

Unveiling GH7 cellulases from *Malbranchea cinnamomea* for enhanced saccharification of biorefinery relevant pretreated lignocellulosic biomass

Neha Basotra

Shaheed Rajguru College of Applied Sciences for Women, Delhi University

Mohit Bibra

Prairie Aqua Tech LLC, Brookings,

Gaurav Sharma

DAV University

Vivek Kumar Gaur

Amity University,

Adrian Tsang

Center for Structural and Functional Genomics, Concordia University, 7141 Sherbrooke Street, West, Montreal, Quebec H4B 1R6,

Bhupinder Singh Chadha

Guru Nanak Dev University,

Yashika Raheja

dr.yashikaraheja@gmail.com

Guru Gobind Singh Indraprastha University

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Abstract

Malbranchea cinnamomea harbors a large yet under-explored cellulolytic repertoire. Two GH7 core enzymes, endo- β -1,4-glucanase EG7A_MALCI and cellobiohydrolase CBH7A_MALCI were heterologously expressed in *Pichia pastoris* and purified as ~ 48 kDa and ~ 51 kDa proteins, respectively. EG7A_MALCI displayed maximal activity at pH 7.0 and 60°C, hydrolysing barley β -glucan most efficiently ($K_m = 2.27 \text{ mg ml}^{-1}$, $V_{max} = 81 \text{ U mg}^{-1}$) while retaining appreciable activity on carboxymethyl-cellulose, lichenan and phosphoric-acid-swollen cellulose. CBH7A_MALCI operated optimally at pH 7.0 and 40°C, showed highest turnover on p-nitrophenyl- β -lactoside ($K_m = 0.284 \text{ mM}$, $V_{max} = 156 \text{ U mg}^{-1}$), and exhibited moderate activity towards crystalline substrates, consistent with its processive mode of action. Homology models confirmed conserved GH7 catalytic triads and revealed endo- versus exo-orientated substrate tunnels. Replacing 10–20% of a commercial cellulase cocktail (Celluclast 1.5 L + β -glucosidase) with either recombinant enzyme enhanced glucose release from acid- or alkali-pre-treated rice straw and bagasse by up to 1.5-fold. Cocktail prepared by replacing two parts of commercial CellicCTec2 with either EG or CBH enhanced the hydrolytic potential resulting in 1.30, 1.50, 1.35 and 1.31 folds increase in release of sugars after saccharification of acid and alkali treated rice straw and bagasse, respectively, using EG7A_MALCI. These results provide the first functional description of *M. cinnamomea* GH7 cellulases and introduce robust, thermostable biocatalysts for designing high-performance enzyme blends in lignocellulose biorefineries.

1 Introduction

Efficient enzymatic deconstruction of lignocellulosic biomass is fundamental to the economic viability of second-generation (2 G) bioethanol. Current industrial processes still require enzyme loadings of roughly 20 mg g⁻¹ pre-treated solids because lignin, residual hemicellulose, and crystalline cellulose restrict enzyme access and lower catalytic turnover. Complete hydrolysis relies on the synergy of endo- β -1,4-glucanases (EGs), processive cellobiohydrolases (CBH I/II) and β -glucosidases, assisted by hemicellulases that remove xylan coatings and oxidative lytic polysaccharide mono-oxygenases (LPMOs) that loosen recalcitrant fibers. Commercial cocktails derived from *Trichoderma reesei* or *Aspergillus niger* deliver large quantities of GH7 CBH I, yet they remain limited in β -glucosidase activity, oxidative partners and thermostability prompting continued bioprospecting for thermophilic enzyme sources that can outperform existing formulations under industrial conditions [1].

The thermophilic ascomycete *Malbranchea cinnamomea* represents a compelling alternative. Its 35 Mb genome encodes 301 carbohydrate-active enzymes (CAZymes) (137 GHs, 62 GTs, 24 CEs, 42 AAs, 32 CBMs, 4 PLs) with single CBM1 family domain fused with GH10 xylanase reflecting its reducing affinity towards crystalline cellulose. However, the genomic analysis unveils the diverse suite of hemicellulolytic (GH10 and GH11) and auxiliary activity enzymes (AA7, AA9 and AA11 enzymes) highlighting its specific nature in lignocellulosic biomass hydrolysis in comparison to other filamentous fungi reported [2]. Biochemically, several *M. cinnamomea* enzymes are exceptionally robust such as GH11 xylanase rXYN11A_MALCI retains activity at 70°C and show broad substrate specificity towards substituted and unsubstituted xylan and PMO9A_MALCI is the only documented LPMOs showing dual catalytic behavior towards xylan as well as cellulose [3, 4]. Further, multi-omics data also affirms that temporal proteome profile of *M. cinnamomea* on wheat-straw based culture medium revealed the enhanced secretion of GH11 xylanases followed by GH7 endoglucanase as predominant enzymes. Previous study on supplementing a commercial Celluclast 1.5 L blend with the crude *M. cinnamomea* secretome showed the release of increased glucose from alkali-pre-treated wheat straw from 54 % to 71 underscoring the strain's industrial potential in biomass hydrolysis [5]. Despite extensive work on its hemicellulases and auxiliary enzymes, the core cellulases of *M. cinnamomea* have not yet been characterized. To fill this research gap, two GH7 enzymes from *M. cinnamomea* i.e cellobiohydrolase and endoglucanase were heterologously expressed in *Pichia pastoris*. Their molecular characteristics, pH and temperature optima, thermostability, substrate range, and kinetic parameters were defined, and their performance was assessed in enzyme cocktails for saccharifying acid and alkali-pre-treated rice straw and bagasse. This first functional characterisation of *M. cinnamomea* GH7 cellulases expands the pool of thermostable biocatalysts available for cost-effective lignocellulose biorefineries and designing tailor made cellulase enzyme cocktail.

2 Experimental procedures

2.1 Strain, plasmid and medium

M. cinnamomea, thermophilic fungus isolated from composting soil[6] was used as a source of endo- β -1, 4-glucanase and probable 1, 4- β -D-glucan cellobiohydrolase genes designated as EG7A_MALCI and CBH7A_MALCI, respectively. Both genes belong to glycosyl hydrolase family 7 (GH7). *Escherichia coli* DH5 α was used as a host for sub-cloning. Plasmid pPICZ α A and *P. pastoris* X-33 were used as vector and expression host, respectively. To induce the production of cellulase, *M. cinnamomea* was grown in cellulase inducing CWR medium containing cellulose, wheat bran and rice straw in 3:1:1 combination, respectively, as carbon source[7] at 40 °C on a rotary shaker using 180 rpm. *E. coli* cultures were grown at 37°C in low-salt Luria–Bertani medium (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl) or plated on LSLB agar supplemented with 25 μ g/mL zeocin. *P. pastoris* was maintained at 30°C in YPD agar (1 % yeast extract, 2 % peptone, 2 % dextrose, 2 % agar), propagated in BMGY (1 % yeast extract, 2 % peptone, 100 mM potassium phosphate pH 6.0, 1.34 % yeast nitrogen base, 0.00004 % biotin, 1 % glycerol), and induced in BMMY (same composition as BMGY but with 1 % methanol in place of glycerol).

2.2 Construction of recombinant expression vector

After 48 h of incubation in CWR medium, *M. cinnamomea* mycelium was collected, washed and frozen in liquid nitrogen. Total RNA was isolated from finely ground (100 mg) mycelium using TRIzol (Ambion). Full length cDNA was synthesized by reverse transcription (RT) PCR using mRNA template extracted from total RNA by employing maxi mRNA isolation kit (Invitrogen, USA) [3]. Based on genome sequence of *M. cinnamomea* (Prof. Tsang, Concordia University, Montreal, Canada) available at <http://www.fungalgenomics.ca>, gene specific primers (forward and reverse) were designed for both EG7A_MALCI and CBH7A_MALCI corresponding to gene model ID Malci1p7_008884 and Malci1p7_003444, respectively. The PCR conditions used for amplification were as follows: initial denaturation at 95 °C for 5 min followed by 34 cycles of denaturation at 95 °C for 45 sec, annealing at 55 °C for 45 sec, extension at 72 °C for

1 min and 30 sec and final extension at 72 °C for 10 min. Size of PCR product was confirmed through agarose gel electrophoresis (1% w/v agarose) and desired amplicon was eluted from gel using Gene clean®Turbokit (MP). The amplified inserts were each digested with their corresponding restriction enzymes and then ligated into similarly digested pPICZαA vector to generate pPICZαA-EG7A_MALCI and pPICZαA-CBH7A_MALCI. In these constructs, the target gene is fused to the α-factor secretion signal and driven by the AOX1 promoter. Each plasmid was linearized with PmeI and introduced into *P. pastoris* X-33 competent cells by electroporation (Invitrogen, Carlsbad, CA, USA) [4]. Transformed cells were plated on YPDS agar (1 % yeast extract, 2 % peptone, 2 % dextrose, 1 M sorbitol) supplemented with 100 µg/mL zeocin for selection.

