme activity incubated without organic solvent was defined as 100%. Results are expressed as mean of triplicates \pm standard deviation for all the values that are $< \pm 5.0\%$

Table 5 Stability of β -glucosidase in the presence of various organic solvents

Organic solvents (30%)	Log <i>P</i>	Relative activity (%)							
		1 h	24 h	48 h	72 h	96 h	120 h	144 h	168 h
2-Propanol	0.16	35.8	46.9	58.7	67	55.9	48.2	43.5	40.7
Propan-1-ol	0.32	50.9	55.7	58.6	69.5	63	60.5	57.9	54.5
Isoamyl alcohol	1.28	120.4	138.2	154.2	187.9	132.6	125.3	113.1	109.2
1-Butanol	0.8	43.8	51.7	66.5	74.8	63.8	60.4	49.3	45.6
<i>n</i> -pentane	3.2	52.3	56	69.8	76.4	71.2	68.5	65.4	64.2
Heptane	4.0	114.6	132.7	145.2	158.3	146.8	138.7	126	117.2
Ethanol	−0.24	54.2	66.5	74.9	86.5	70.2	66.8	58.3	52
Methanol	−0.76	42.3	53.5	66.8	79.2	74.6	63.2	56.5	54.7
Acetone	−0.2	35.5	48.4	56.5	63.3	57.9	52.6	49.3	45.2
Xylene	3.1	45.7	50.2	58.7	69.7	68.5	63.2	52.7	50.5
Benzene	2.13	30.3	36.6	38.4	47.5	45.3	40.5	37.5	35.8
Toluene	2.5	114.7	127	148	164.2	157	142.2	126.3	120.3
Ethylene glycol	−1.6	41.9	58.5	62.5	64.9	53.6	49.5	42.9	40.5
Chloroform	1.8	47.6	56.9	60.9	68.2	66.3	54.9	47.7	43.4
Ethyl acetate	0.7	119.4	130	159.8	182	173.9	131.9	126.6	121.4

β -glucosidase was pre-incubated with different organic solvents at a concentration of 30% (v/v) at 60 °C for 168 h and assayed under standard conditions. The enzyme activity incubated without organic solvent was defined as 100%. Results are expressed as mean of triplicates \pm standard deviation for all the values that are $< \pm 5.0\%$

among various organic solvents. The prolonged incubation of 30% acetone with purified CMCCase exhibited relative activity to 106 (1 h), 110.4 (24 h), 115.7 (48 h), 119.3 (72 h), 114.2 (96 h), 107.5 (120 h), 104.3 (144 h), and 101.8% (168 h). However, CMCCase in the presence of other organic solvents did not retain its activity. Most of the organic solvents with purified CMCCase showed relative activity over 50% after 24 h of incubation.

In the same approach, the purified β -glucosidase was incubated with 30% (v/v) organic solvents at 60 °C for a week, and obtained values are summarized in Table 5. In this study, the purified β -glucosidase in the presence of isoamyl alcohol, heptane, toluene, and ethyl acetate retained the activity throughout 168 h of the incubation period. The purified β -glucosidase with isoamyl alcohol, heptane, toluene, and ethyl acetate demonstrated optimum relative activities increased to 187.9, 158.3, 164.2, and 182% of the activity at 72 h, respectively. While in the presence of other organic solvents, the β -glucosidase was unable to retain its activity. The β -glucosidase relative activities were decreased to 35–50% approximately by 2-propanol, propan-1-ol, 1-butanol, *n*-pentane, ethanol, methanol, acetone, xylene, ethylene glycol, and chloroform after 24 h of incubation. The greater value of log *P*, the partition coefficient, demonstrates that the higher hydrophobic nature of solvents caused higher enzyme activity than that in the presence of hydrophilic solvents [93–95]. The enzyme activity is influenced by the addition of solvent, which interacts with the water molecules around the enzyme. The enzymes lose the catalytic activities in the presence of highly polar solvents (like ethanol, methanol, and 1-propanol) due to various factors such as their high degree of partitioning, hydrophobic interactions, disruption of native H-bonds, and stripping off eminent water layer [96–99]. Besides, the enzymes from thermophiles are more tolerant to organic solvents than mesophilic enzymes [100, 101].

The study of purified cellulase from *Aspergillus tubingensis* NKBP-55 showed inhibition by 5% of solvents [86]. The cellulase activity from *Ganoderma lucidum* was partially or severely inhibited by 1% of acetone, butanol, ethanol, methanol, isopropanol, and toluene [2]. Similarly, the cellulase from *Peniophora* sp. NDVN01 demonstrated low activity at 10 to 20% of methanol, ethanol, and 1-butanol, in which loss of activity is up to 36% [102]. The β -glucosidase (BglH) from *Aspergillus oryzae* was inactivated by 25% ethanol [103]. The activity of β -glucosidase (BG2) from *Trichosporon asahii* had slightly declined by 10% of methanol, ethanol, and isopropanol [104]. The study described that the β -glucosidase activity was reduced when exposed to (30%) methanol and (20%) 2-propanol [105]. Thus, large doses of organic solvents reduced the enzyme activity. This decrease may be due to the denaturing effect of organic solvents at high concentrations [69].

