Thermostable Recombinant β-(1→4)-Mannanase from C. thermocellum: Biochemical Characterization and Manno-Oligosaccharides Production

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Ⓢ Supporting Information

ABSTRACT: Functional attributes of a thermostable β-(1→4)-mannanase were investigated from *Clostridium thermocellum* ATCC 27405. Its sequence comparison the exhibited highest similarity with Man26B of *C. thermocellum* F1. The full length *Ct*Manf and truncated *Ct*ManT were cloned in the pET28a(+) vector and expressed in *E. coli* BL21(DE3) cells, exhibiting 53 kDa and 38 kDa proteins, respectively. On the basis of the substrate specificity and hydrolyzed product profile, *Ct*Manf and *Ct*ManT were classified as β-(1→4)-mannanase. A 1.5 fold higher activity of both enzymes was observed by Ca^{2+} and Mg^{2+} salts. Plausible mannanase activity of *Ct*Manf was revealed by the classical hydrolysis pattern of carob galactomannan and the release of manno-oligosaccharides. Notably highest protein concentrations of *Ct*Manf and *Ct*ManT were achieved in tryptone yeast extract (TY) medium, as compared with other defined media. Both *Ct*Manf and *Ct*ManT displayed stability at 60 and 50 °C, respectively, and Ca^{2+} ions imparted higher thermostability, resisting their melting up to 100 °C.

KEYWORDS: *Man26B, carob galactomannan, manno-oligosaccharides, thermostability*

■ INTRODUCTION

Plant cell wall is mainly composed of complex structural polysaccharides like celluloses and hemicelluloses. Polysaccharides of the primary cell wall are cellulose, hemicelluloses such as xyloglucans, mannans, galactomannans, glucomannans, laminarin, glucuronoarabinoxylans, and arabinoxylan, etc. Mannans are the polysaccharides with a backbone chain of β-(1 → 4)-linked mannose units. They constitute a major portion of hemicelluloses in hardwoods. The major distribution of mannan in combination with galactose and glucose units in plant hemicellulose reservoir is abundant in nature. Carob galactomannan (from the *Ceratonia siliqua* plant) contain β-(1→4)-D-mannan backbone (78%) and galactose as α-(1→6)-linked (22%) single units, where as guar gum (from endosperm of guar seeds) backbone is a linear chain of β-(1→4)-linked mannose residues to which galactose residues are (1→6)-linked at every second mannose, forming short side-branches.[1] Glucomannan (from *Amorphophallus konjac*) is a water-soluble polysaccharide that is considered a dietary fiber. The component sugars in konjac glucomannan are β-(1→4)-linked D-mannose and D-glucose residues in a molar ratio of 1.6:1 and branched chain composed of β-(1→6)-linked D-glucosyl units.[2]

β-D-Mannanase [endo β-(1→4)-mannan mannohydrolase, E.C. 3.2.1.78] hydrolyzes β-(1→4)-D-mannopyranosyl linkages within the main chain of mannans, glucomannans, galacto-mannans, and galactoglucomannans.[3] Mannanases have been listed within glycoside hydrolase (GH) families viz. GH26, GH5, and GH113 in carbohydrate-active enzyme database (http://www.cazy.org/Glycoside-Hydrolases.html) based on sequence similarity.[4] β-D-Mannanases belong to families GH5, GH26, GH113 and display a $(\beta/\alpha)_8$ barrel-shaped protein folding pattern, and the acid−base-assisted catalysis via a double displacement mechanism involving a covalent glycosyl-enzyme intermediate.[5,6] The mechanism of glycosidic bond cleavage is found conserved within these families. These are the characteristic patterns of clan GH-A protein families and helped β-D-mannanases of families GH5, GH26, and GH113 to group into this clan.[5,6] Due to the retaining double-displacement mechanism, these enzymes can perform transglycosylation. Transglycosylation may lead to the synthesis of new glycosides or oligosaccharides longer than the original substrate. GH5 and GH113 mannanases have been described as able to catalyze transglycosylation reactions,[5,6] while to date no evidence of transglycosylation has been reported for GH26 mannanases.[7]