2.3 Expression and purification of recombinant enzymes

Colonies of *Pichia pastoris* X-33 carrying the recombinant plasmids were inoculated into 25 mL of BMGY medium (buffered minimal glycerol complex) and cultivated at 250 rpm for 48 h. Cells were harvested by centrifugation (10,000 × g, 10 min, 4°C), resuspended in 50 mL of BMMY medium (buffered minimal methanol), and incubated for 96 h with 1% (v/v) methanol added every 24 h to sustain induction. After induction, cells were pelleted again (10,000 × g, 10 min, 4°C), and the culture supernatant was collected as crude enzyme extract for subsequent EG7A_MALCI and CBH7A_MALCI activity assays.

The transformants exhibiting the highest EG7A_MALCI and CBH7A_MALCI activity were selected for bulk production of the enzyme and purification. The crude enzyme was concentrated and desalted using a stirred-cell ultrafiltration device equipped with a 10 kDa PES membrane (Permionics, India). For the purification of EG7A_MALCI, the concentrated crude enzyme was equilibrated with 10 ml of 50 mM phosphate buffer (pH 8.0) containing 20 mM imidazole and 300 mM NaCl and the protein sample was loaded onto 1 ml immobilized nickel-NTA ion affinity chromatography column (Qiagen). The bound EG7A_MALCI was eluted with 50 mM phosphate buffer (pH 8.0) containing 250 mM imidazole and 300 mM NaCl at a flow rate of 0.5 mL min⁻¹. Whereas, concentrated and desalted extract of recombinant CBH7A_MALCI was subjected to anion exchange chromatography using UNOsphere™ Q column (Bio-Rad). Equilibration of the crude enzyme was carried out with buffer A (Tris-HCl; 25 mM; pH 8.0). Linear salt gradient from 100% buffer A to 50% buffer B (buffer A with 1M NaCl) at a flow rate of 1 mL min⁻¹ was applied to elute the bound protein using AKTA prime fast protein liquid chromatography system (GE Healthcare, USA). The purity and molecular mass of recombinant protein was checked by 12% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) [8].

2.4 Enzyme and protein assay

EG7A_MALCI activity was assayed by quantifying reducing sugars released from barley β-glucan. In a 1 mL reaction, 250 µL of suitably diluted enzyme and 250 µL of 1.0% (w/v) barley β-glucan (in 50 mM sodium citrate buffer, pH 6.0) were combined and incubated at 50°C for 10 minutes. The reaction was stopped by adding 3 mL of DNS reagent, followed by boiling for 10 minutes. After cooling to room temperature, absorbance was read at 540 nm. One unit of endo-β-1,4-glucanase activity is defined as the amount of enzyme that liberates 1 µmol of glucose equivalent per minute under these conditions [9]. For screening of colonies expressing CBH7A_MALCI gene, a micro-titre plate-based method was used. The reaction mixture (100 µl) was prepared containing 50 µl sodium acetate buffer (50 mM; pH 5.0), 25 µl diluted enzyme and 25 µl of 3mM pNP-β-cellobioside (pNPC) as substrate (Sigma Chemicals) for the assay of cellobiohydrolase (CBHI). The reaction mixture was incubated at 50°C for 30 min. The reaction was stopped thereafter, by adding 100 µl NaOH-glycine buffer (0.4M, pH 10.8). The amount of released *para* nitro-phenol (pNP) was determined by measuring the intensity of developed yellow colour at 405 nm using a plate reader (Biorad 580XR) [10]. The enzyme activity was defined as the amount of enzyme required to release 1 µmol of the pNP under the assay conditions. The protein content in the crude extract and in the chromatographic fractions was determined using Lowry's method [11].

2.5 Biochemical characterization of recombinant proteins

Recombinant EG7A_MALCI and CBH7A_MALCI were characterized using barley β-glucan and pNPC, respectively. To determine their optimal pH, EG7A_MALCI was assayed at 50°C for 10 min and CBH7A_MALCI for 30 min in 50 mM buffers: sodium acetate (pH 3.0–5.0), sodium citrate (pH 6.0), sodium phosphate (pH 7.0–8.0), Tris-HCl (pH 9.0), and glycine–NaOH (pH 10.0). Activity at pH 6.0 (EG7A_MALCI) and pH 5.0 (CBH7A_MALCI) was set as 100%. Temperature optima were measured in 50 mM sodium phosphate (pH 7.0) over 30–80°C. Thermal stability was evaluated by pre-incubating EG7A_MALCI at 60°C and CBH7A_MALCI at 50°C (both in their respective optimal pH) for various times, followed by residual activity assays. To assess the influence of metal ions and inhibitors, each enzyme was incubated at room temperature for 30 min in 50 mM sodium phosphate (pH 7.0) containing 5 mM of NaCl, KCl, CuCl₂, HgCl₂, MnCl₂, MgCl₂, CaCl₂, CoCl₂, FeCl₃, or ZnCl₂, and 1 mM of EDTA, β-mercaptoethanol, N-bromosuccinimide (NBS), dithiothreitol, PMSF, or 1% (w/v) SDS. Activity without additives was defined as 100% relative activity.

2.5.1 Substrate specificity and kinetic parameters

The substrate specificity of recombinant enzymes was assessed using the following substrates: carboxyl methyl cellulose (CMC @ 2% w/v), avicel, lichenin, laminarin, xyloglucan, birchwoodxylan, debranched arabinan (1.0% w/v), 3mM *para*-nitrophenyl (pNP), 3mM pNP β-D-lactopyranoside (pNPL), 3mM pNP β-cellobioside (pNPC), 3mM pNPβ-xylopyranoside (pNPX), 3mM pNPα-L-arabinofuranoside (pNPA), 3mM pNP α-D-galactopyranoside (pNPGL) [12]. Evaluation of kinetic parameters (K_m and V_{max}) were carried out using 50 mM sodium phosphate buffer (pH 7.0) containing 0.5 – 2.0 mg/ml barley β-glucan at 50°C for 10 min (in case of EG7A_MALCI) and 0.3-3.0 mM pNPL at 50°C for 30 min (in case of CBH7A_MALCI). All assays were carried out in triplicate.

2.6 Homology modelling and molecular docking

The amino acid sequence of EG7A_MALCI and CBH7A_MALCI were compared to other homologues via multiple sequence alignment and the conserved domain analysis, performed using EMBI's Clustal Omega tool and NCBI's database, respectively [13, 14]. The initial structures for EG7A_MALCI and CBH7A_MALCI were modelled using Iterative Threading ASSEmbly Refinement (I-TASSER) webserver [15, 16] and visualized using PyMOL (<https://pymol.org/2/>). The endoglucanase from *Fusarium oxysporum* (1ovwa) and cellobiohydrolase from *Dictyostyleum purpureum* 4zzpA, respectively, were aligned to the structure of enzymes under study and root mean square deviation (RMSD) values were calculated. For EG7A_MALCI and CBH7A_MALCI, the ligand prediction and molecular docking of proteins and the ligands was done using AutoDock Vina to compute binding energy for the ligand [13, 17]. The

proteins were considered as the rigid body with minimum force field energy and the ligands as the flexible entities with ability to rotate around torsional angles. Several docking models were studied with the most probable ligand to determine the best configuration and the one with least binding energy were selected for further studies.

2.7 Designing of cocktails using recombinant enzymes and commercial CelliCTec2 for improved hydrolysis of lignocellulosics.

To evaluate the synergy between EG7A_MALCI and CBH7A_MALCI with the commercial cellulase Cellic CTec2 (Novozymes), saccharification assays were performed on four types of pretreated biomass acid and alkali treated rice straw (AcRS, AIRS) and bagasse (AcBG, AIBG). Each 5 mL capped glass vial contained 7% (w/v) substrate in 900 μ L of 50 mM sodium acetate buffer (pH 5.0). As a control, 100 μ L of appropriately diluted Cellic CTec2 (providing 6.6 mg protein per gram of substrate) was added. To assess synergy, either 10 μ L or 20 μ L of this Cellic CTec2 solution was substituted with the corresponding volume of recombinant enzyme preparation (at $\sim$ 5.8 mg protein/mL). Samples were incubated at 50°C, 150 rpm for 96 h. After incubation, 1 mL of each reaction was diluted fivefold with deionized water and centrifuged at 10,000 rpm for 5 min. Total reducing sugars and released glucose in the supernatants were quantified by the DNS method and the GOD-POD assay [4], respectively. All experiments were carried out in triplicate.