Effect of Surfactants on Enzyme Activity

Surfactants are an essential constituent of detergents industries and can alter the properties of the enzyme. The CMCCase activity was measured using surfactants at concentrations of 1% and 5% (v/v). In this study, Tween-20, Tween-80, and DMSO were retained 102%, 97.8%, and 105.2% of the activity at 1%, respectively (Fig. 6A). The CMCCase activity was enhanced by Tween-20 and DMSO, probably due to increasing substrate solubility or stabilizing enzyme structure [69, 106]. However, a slightly decline in the CMCCase activity by Tween 80 at concentrations of 1% and 5% approximately 2–4% loss of activity was determined. The CMCCase activity was enhanced with Triton X-100 by 180.6% and 206.5% at concentrations of 1% and 5%, respectively. The permeability of the cell membrane could increase by Triton X-100, leading to improve membrane transport and, thus, higher enzyme activity [46]. The stimulating effect of surfactants may result from their action on the cell membrane, causing enhanced permeability by promoting the release of cell-bound enzymes [107, 108]. The previous reports demonstrated that the cellulase activities from

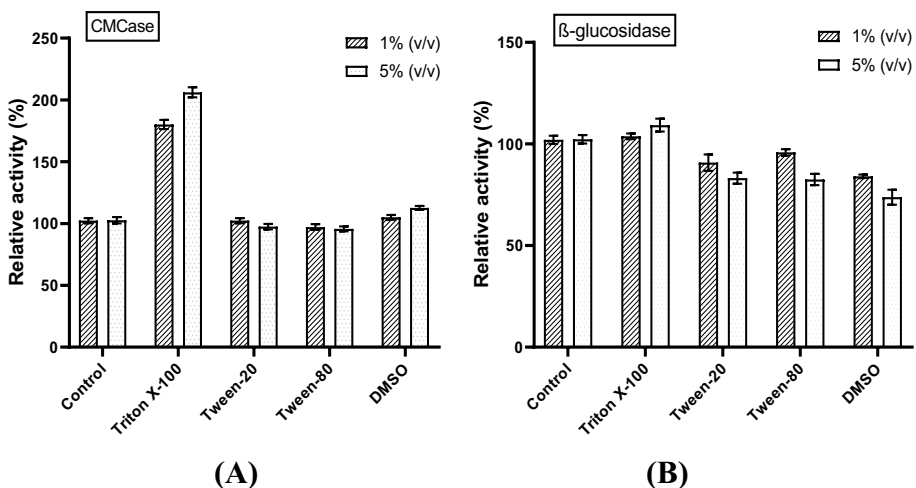


Fig. 6 Effect of surfactants on cellulase activity. The purified CMCCase (A) and β -glucosidase (B) were pre-incubated with different surfactants at 50 and 60 °C for 1 h, respectively, and assayed under standard conditions. The enzyme activity without incubation with surfactants was defined as 100%. Results are the mean of triplicate experiments with standard deviation represented by error bars

Trichoderma reesei Rut C30 and *T. longibrachiatum* were enhanced by Triton X-100 [46, 109]. The report also described that the CMCase activity was improved by Triton-X-100 [108].

On the other hand, Tween-20 (90.4%), Tween-80 (95.5%), and DMSO (84.2%) slightly declined the β -glucosidase activity, whereas Triton X-100 retained 103.5% at a concentration of 1%. The β -glucosidase activity was inhibited by Tween-20 (83.5%), Tween-80 (82.5%), and DMSO (73.8%), while Triton X-100 retained 109% of the activity at a concentration of 5% (Fig. 6B). As a result, surfactants have no remarkable impact on the β -glucosidase activity [110]. The β -glucosidase of *Myceliophthora thermophila* M.7.7 showed a loss of activity by 7% with DMSO [80]. The previous report described that the β -glucosidase from *Chalara paradoxa* CH32 was inhibited by Tween 80 and Triton X-100 [111]. Wang et al. [104] described that two purified β -glucosidases from *Trichosporon asahii*, named BG1 and BG2, exhibited less susceptibility by Triton X-100. The β -glucosidase BGL2 from *Aspergillus* sp.YDJ216 was inhibited by 1% Triton X-100, while β -glucosidase BGL1 had no significant effect [49]. Similarly, β -glucosidase from *Aspergillus oryzae* was inactivated by DMSO [103]. The previous studies revealed that Tween 20, Tween 80, and Triton-X 100 had no significant effects on cellulases from *Ganoderma lucidum* and *Aspergillus tubingensis* NKBP-55 [2, 86]. In the hydrolysis process, the non-ionic surfactants have the ability to alter the enzyme's surface property and assist in reducing the irreversible inactivation of cellulase or compensating the negative effects during larger conversions which are greater advantages to the paper industry [112, 113].