The benefit of employing novel enzymes for specific industrial processes is well-recognized with the discovery of β-mannanases. β-Mannanases (EC 3.2.1.78) hydrolyze mannan-based hemicelluloses and liberate short β-(1→4)-manno-oligosaccharides, which can be further hydrolyzed to mannose by β-mannosidases (EC 3.2.1.25). There are currently around 50 β-mannanase gene sequences in families 5 and 26 GHs from various microbial origins. The family 26 Glycoside Hydrolase (GH26) mannanase has narrow substrate specificity hydro-lyzing (1→4)-β-D-linkages in mannans, galacto-mannans, glucomannans, and galactoglucomannans but does not show activity against β-glycan chain of soluble cellulose derivatives.[8] Several studies exhibited the presence of distinct types of mannanases (GH5A, GH5B, GH5C, GH26A, GH26B, and GH26C) expressed on the cell surface of *Cellvibrio japonicas*[9]

having different substrate specificity. GH5 mannanase exhibits some activity for cellulosic substrates.[10] By contrast, Man26B displays canonical endomannanase activity and linked to the cell membrane via ~70 residue linker sequence of *C. japonicas*.[10] Thus, Man26B gets enough space via the linker sequence to adsorb on the natural substrates galactomannan and glucomannan than other surface-expressed mannanases. Man26B rapidly generates large amounts of mannose, even in the early stages of galactomannan or manno-oligosaccharide hydrolysis. But Man26A displayed a typical endo-β-(1→4)-bond cleavage activity against small manno-oligosaccharides, hydrolyzing mannotriose approximately, 10000 times more efficiently than Man26B.[10] There are distinct differences in topology of the substrate-binding cleft and substrate specificity among the mannnases (Man26A, Man26B, and Man26C) within GH26 family.[5]

There are several reports of manno-oligosaccharides synthesis by the utilization of mannanases from manno-configured substrates.[10,11] Though the manno-oligosaccharides are indigestible inside the human gut, their potential role as dietary fiber and prebiotics were attributed in various studies.[12] It was evident from earlier reports that the efficient prebiotics role of manno-oligosaccharides that supports the growth of human intestinal beneficial microflora viz. Bifidobacteria and *Lactobacilli*.[13,14] In addition, manno-oligosaccharides can prevent the probability of high blood pressure and higher intestinal absorption of fatty acid substance from a high fat diet.[15]

Clostridium thermocellum expresses a large number of hemicellulases in its multienzyme complex, targeting various hemicellulosic components such as mannans and xylans, removes the hemicellulosic polysaccharides exposing the cellulose microfibrils and uses it as primary carbon and energy sources and releases soluble sugars.[16] The carbohydrate binding modules (CBMs) are the noncatalytic modules known to help or bring the catalytic modules in close proximity to its substrates, and also some CBMs are known to stabilize the enzyme (catalytic module) structure and increase its thermostability.[17,18] The majority of *C. thermocellum* cellulosomal enzymes display rather complex multimodular architectures containing CBMs either at the N- or C- terminal domain. These CBMs potentiate the interaction of the multifunctional complex with the diversity of polysaccharides in the plant cell wall by directing the appended catalytic domains to their target substrates.[16] The CBMs may be found to contain up to 200 amino acids and can be found attached as single, double, or triple domains in one protein, located at both the C- or N-terminal within the parental protein.[19] A family 35 carbohydrate binding module (CBM35) often appended with mannanase that binds to the galactose-decorated mannanas and facilitates their efficient hydrolysis.[20] There are few reports of Man26B functions explored earlier from *Paenibacillus* sp. BME14,[21] *C. japonicas*,[22] *C. thermocellum* strain F1,[23] and *Bacillus licheniformis* DSM13.[24] In the present report, we studied the molecular and biochemical characterization of family 26 glycoside hydrolase (GH26) mannanase B (Man26B) from *C. thermocellum* ATCC 27405. Its potential in manno-oligosaccharide production by the hydrolysis of the manno-configured substrate was analyzed.