3 Results and Discussion

3.1 Heterologous expression and purification of EG7A_MALCI and CBH7A_MALCI

The open reading frame (ORF) of EG7A_MALCI and CBH7A_MALCI was observed to be 1,251 and 1,377 bp in size, respectively, encoding proteins of 416 and 458 amino acid residues (Fig.S1). The recombinant clone EG7A_MALCI exhibited the highest activity (62.61 U/mL) against β -barley glucan. This elevated β -glucanase activity is commercially important, as it enhances wort filtration during lautering and facilitates efficient separation of the liquid wort from spent grain in beer production. Further, it was observed that CBH7A_MALCI displayed greater affinity towards pNPL (1.732 U/ml) followed by pNPC (0.34 U/ml) (Fig.S2) indicating broader exo-acting specificity rather than strict preference for cellobiose-like chain ends. The estimated recombinant cellobiohydrolase activities were quite higher than the other reported in commercial fungi [18–21]

Both the expressed proteins were further purified and was examined on SDS-PAGE (Fig. 1). The molecular weight of purified EG7A_MALCI and CBH7A_MALCI was estimated to be $\sim$ 48 and 50 kDa, respectively, which appeared to be higher than the predicted value. The theoretical molecular mass and isoelectric point for EG7A_MALCI and CBH7A_MALCI were predicted to be 43.7 kDa & 5.02, and 47.8 kDa & 4.12, respectively, using the ProtParam tool of ExPASy. The difference in molecular weight can be attributed to the presence of a c-myc epitope and a polyhistidine tag, contributing 2.8 kDa to the recombinant enzyme size. Additionally, the nominal mass discrepancy observed between EG7A_MALCI and CBH7A_MALCI may be due to Asn-Xaa-Ser/Thr sequons, which are essential for N-glycosylation. Three N-glycosylation sites were predicted in EG7A_MALCI, while one of these sites (Asn-Pro-Ser) is unlikely to undergo glycosylation due to the presence of a Pro residue that typically prevents N-linked glycosylation. In CBH7A_MALCI, only two N-glycosylation sites were predicted [22]. Both enzymes also showed potential O-glycosylation sites in the flexible linker region rich in Ser and Thr residues, common in yeast expression systems. One potential O-glycosylation site (Thr) was identified in EG7A_MALCI, while CBH7A_MALCI contained three potential sites (two Ser and one Thr) [23]. Sequence analysis revealed that both enzymes feature a hydrophobic signal sequence, a GH7 catalytic domain, a Thr/Ser-rich linker region which are typical of other endoglucanases and cellobiohydrolases, such as MtEG7a from *Myceliophthora thermophila* [22], Cel7B from *Penicillium decumbens* [24], StCel61a from *Sporotrichum thermophile* [25], and Man5XZ3 from *Aspergillus nidulans* [26].

3.2 Effect of pH and temperature

The effect of pH on the enzyme activity of purified EG7A_MALCI and CBH7A_MALCI was evaluated using β -barley glucan and pNPL as substrates, respectively (Fig. 2a & 2b). Both enzymes demonstrated optimal activity at pH 7.0. EG7A_MALCI showed significant activity at pH 8.0, with 136% of its activity at pH 7.0, while it exhibited 83.1% and 96.6% of activity at pH 4.0 and pH 5.0, respectively, relative to the control at pH 6.0. This is consistent with the general trend observed for fungal endo- β -1,4-glucanases and cellobiohydrolases from the GH7 family, which typically show optimal activity in the acidic pH range of 4.0–6.0 [27, 28]. However, MtEG7a from *M. thermophila* ATCC 42464 [22] and some bacterial endo- β -1,4-glucanases are optimally active at pH > 8.0. Notably, recombinant EG7A_MALCI and CBH7A_MALCI from *M. cinnamomea* exhibited maximum activity at pH 7.0 and remained stable over a broad pH range (pH 4.0–8.0). This stability is an advantageous feature for industrial applications, as many enzymes exhibit limited stability outside their optimal pH ranges. In comparison, CBHI enzymes from thermophilic fungi such as *Acremonium thermophilum*, *Thermoascus aurantiacus*, *Chaetomium thermophilum*, and *Trichoderma reesei* are typically active at more acidic pH values [29].

The temperature-dependent activity of purified EG7A_MALCI and CBH7A_MALCI was also assessed (Fig. 3a & 3b). EG7A_MALCI displayed maximum activity at 60°C, with notable activity even at 70°C. This is consistent with other fungal endoglucanases that exhibit optimal activity at 60°C, such as those from *Fomitopsis pinicola* [30] and *Bispora sp. MEY-1* [27]. In contrast, CBH7A_MALCI showed its highest activity at 40°C, exhibiting 2.62 times higher activity at this temperature compared to 50°C. The temperature optimum for CBH7A_MALCI is relatively low when compared to other cellobiohydrolases, which typically exhibit optimal activity between 60–65°C [28]. For both enzymes, the relative activity was considered 100% when incubated at 50°C, demonstrating that both EG7A_MALCI and CBH7A_MALCI retain substantial activity within a broad temperature range. The findings indicate that EG7A_MALCI and CBH7A_MALCI from *M. cinnamomea* display robust activity and stability over a broad spectrum of pH values and temperatures, highlighting their potential for diverse industrial applications. The findings indicate that EG7A_MALCI and CBH7A_MALCI from *M. cinnamomea* display robust activity and stability over a broad spectrum of pH values and temperatures, highlighting their potential for diverse industrial applications.

3.3 Thermostability

The recombinant EG7A_MALCI retained 46.1% and 50.2% activity at 60 °C after 6 h at pH 5.0 and 7.0, respectively. Whereas, CBH7A_MALCI was appreciably stable at 40 °C, where no loss of activity was observed after 24 h of incubation at pH 7.0, however, the observed residual activity was 52% at pH 5.0 (Fig. 4a & 4b), respectively. Egl7A (from *T. emersonii*) and Cel7A (from *N. fisheri*) retained > 40% and ~ 16% of their maximum activity, respectively, after 1 h at 70°C [31, 32]. Our results revealed that EG7A_MALCI was appreciably thermostable. Thermostable enzymes can be added during cooling of the heated steam pre-treated lignocellulosic biomass which has been the most frequently used pretreatment method, saving time, cost as well as exhibiting enhanced catalytic rates at elevated temperatures [33, 34].

3.4 Assessment of enzyme sensitivity to metal ions and chemical substances

The effect of various metal cations and chemical reagents (5mM) on enzyme activity was also tested (Table 1). EG7A_MALCI activity was enhanced in presence of Ca²⁺ (121.40% relative activity) when compared to control. The metal ions such as Fe³⁺, Hg²⁺ led to appreciable loss in activity while moderate loss was observed in presence of K⁺, Cu²⁺ and Co²⁺. Similar findings were observed in a previous study where Ca²⁺ divalent ions were reported to positively modulate the activity of endoglucanase from *Bacillus subtilis* UMC7 expressed in *E. coli*, however, Fe³⁺ and Cu²⁺ ions were found to inhibit the endoglucanase activity [35]. [36] also found that the addition of Ca²⁺ enhances endoglucanase activity. It is suggested that addition of Ca²⁺ ions may increase the binding affinity of the enzyme with the substrate by stabilizing the conformation of the catalytic site [37]. In contrast, Fe³⁺ ions were found to impair enzyme function. While the ionic detergent SDS and EDTA moderately inhibited the enzymatic activity like the endoglucanase from *Citrobacter farmeri* A1 which showed decreased activity with SDS and EDTA [38]. On the other hand, CBH7A_MALCI activity was positively modulated in the presence of Fe³⁺, Mn²⁺ and K⁺ resulting in 170.5, 147.3 and 135.6% relative activity when compared to control, respectively (Table 1). Contrasting results were obtained by Vanitha and co-workers (2011) [39] where Ca²⁺ ions were found to enhance the activity of CBHI (GH7) from *Penicillium funiculosum* NCL1. N-bromosuccinide resulted in nearly complete loss of EG7A_MALCI activity and appreciable loss of CBH7A_MALCI activity indicating the presence of tryptophan residues in the active site of EG7A_MALCI and CBH7A_MALCI [40].

Table 1
Effect of additives on the activity of recombinant EG7A_MALCI and CBH7A_MALCI.

Agent ^a	Concentration	Relative activity (%)	
		EG	CBH I
Control ^b	-	100.00 ± 1.23 ^c	100.00 ± 1.20
K ⁺	5mM	85.33 ± 2.01	135.6 ± 2.82
Cu ²⁺	5mM	88.58 ± 0.98	25.67 ± 0.56
Hg ²⁺	5mM	68.78 ± 0.75	41.62 ± 0.59
Mn ²⁺	5mM	94 ± 0.82	147.29 ± 2.01
Mg ²⁺	5mM	100 ± 2.22	124.59 ± 0.99
Ca ²⁺	5mM	121.40 ± 1.87	67.29 ± 0.81
Co ²⁺	5mM	87.12 ± 0.65	79.54 ± 0.34
Fe ³⁺	5mM	64.99 ± 0.54	170.5 ± 1.87
Zn ²⁺	5mM	100 ± 0.83	31.08 ± 0.32
EDTA	1mM	89.76 ± 1.01	155.40 ± 2.02
β-mercaptoethanol	1mM	105.4 ± 0.99	126.2 ± 1.54
Bromosuccinide	1mM	2.99 ± 0.27	20.21 ± 0.25
Dithiothreitol	1mM	99.11 ± 0.56	120 ± 0.45
PMSF	1mM	100 ± 0.89	95 ± 0.23
SDS	1%	88.73 ± 0.63	94.5 ± 0.47
a Metal ions tested were assayed as chloride salts			
b The relative activity in the control reaction without additives was defined as 100%			
c ± Values represent standard error @ 5%.			
All of the reactions were performed at pH 7.0 and 50°C			