Substrate Specificity

The substrate specificity of the purified cellulases showed broad substrate cleaving activities in Table 6. The purified CMCase exhibited high activity towards CMC (100%), followed by Avicel. The hydrolyzing activity on *p*NPG, cellobiose, and filter paper demonstrated a low correlation with purified CMCase. In addition, CMCase exhibited the least hydrolyzing activity with the beechwood xylan substrate. The β -glucosidase showed utmost activity against *p*NPG (100%) and hydrolyzed dimeric compound cellobiose up to 92.5% despite the main substrate. However, β -glucosidase was not reactive towards substrates

Table 6 Substrate specificity of purified cellulases against different substrates

Substrates	Relative activity (%)	
	CMCase	β -glucosidase
CMC	100	-
<i>p</i> NPG	10.6	100
Cellobiose	13.8	92.5
Avicel	65.2	-
Filter paper	15.34	-
Beechwood xylan	7.69	-

(-) Not detected

The CMCase and β -glucosidase activities on different substrates were measured at 50 and 60 °C under standard assay conditions. Relative activities were considered 100% with CMC and *p*NPG as substrates, respectively. Results are expressed as mean of triplicates \pm standard deviation for all the values that are $< \pm 5.0\%$

CMC, filter paper, Avicel, and beechwood xylan [54, 114]. This finding agreed with previously reported purified endoglucanase (AS-HT-Celuz A) and β -glucosidase (AS-HT-Celuz B) from *Aspergillus ochraceus* MTCC 1810 [48].

Kinetic Studies

Kinetic parameters of purified CMCase and β -glucosidase from *A. fumigatus* JCM 10253 revealed K_m values of 10.79 and 32.59 mg/mL, respectively. The V_{max} values for CMCase and β -glucosidase were 128.7 and 9.36 $\mu\text{mol}/\text{mg}/\text{min}$, respectively. The previous reports demonstrated the K_m values of cellulases from *Aspergillus niger* (0.23 mg/mL towards CMC) [54] and *Thermoascus aurantiacus* RBB-1 (37 mg/mL towards CMC) [115]. The β -glucosidase from *A. fumigatus* JCM 10253 showed lower affinity towards pNPG in comparison with K_m values of β -glucosidases from *Aspergillus flavus* (0.124 mg/mL towards pNPG) [116] and *Fusarium chlamydosporum* HML278 (2.76 mg/mL towards pNPG) [67]. When the K_m value is lesser, it has been suggested that the enzyme has a stronger affinity with the substrate [73]. Therefore, the substrate is a significant factor that affects enzyme activity. Enzymes are complex machines that work with a wide range of chemical mechanisms [117]. In an enzyme-catalyzed reaction, enzyme activity increases as the substrate expand [117]. Although at the same time a continuous increase in substrate concentration will have minor or no effect on the enzyme activity, at that time, the enzyme is said to be saturated with its substrate [54, 117]. Moreover, the lower K_m of the substrate is a significant factor for the industrial saccharification process because it can reduce the end-product inhibition in the enzymatic system [118, 119].

Conclusion

The present study was carried out for the biochemical characterization of purified CMCase and β -glucosidase from *Aspergillus fumigatus* JCM 10253. The production rate and cost reduction are essential factors in converting cellulose into glucose. The synergistic action of β -glucosidases with multiple cellulase enzymes could efficiently enhance the biomass transformation process. The CMCase and β -glucosidase exhibited optimum activities at 50 and 60 °C, respectively, and stabilities under elevated temperatures indicated the enzymes' thermo-tolerant nature. Furthermore, the purified cellulases showed significant tolerance against various metal ions, denaturing agents, and organic solvents. The present findings demonstrated by the cellulolytic enzymes make ideal candidates for cellulose hydrolysis, which is a crucial step for bioethanol production and other value-added products. Future studies may be designed to assess their potential in the saccharification of lignocellulosic biomass.

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Author Contribution Paramjeet Saroj performed the research experiments, data analysis, and writing of the manuscript. Manasa P contributed to the data collection and manuscript writing. Korrapati Narasimhulu supervised during research experiments and manuscript preparation. All authors contributed to the study conception and design. All authors read and approved the final manuscript.

Availability of Data and Materials Not applicable.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Competing Interests The authors declare no competing interests.

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