■ MATERIALS AND METHODS

Bacterial Strains and Plasmid. The genomic DNA of *C. thermocellum* ATCC 27405 was a gift from Professor Carlos Fontes, Faculdade de Medicina Veterinaria, Lisbon, Portugal. *Escherichia coli* DH5α cells were used for cloning, and *E. coli* BL21 (DE3) was used as the expression host. The plasmids used for cloning and expression were pET-28a (+). All the above-mentioned items were procured from Novagen (Madison, WI).

Fine Chemicals and Natural and Synthetic Substrates for Enzyme Assay. Mannose, xylose, glucose, galactose, EDTA, and NaOH solution (50%, w/v), lichenan (from *Cetraria islandica*) were procured from Sigma Chemical Company (St. Louis, MO). Carob galactomannan, konjac glucomannan, locust bean galactomannan, guar galactomannan, ivory nut mannan, β-(1→4)-mannnan, barley β-glucan, rye arabinoxylan, xyloglucan, mannobiose, and mannotriose were procured from Megazyme International, Ireland. Carboxy methylcellulose (CMC), hydroxyethyl cellulose, Avicel (microcrystalline cellulose) and synthetic pNP-glycosides like pNP-β-mannopyranoside, pNP-α-mannopyranoside, glucuronoxylan, and polygalactouronic acid were purchased from Sigma Chemical Company.

Sequence Analysis. Two mannanase genes (locus name: Cthe_0032 and Cthe_2811) (http://www.cazy.org/GH26_bacteria. html) belonging to family 26 glycoside hydrolase (GH26), were identified in the native host *C. thermocellum* ATCC 27405 (16S rDNA sequence ID: CP000568, http://www.straininfo.net/strainPassport. action?page=34&cultureId=40680). Mannanase encoding ORF region was identified using the protein sequence (gene bank protein accession ABN51273.1, locus name: Cthe_0032) of *C. thermocellum* ATCC 27405 in tBLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) tool. This sequence was used to design the desired oligonucleotide primers for amplification of full length *Ct*Manf (CBM35-*Ct*ManT) and truncated catalytic module *Ct*ManT (nucleotide accession: CP000568.1). The sequence for amplification was devoid of signal peptide and dockerin (Doc I). The protein sequence of *Ct*ManT was analyzed for the type of enzyme synthesized using multiple sequence alignment using MULTALIN (http://multalin.toulouse.inra.fr/multalin/cgi-bin/multalin.pl) and viewed in the ESPript (http://espript.ibcp.fr/ESPript/ESPript/) tool. Multiple sequence alignment was performed using the different types of mannanase (Man26A and Man26B) sequences of glycoside hydrolase family 26 (GH26) from *Paenibacillus* sp BME14,[21] *Cellvibrio japonicas*,[22] *Clostridium thermocellum* strain F1,[23] *Bacillus licheniformis* DSM13,[24] *Cellulomonas fimi*,[25] *Rhodothermus marinus*,[26] *Bacillus* sp JAMB750,[27] *Paenibacillus polymyxa* GS01.[28] Evaluation of the functional property of this protein sequence was performed using InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/), and the molecular architecture of the entire protein sequence to be cloned was drawn.