3.5 Substrate specificity and kinetic characterization

The substrate specificity of recombinant EG7A_MALCI and CBH7A_MALCI was assayed against different complex and pNP substrates (Table 2). EG7A_MALCI exhibited the highest specific activity against barley β-glucan followed by CMC > lichenan. Like most GH 7 endo-1, 4-β-glucanases, EG7A_MALCI exhibited higher activity on barley β-glucan as compared to CMC. The reason might be that CMC is highly substituted with methoxy side chains

besides being highly viscous cause diffusion limitation and interfere with the enzyme activity [41, 42]. In addition, it showed weak activity on crystalline substrate avicel due to lack of CBM1. Negligible amount of activity was detected against *p*NP substrates (Table 2). While GH5 endoglucanases are commonly used in brewing for their broad substrate specificity, GH7 endoglucanases, such as EG7A_MALCI, offer superior substrate specificity and activity. EG7A_MALCI exhibits exceptionally high GH7 activity against barley β -glucan, outperforming GH7 enzymes from commercial fungi such as Rem_GH7EG from *Rasamsonia emersonii* (5 U/mL) [43]. Its high efficiency makes it a more effective choice for brewing applications, offering better control over mash fluidity and beer clarity than GH5 enzymes. On the other hand, purified CBH7A_MALCI exhibited activity against specifically against *p*NPL (0.65 U/mg) followed by *p*NPC (0.45 U/mg) (Table 2).

Table 2
Substrate specificity of EG7A_MALCI and CBH7A_MALCI against complex and *p*NP substrates.

Substrates	Main Linkage (monomer)	Specific activity (U/mg)	
		EG7A_MALCI	CBH 7A_MALCI
Barley- β -glucan	1,3–1,4- β -(glucose)	156.2 $\pm$ 2.32	n.d.
CMC	1,4- β -(glucose)	59.5 $\pm$ 0.08	n.d.
Lichenan	1,3–1,4- β -(glucose)	56 $\pm$ 0.67	n.d.
Laminarin	1,3- β -(glucose)	15 $\pm$ 0.03	n.d.
Avicel	1,4- β -(glucose)	n.d.	0.09 $\pm$ 0.001
Xylan	1,4- β -D-(xylan)	n.d.	n.d.
Xyloglucan	1,4- β -D-(glucose) linked with α -D xylose	n.d.	n.d.
Starch	1,4or 1,6- α -(glucose)	n.d.	n.d
<i>p</i> NPL	β -1,4 (Galactose–Glucose)	n.d.	0.65 $\pm$ 0.003
<i>p</i> NPC	β -1,4 (Glucose–Glucose)	n.d.	0.45 $\pm$ 0.002
<i>p</i> NPG	β -(glucose)	n.d.	n.d
<i>p</i> NPX	β -(xylose)	n.d.	n.d
<i>p</i> NPA	α -(arabinose)	n.d.	n.d
n.d.-no enzyme activity was detected. All the reactions were performed at pH 7.0 and 50 $^{\circ}$ C			

The kinetic parameters were also determined using barley β -glucan for EG7A_MALCI and *p*NPL for CBH7A_MALCI as substrates. The results revealed that both the purified enzymes, EG7A_MALCI and CBH7A_MALCI, showed higher affinity for their respective substrates as evidenced by low K_m values of 2.27 mg/ml and 0.284 mM, respectively. V_{max} values were calculated to be 80.95 U/mg for EG7A_MALCI and 155.542 U/mg for CBH7A_MALCI using Lineweaver Burk plot. To rationalize the observed substrate preferences and kinetic behaviour (Table 2), homology modelling and docking were performed to examine ligand interactions within the GH7 catalytic cleft.

3.6 Homology modelling and molecular docking

The multiple alignment comparison of EG7A_MALCI from *M. cinnamomea* with several other family 7 endoglucanases showed the presence of conserved catalytic residues E215, D217, E220 and H231. The catalytic residues were conserved in the organisms selected for multiple alignments and observed as E197, D199, E202 and H213 in the template endoglucanase from *F. oxysporum* [44]. The structural prediction for family 7 endoglucanase (EG7A_MALCI) from *M. cinnamomea* was done using homology modelling in I-TASSER. The structural model was aligned to the endoglucanase from *F. oxysporum* (1ovwA). Figure S3A and 3B show the predicted model of the EG7A_MALCI and its superimposition by 1ovwA, respectively. The predicted model shared 85% of the amino acid sequence similarity with the template with a root-mean square value (RMSD) of 0.376 [13]. At minimum probability distribution field (PDF) energy of the protein model, several ligands viz. DP5, cellobiose, cellotriose (in the order of confidence score (c-score) for binding to the protein) were tested for binding to EG7A_MALCI. DP5 was the most probable ligand for EG7A_MALCI with a confidence score (c-score) of 0.53 and binding energy of -8.7 kcal/mol (Fig. 5a). This preference for a longer cello-oligosaccharides is consistent with EG7A_MALCI showing highest activity on polymeric mixed-linkage β -glucans (barley β -glucan > CMC $\approx$ lichenan) and a low K_m on barley β -glucan, indicating efficient substrate engagement. The docking of the ligand to the protein showed H-bond formation with amino acid residues Y72, Y189, Q220, and S360 (Fig. 5b). The length of all the H-bonds was within 3Å. The various docking models tested showed the involvement of Q220 and S360 depicting the importance of the two amino acids in catalysis. Several aromatic residues Y72, Y165, Y195, W362 and W371 were also observed closer to the ligand binding site. These amino acids assist in the coordination and proper orientation of long-chain substrates within the active site via hydrophobic interactions [44, 45]. However, the presence of only two tryptophan residues in the active site when compared to that of *T.hcel7B* harbouring three residues [45] may affect the catalytic efficiency of this enzyme. Although DP5 is a β -1,4 glucan fragment, the observed hydrogen-bonding and aromatic interactions are likely transferable to mixed-linkage β -glucans, supporting efficient binding and thereby aligning with the high activity on barley β -glucan and lichenan. The structural properties of EG7A_MALCI were closely related to the other GH7 endoglucanases viz. *F. oxysporum* [44], *Trichoderma harzianum* [45]. The family 7 endoglucanases carryout hydrolysis of β -1, 4-glycosidic bond via a double displacement mechanism retaining the anomeric C1 configuration [45]. The Glu amino acids in the conserved triad formed by Q_{nuc} -X-X-D_X- $Q_{a/b}$ act as proton donor and a

nucleophile that contact with – 1 and + 1 glucose moieties in the substrate (Fig. 5b). In the active site pocket, β sheets fabricate the β sandwich bearing the cellulose binding cleft which was found to be deep. However, in other GH7 endoglucanases this binding cleft is more open [44, 45]. A long tunnel or deep cleft is essential for processivity [46].

The CBH7A_MALCI enzyme shared structural similarities with cellobiohydrolases reported from other organisms such as *Melanocarpus albomyces* [47] and *Dictostyleum purpureum* [48]. The multiple sequence alignment of the protein sequences for cellobiohydrolase from *D. purpureum* showed the presence of conserved catalytic sequences Q230, D232, Q235 and H246. Previous studies have shown that these amino acids remain conserved among the cellobiohydrolases of GH 7 family even between distant life forms [48]. The active site residues in *TrCel7A* from *T. reesei* were shown to be Q212, Q217 and D173 [49]. The model obtained by homology modelling in I-TASSER was visualized in PyMOL and aligned to cellobiohydrolase from *D. purpureum* as shown in the Fig.S4 A and 4 B, respectively. 89.5% of the predicted model aligned to the template with a RMSD value of 0.205.

Under minimized energy, cellobiose was docked to the CBH7A_MALCI with a binding energy of -9.2 kcal/mol (Fig. 6a and 6b). The predicted docking showed H bonds with the amino acid residues Q193, Q235, H246 and W392 (Fig. 6b). All the H-bonds were within 3 Å as was observed for EG7A_MALCI. Several tyrosine and tryptophan residues were observed in CBH7A_MALCI as well as EG7A_MALCI. The CBH7A_MALCI from *M. cinnamomea* also had 9 sulfide bridges as observed in cellobiohydrolase of *D. purpureum* [48]. Furthermore, the distance between the carboxylate groups of Q230 and Q235 was 8 Å, close to 6 Å reported for cellobiohydrolase in *M. albomyces* [47]. The active site appeared as a tunnel where on one side the substrate enters, gets hydrolyzed and the products were released from the other end. The docking results showed better binding of substrates against cellobiose with a binding energy of -9.2 kcal/mol. The tunnel shaped catalytic cleft is characteristic of processive GH7 cellobiohydrolases and is consistent with an exo-acting mode of action, providing a structural basis for CBH7A_MALCI detectable albeit low hydrolysis of crystalline cellulose (Avicel), in contrast to EG7A_MALCI.

3.7 Synergy studies using commercial CellicCTec2 with the recombinant enzymes

Four biomass substrates rice straw and bagasse pretreated with either acid (AcRS, AcBG) or alkali (AIRS, AIBG) were employed to assess how supplementing Cellic CTec2 with recombinant enzymes (replacing 10% or 20% of the commercial preparation at 9:1 and 8:2 ratios, respectively) enhances cellulolytic performance. Results in Fig. 7 (a and b) shows that, when compared to benchmark enzyme, elevated levels of glucose release after hydrolysis of acid and alkali treated rice straw when a cocktail of CellicCTec2 + EG7A_MALCI (1.18 and 1.39 folds, respectively) and CellicCTec2 + CBH7A_MALCI (1.16 and 1.28 folds, respectively) was used in 9:1 ratio were observed. Similarly, observed improvement in the yield of glucose when acid and alkali treated bagasse hydrolysed by cocktail (9:1 ratio) was 1.27 and 1.17 folds (for EG7A_MALCI), 1.23 and 1.27 folds for CBH7A_MALCI. The boosting effect was comparatively more pronounced in cocktails containing CellicCTec2 and respective enzymes in 8:2 ratio when compared to benchmark cellulase that resulted in increased glucose in the hydrolysate by 1.30, 1.50, 1.35 and 1.31 folds for AcRS, AIRS, AcBG and AIBG, respectively, in a cocktail containing CellicCTec2 + EG7A_MALCI, whereas, for CBH7A_MALCI, the fold increase was 1.27, 1.36, 1.23 and 1.27, respectively, was observed. The improved level of hydrolysis of different substrates using cocktails of cellulases with recombinant EG7A_MALCI and CBH7A_MALCI strongly advocates its importance in formulating cocktails applicable to lignocellulosic substrates [50–52]. Therefore, it is of interest to prospect diverse microbial strains for novel lignocellulolytic enzymes for enhancing the efficacy of existing benchmark enzymes as well as custom designing of cocktails specific to the treated substrates being used in 2G ethanol plants.

4 Conclusions

This work delivers the first functional insight into two GH7 cellulases from the thermophilic fungus *Malbranchea cinnamomea*. By heterologously producing the enzymes, defining their catalytic and stability profiles, and demonstrating their compatibility with commercial cocktails, the study adds robust, high-temperature endoglucanase and cellobiohydrolase activities to the current biocatalyst repertoire. These enzymes enhance the flexibility of enzyme-mix design for lignocellulose processing and underscore the value of thermophilic fungal diversity for next-generation, cost-efficient bio-refinery applications.

Abbreviations

GH
glycosyl hydrolase
2G
second generation
CMC
carboxy methyl cellulose
EG
endoglucanase
CBH
cellobiohydrolase
LPMO
lytic polysaccharide mono oxygenase
AOX
alcohol oxidase
SDS
sodium dodecyl sulphate
DNS

di nitro salicylic acid
GOD-POD
glucose oxidase-per oxidase.

Declarations

Competing interests

The author(s) declare no competing interests.

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Author Contribution

NB: Data curation; Literature survey, Investigation; Methodology; Writing- Original draft preparation; MB: Reviewing and Editing; GS: Methodology; Writing- Reviewing and Editing; VKG: Methodology; Writing-Reviewing and Editing; AT: Supervision; BSC: Conceptualization; Project administration; Resources; Supervision; Writing-Reviewing and Editing; YR: Conceptualization, Methodology, Writing- Original draft preparation, Investigation; Validation.

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Data Availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

References

1. Agrawal D, Basotra N, Balan V et al (2020) Discovery and Expression of Thermostable LPMOs from Thermophilic Fungi for Producing Efficient Lignocellulolytic Enzyme Cocktails. *Appl Biochem Biotechnol* 191:463–481. <https://doi.org/10.1007/S12010-019-03198-5/METRICS>
2. Bai W, Cao Y, Liu J et al (2016) Improvement of alkalophilicity of an alkaline xylanase Xyn11A-LC from *Bacillus* sp. SN5 by random mutation and Glu135 saturation mutagenesis. *BMC Biotechnol* 16:77
3. Basotra N, Dhiman SS, Agrawal D et al (2019) Characterization of a novel lytic polysaccharide monooxygenase from *Malbranchea cinnamomea* exhibiting dual catalytic behavior. *Carbohydr Res* 478:46–53
4. Basotra N, Joshi S, Satyanarayana T et al (2018a) Expression of catalytically efficient xylanases from thermophilic fungus *Malbranchea cinnamomea* for synergistically enhancing hydrolysis of lignocellulosics. *Int J Biol Macromol* 108:185–192
5. Basotra N, Joshi S, Satyanarayana T et al (2018b) Expression of catalytically efficient xylanases from thermophilic fungus *Malbranchea cinnamomea* for synergistically enhancing hydrolysis of lignocellulosics. *Int J Biol Macromol* 108:185–192. <https://doi.org/10.1016/J.IJBIOMAC.2017.11.131>
6. Basotra N, Kaur B, Raheja Y et al (2023) Developing and evaluating lignocellulolytic hyper producing deregulated strains of *Mycothermus thermophilus* for hydrolysis of lignocellulosics. *Biomass Convers Biorefin* 13:5059–5071. <https://doi.org/10.1007/S13399-021-01539-1/FIGURES/6>
7. Borisova AS, Eneyskaya EV, Jana S et al (2018) Correlation of structure, function and protein dynamics in GH7 cellobiohydrolases from *Trichoderma atroviride*, *T. reesei* and *T. harzianum*. *Biotechnol Biofuels* 11(11):5–. <https://doi.org/10.1186/S13068-017-1006-7>
8. Brar KK, Raheja Y, di Falco M et al (2023) Novel β -glucanases along with xylanase identified in *Thermomyces lanuginosus* secretome for enhanced saccharification of different lignocellulosics. *Biomass Convers Biorefin* 13:273–286. <https://doi.org/10.1007/s13399-020-01152-8>
9. Brodeur G, Yau E, Badal K et al (2011) Chemical and physicochemical pretreatment of lignocellulosic biomass: a review. *Enzyme Res* 2011:787532
10. Carninci P, Nishiyama Y, Westover A et al (1998) Thermostabilization and thermoactivation of thermolabile enzymes by trehalose and its application for the synthesis of full length cDNA. *Proc Natl Acad Sci U S A* 95:520–524. <https://doi.org/10.1073/PNAS.95.2.520;SUBPAGE:STRING:ABSTRACT;WEBSITE:WEBSITE:PNAS-SITE;WGROU:STRING:PUBLICATION>
11. Conde R, Cueva R, Pablo G et al (2004) A search for hyperglycosylation signals in yeast glycoproteins. *J Biol Chem* 279:43789–43798
12. De Castro AMH, De Carvalho A, Leite ML, Pereira SGF N (2010) Cellulases from *Penicillium funiculosum*: production, properties and application to cellulose hydrolysis. *J Ind Microbiol Biotechnol* 37:151–158. <https://doi.org/10.1007/S10295-009-0656-2>
13. Dimarogona M, Topakas E, Olsson L, Christakopoulos P (2012) Lignin boosts the cellulase performance of a GH-61 enzyme from *Sporotrichum thermophile*. *Bioresour Technol* 110:480–487
14. Dodda SR, Hossain M, Jain P et al (2022) Comparative Biochemical and Structural Properties of an Industrially Important Biocatalyst Cellobiohydrolase Cel7A from Thermophilic *Aspergillus fumigatus*. *Appl Biochem Microbiol* 2022 58(5):564–574. <https://doi.org/10.1134/S0003683822050064>

15. Gao D, Chundawat SPS, Krishnan C et al (2010) Mixture optimization of six core glycosyl hydrolases for maximizing saccharification of ammonia fiber expansion (AFEX) pretreated corn stover. *Bioresour Technol* 101:2770–2781. <https://doi.org/10.1016/J.BIORTECH.2009.10.056>
16. Gusakov AV, Salanovich TN, Antonov AI et al (2007) Design of highly efficient cellulase mixtures for enzymatic hydrolysis of cellulose. *Biotechnol Bioeng* 97:1028–1038. <https://doi.org/10.1002/BIT.21329>
17. Hobdey SE, Knott BC, Haddad Momeni M et al (2016) Biochemical and structural characterizations of two dictyostelium cellobiohydrolases from the amoebozoia kingdom reveal a high level of conservation between distant phylogenetic trees of life. *Appl Environ Microbiol* 82:3395–3409
18. Karlsson J, Momcilovic D, Wittgren B et al (2002) Enzymatic degradation of carboxymethyl cellulose hydrolyzed by the endoglucanases Cel5A, Cel7B, and Cel45A from *Humicola insolens* and Cel7B, Cel12A and Cel45Acore from *Trichoderma reesei*. *Biopolymers: Original Research on Biomolecules* 63:32–40
19. Karnaouri AC, Topakas E, Christakopoulos P (2014) Cloning, expression, and characterization of a thermostable GH7 endoglucanase from *Myceliophthora thermophila* capable of high-consistency enzymatic liquefaction. *Appl Microbiol Biotechnol* 98:231–242
20. Knott BC, Crowley MF, Himmel ME et al (2014) Carbohydrate–protein interactions that drive processive polysaccharide translocation in enzymes revealed from a computational study of cellobiohydrolase processivity. *J Am Chem Soc* 136:8810–8819
21. Kumar P, Barrett DM, Delwiche MJ, Stroeve P (2009) Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Ind Eng Chem Res* 48:3713–3729
22. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685. <https://doi.org/10.1038/227680A0;KWRD=SCIENCE>
23. Li Y, Li H, Li A, Li D (2009) Cloning of a gene encoding thermostable cellobiohydrolase from the thermophilic fungus *Chaetomium thermophilum* and its expression in *Pichia pastoris*. *J Appl Microbiol* 106:1867–1875
24. Liu Y, Dun B, Shi P et al (2015) A novel GH7 Endo- β -1, 4-glucanase from *Neosartorya fischeri* P1 with good thermostability, broad substrate specificity and potential application in the brewing industry. *PLoS ONE* 10:e0137485
25. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275
26. Lu H, Luo H, Shi P et al (2014) A novel thermophilic endo- β -1, 4-mannanase from *Aspergillus nidulans* XZ3: functional roles of carbohydrate-binding module and Thr/Ser-rich linker region. *Appl Microbiol Biotechnol* 98:2155–2163
27. Luo H, Yang J, Yang P et al (2010) Gene cloning and expression of a new acidic family 7 endo- β -1, 3 – 1, 4-glucanase from the acidophilic fungus *Bispora* sp. MEY-1. *Appl Microbiol Biotechnol* 85:1015–1023
28. Mahajan CC, Basotra NN (2016) *Malbranchea cinnamomea*: a thermophilic fungal source of catalytically efficient lignocellulolytic glycosyl hydrolases and metal dependent enzymes. <https://doi.org/10.5555/20163015879>
29. Mansfield SD, Saddler JN, Gübitz GM (1998) Characterization of endoglucanases from the brown rot fungi *Gloeophyllum sepiarium* and *Gloeophyllum trabeum*. *Enzyme Microb Technol* 23:133–140
30. Marchler-Bauer A, Bo Y, Han L et al (2017) CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. *Nucleic Acids Res* 45:D200–D203. <https://doi.org/10.1093/NAR/GKW1129>
31. Ogunmolu FE, Jagadeesha NBK, Kumar R et al (2017) Comparative insights into the saccharification potentials of a relatively unexplored but robust *Penicillium funiculosum* glycoside hydrolase 7 cellobiohydrolase. *Biotechnol Biofuels* 2017 10(1 10):71. <https://doi.org/10.1186/S13068-017-0752-X>
32. Parkkinen T, Koivula A, Vehmaanperä J, Rouvinen J (2008) Crystal structures of *Melanocarpus albomyces* cellobiohydrolase Cel7B in complex with cello-oligomers show high flexibility in the substrate binding. *Protein Sci* 17:1383–1394
33. Payne CM, Jiang W, Shirts MR et al (2013) Glycoside hydrolase processivity is directly related to oligosaccharide binding free energy. *J Am Chem Soc* 135:18831–18839
34. Raheja Y, Singh V, Gaur VK et al (2025a) Fortifying the *Rasamsonia emersonii* secretome with recombinant cellobiohydrolase (GH7) for efficient biomass saccharification. *World J Microbiol Biotechnol* 41:1–14. <https://doi.org/10.1007/S11274-025-04473-W/FIGURES/6>
35. Raheja Y, Singh V, Gaur VK et al (2025b) Fortifying the *Rasamsonia emersonii* secretome with recombinant cellobiohydrolase (GH7) for efficient biomass saccharification. *World J Microbiol Biotechnol* 41:1–14. <https://doi.org/10.1007/S11274-025-04473-W/FIGURES/6>
36. Raheja Y, Singh V, Gaur VK et al (2025c) Heterologous Expression of Thermostable Endoglucanases from *Rasamsonia emersonii*: A Paradigm Shift in Biomass Hydrolysis. *Appl Biochem Biotechnol* 1–18. <https://doi.org/10.1007/S12010-025-05258-5/TABLES/2>
37. Raheja Y, Singh V, Gaur VK et al (2025d) From bench to biorefinery: custom cellulolytic cocktail development for 2G ethanol. *Syst Microbiol Biomanufacturing* 1–9. <https://doi.org/10.1007/S43393-025-00357-0/METRICS>
38. Raheja Y, Singh V, Kumar N et al (2024a) Transcriptional and secretome analysis of *Rasamsonia emersonii* lytic polysaccharide mono-oxygenases. *Appl Microbiol Biotechnol* 108:1–17. <https://doi.org/10.1007/S00253-024-13240-0/FIGURES/5>
39. Raheja Y, Singh V, Sharma G et al (2024b) A thermostable and inhibitor resistant β -glucosidase from *Rasamsonia emersonii* for efficient hydrolysis of lignocellulosics biomass. *Bioprocess Biosyst Eng* 47:567–582. <https://doi.org/10.1007/S00449-024-02988-4/FIGURES/1>
40. Rai R, Kaur B, Singh S et al (2016) Evaluation of secretome of highly efficient lignocellulolytic *Penicillium* sp. Dal 5 isolated from rhizosphere of conifers. *Bioresour Technol* 216:958–967. <https://doi.org/10.1016/J.BIORTECH.2016.06.040>
41. Roy A, Kucukural A, Zhang Y (2010) I-TASSER: A unified platform for automated protein structure and function prediction. *Nat Protoc* 5:725–738. <https://doi.org/10.1038/NPROT.2010.5;SUBJMETA=114,1647,2410,2411,48,631;KWRD=BIOINFORMATICS,PROTEIN+FUNCTION+PREDICTIONS,PROTEIN>
42. Sharma M, Chadha BS, Saini HS (2010) Purification and characterization of two thermostable xylanases from *Malbranchea flava* active under alkaline conditions. *Bioresour Technol* 101:8834–8842

43. Sievers F, Wilm A, Dineen D et al (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol* 7:539. https://doi.org/10.1038/MSB.2011.75/SUPPL_FILE/MSB201175.REVIEWER_COMMENTS.PDF
44. Singh V, Raheja Y, Basotra N et al (2023) CRISPR/Cas9 mediated gene editing of transcription factor ACE1 for enhanced cellulase production in thermophilic fungus *Rasamsonia emersonii*. *Fungal Biol Biotechnol* 10:1–14. <https://doi.org/10.1186/S40694-023-00165-Y/TABLES/2>
45. Sonoda MT, Godoy AS, Pellegrini VOA et al (2019) Structure and dynamics of *Trichoderma harzianum* Cel7B suggest molecular architecture adaptations required for a wide spectrum of activities on plant cell wall polysaccharides. *Biochim et Biophys Acta (BBA)-General Subj* 1863:1015–1026
46. Sulzenbacher G, Driguez H, Henrissat B et al (1996) Structure of the *Fusarium oxysporum* endoglucanase I with a nonhydrolyzable substrate analogue: substrate distortion gives rise to the preferred axial orientation for the leaving group. *Biochemistry* 35:15280–15287
47. Trott O, Olson AJ (2010) AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem* 31:455–461
48. Vanitha MC, Meera B, Ramani G et al (2011) Molecular cloning and expression of a family 7 cellobiohydrolase gene *cbh1* from *Penicillium funiculosum* NCL1. *Int J Microbiol Res* 3:97
49. Voutilainen SP, Puranen T, Siika-aho M et al (2008) Cloning, expression, and characterization of novel thermostable family 7 cellobiohydrolases. *Biotechnol Bioeng* 101:515–528
50. Wang K, Luo H, Bai Y et al (2014) A thermophilic endo-1, 4- β -glucanase from *Talaromyces emersonii* CBS394. 64 with broad substrate specificity and great application potentials. *Appl Microbiol Biotechnol* 98:7051–7060
51. Wei KSC, Teoh TC, Koshy P et al (2015) Cloning, expression and characterization of the endoglucanase gene from *Bacillus subtilis* UMC7 isolated from the gut of the indigenous termite *Macrotermes malaccensis* in *Escherichia coli*. *Electron J Biotechnol* 18:103–109
52. Wei X-M, Qin Y-Q, Qu Y-B (2010) Molecular cloning and characterization of two major endoglucanases from *Penicillium decumbens*. *J Microbiol Biotechnol* 20:265–270
53. Yoon J-J, Cha C-J, Kim Y-S, Kim W (2008) Degradation of cellulose by the major endoglucanase produced from the brown-rot fungus *Fomitopsis pinicola*. *Biotechnol Lett* 30:1373–1378
54. Zafar M, Ahmed S, Khan MIM, Jamil A (2014) Recombinant expression and characterization of a novel endoglucanase from *Bacillus subtilis* in *Escherichia coli*. *Mol Biol Rep* 41:3295–3302
55. Zhang C, Freddolino PL, Zhang Y (2017) COFACTOR: improved protein function prediction by combining structure, sequence and protein–protein interaction information. *Nucleic Acids Res* 45:W291–W299. <https://doi.org/10.1093/NAR/GKX366>

Figures

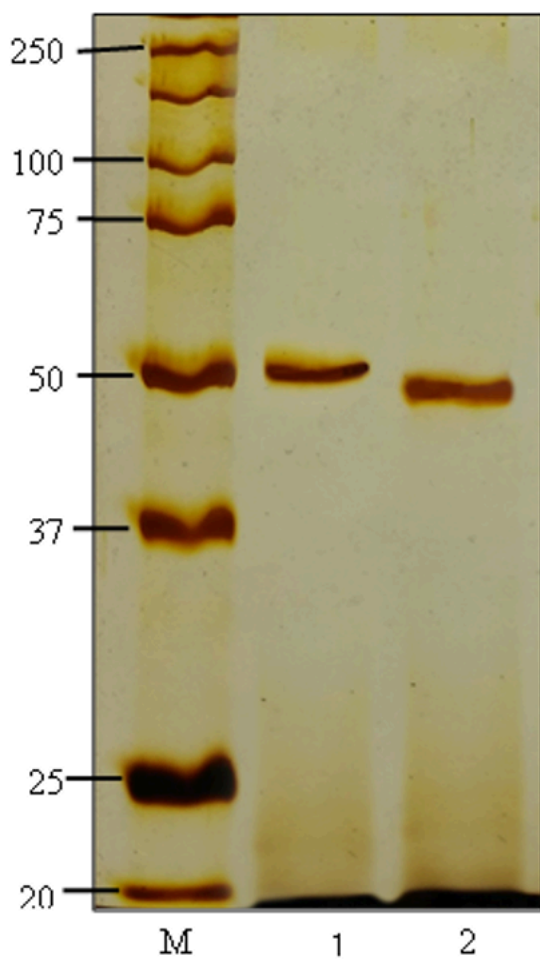


Figure 1

SDS-PAGE of purified enzymes. Lane M, standard protein marker in the order of increasing molecular mass in kDa (Precision Plus Protein™ Dual Color Standard molecular weight marker, BioRad); Lane 1: purified CBH7A_MALCI (~50KDa); Lane 2: purified EG7A_MALCI (~48 KDa)

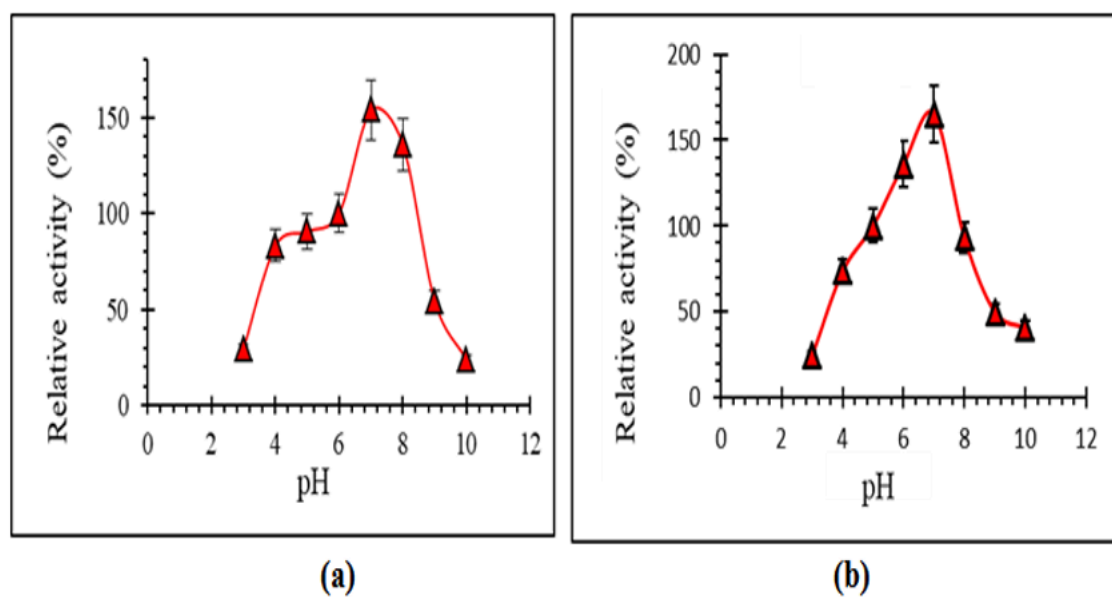


Figure 2

Effect of pH on enzyme activity of purified (a) EG7A_MALCI and (b) CBH7A_MALCI

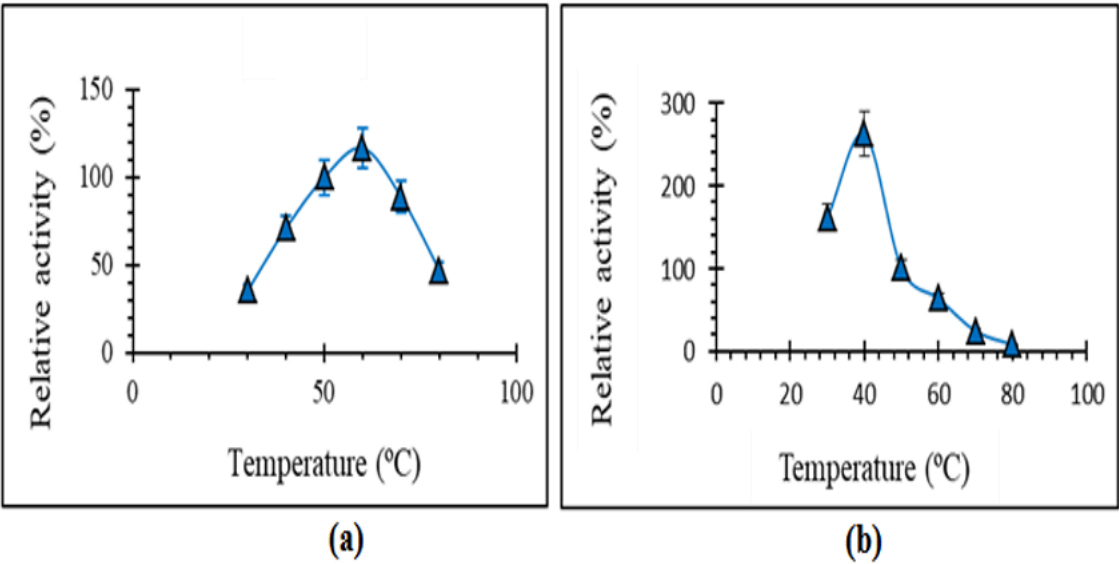


Figure 3
Effect of temperature on enzyme activity of purified (a) EG7A_MALCI and (b) CBH7A_MALCI

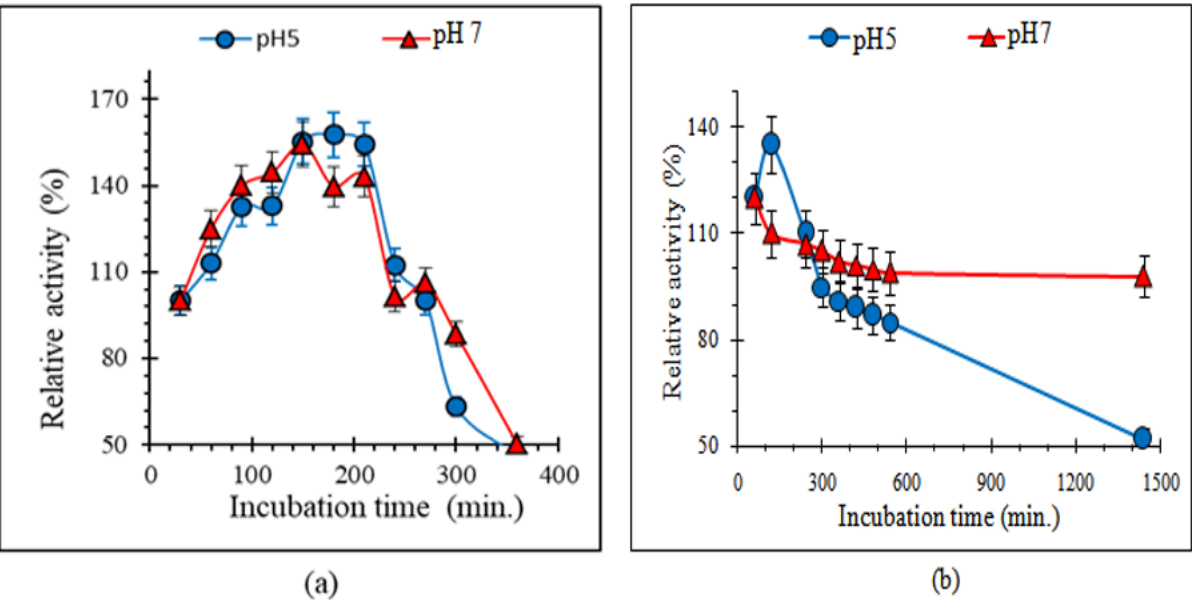
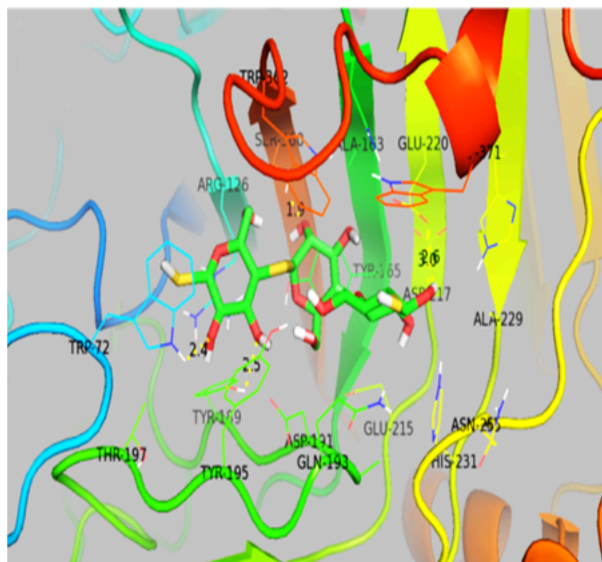
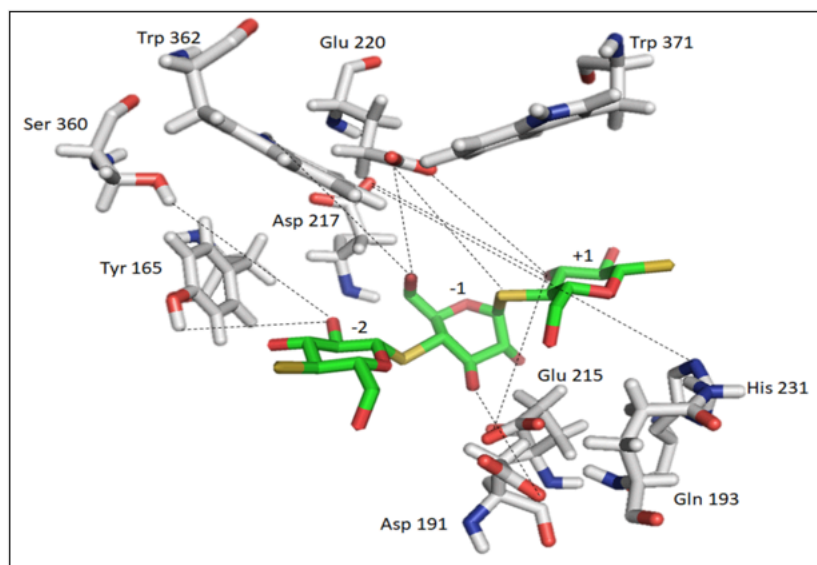


Figure 4
Thermostability of purified (a) EG7A_MALCI and (b) CBH7A_MALCI



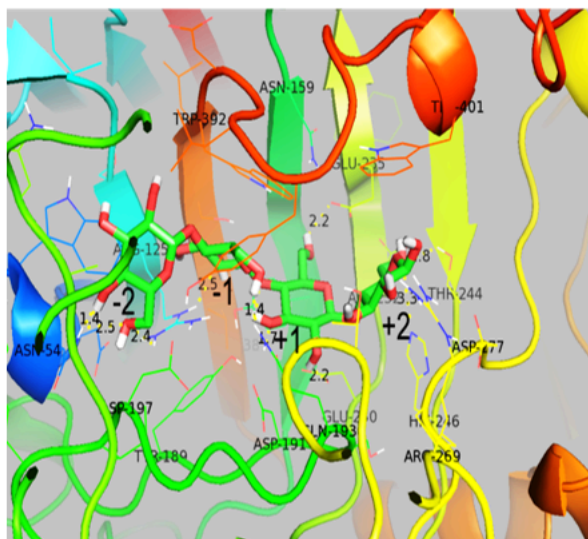
(a)



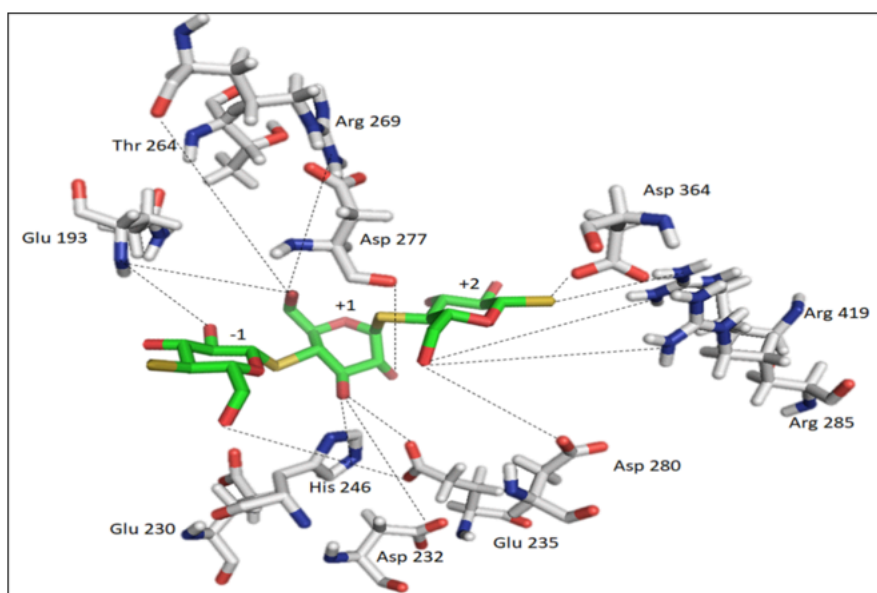
(b)

Figure 5

Predicted molecular protein-ligand docking for EG7A_MALCI with three sugar units of DP5 (cellopentaose) as the ligand. The representative of the ligand shows its interaction with the residues. E 215, D 217, E 220 and H 231 are the active site residues.



(a)



(b)

Figure 6

Predicted molecular docking of cellulose with the CBH7A_MALCI, Q 230, D 232, Q235, and H 246 represent the residues in the active sites. The other residues aid in the hydrolysis by rendering conformational stability.

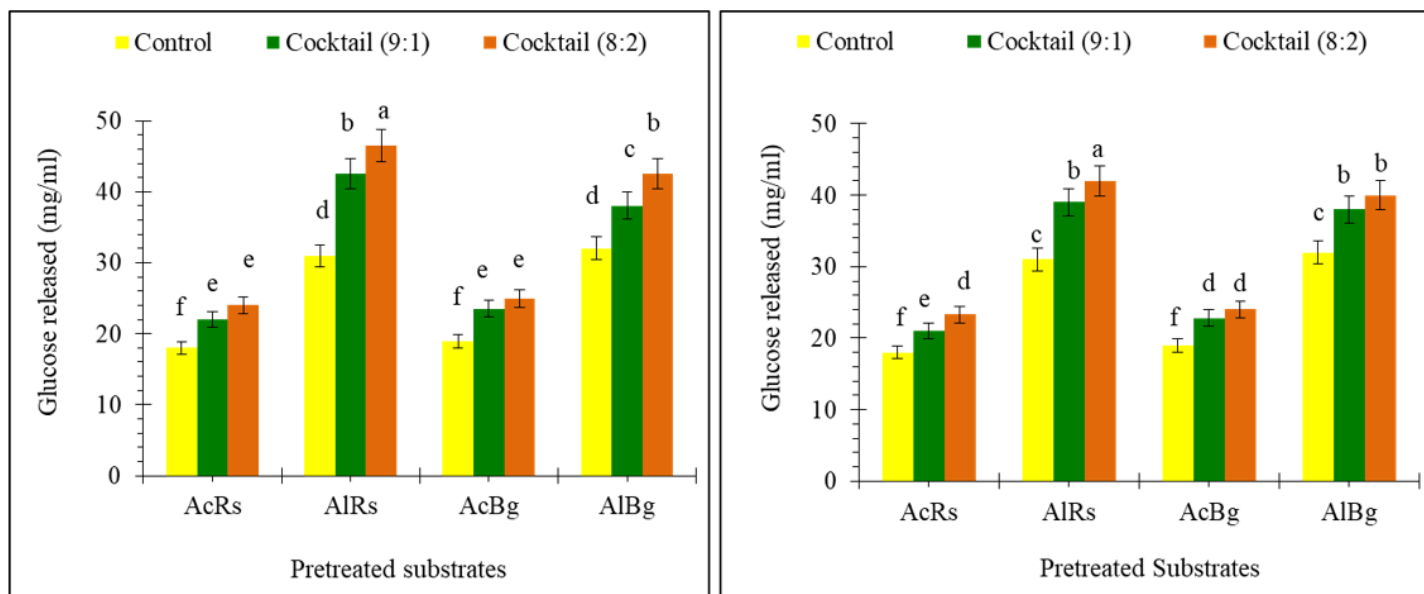


Figure 7

Synergy of recombinant hydrolases with commercial CelliCTec2 in hydrolysis of differently pre-treated substrates @7% substrate loading (a) glucose released using cocktails of CelliCTec2 with recombinant EG7A_MALC1 (b) glucose released using cocktails of CelliCTec2 with recombinant CBH7A_MALC1

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