Gene Amplification and Cloning. The ORF region encoding full length *Ct*Manf (CBM35-*Ct*ManT) containing family 35 carbohydrate binding module (nucleotide accession: CP000568.1) at the N-terminal and the family 26 glycoside hydrolase (GH26), a mannanase B (Man26B) catalytic module, *Ct*ManT (nucleotide accession: CP000568.1) were amplified from the genomic DNA of *C. thermocellum* ATCC 27405, using two oligonucleotide primers having *Nhe*I and *Xho*I restriction sites. The 50 μL PCR reaction mixture contained Mg^{2+} ions (2.5 mM), dNTPs (0.2 mM), primers (1.5 μM), 1.0 μL of Taq DNA polymerase (1 μL of 1 Unit/μL), and 1 μL of genomic DNA (10 ng) of *C. thermocellum* ATCC 27405 and PCR-grade water (Sigma Chemical Company). The oligonuclecotide primers used for amplifying *Ct*ManT were forward 5′-cacgctagcgca-tattcccttcctg-3′ and reverse 5′-cacctcgagttagctaaagtatattttg-3′. The oligonuclecotide primers for *Ct*Manf used were: forward 5′-cacgctagc-gcatattcccttcctg-3′ and 5′-cacctcgagttaaagttcatccaagctgc-3′. The PCR amplification cycles used were denaturation at 94 °C for 4 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 60 s and extension at 72 °C for 2 min, and final extension at 72 °C for 10 min. The amplified products were run on 0.8% agarose gel and purified by a gel extraction kit (Qiagen). The PCR-amplified *Ct*Manf and catalytic *Ct*ManT were cloned into *Nhe*I/*Xho*I digested pET-28a (+) expression vector containing kanamycin as a resistant marker, resulting in cloned plasmids pMan*f* and pManGH26, respectively. The *E. coli* DH5α cells were transformed with above recombinant plasmids. These transformed cells were grown on LB agar plates,[29] supplemented with kanamycin (50 μg mL^{-1}) at 37 °C

for growth of recombinant clones. The positive clones were selected by restriction digestion analysis of the recombinant plasmids.

Expression and Purification of *Ct*Manf and *Ct*ManT. *E. coli* BL-21(DE3) (Novagen) cells were transformed for expression of *Ct*Manf and *Ct*ManT as described elsewhere.[29] The cells were grown in LB medium containing kanamycin (50 μg mL^{-1}) at 37 °C with 180 rpm to the midexponential phase (A$_{600\ nm} \approx 0.6$). Then the cells were induced with 1.0 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and incubated at 24 °C with 180 rpm for 24 h for the hyper-expression of recombinant proteins. The cells were harvested at 9000g, and the resulting pellet was resuspended in 50 mM sodium phosphate buffer pH 7.0, containing 1 mM phenylmethanesulfonyl fluoride (PMSF). Then the cells were sonicated (Sonics, Vibra cell) on ice for 16 min (9 s on/9 s off pulse, 30% amplitude) and again centrifuged using the centrifuge (Sigma, 4K15) at 19000g at 4 °C for 20 min. The cell free supernatant containing the soluble protein was purified by immobilized metal ion chromatography (IMAC).[29] The recombinant proteins containing *Ct*Manf and *Ct*ManT appended by the His$_6$ tag were purified in a single step using 1 mL HiTrap chelating columns (GE Healthcare) as recommended by the manufacturer. The purity and molecular mass of recombinant *Ct*Manf and *Ct*ManT were verified by SDS−PAGE.[30]

Enzyme Assays of *Ct*Manf and *Ct*Mant with Natural and Synthetic Substrates. The enzyme activity of *Ct*Manf and *Ct*ManT was determined by using natural substrates, such as carob galactomannan, locust bean galactomannan, konjac glucomannan, guar galactomannan, ivory nut mannan, β-(1→4)-mannan (an insoluble polysaccharide prepared from carob galactomannan pretreated with *Aspergillus niger* mannanase and subsequently debranched to a high extent containing mannose (97%) and galactose (3%), according to the manufacturer Megazyme International, Ireland), barley β-glucan, lichenan, carboxymethyl cellulose, hydroxyethyl cellulose, Avicel, rye arabinoxylan, glucuronoxylan, arabinogalactan, and polygalactouronic acid (PGA) at (1%, w v^{-1}) in 50 mM sodium phosphate buffer (pH 7.0) and by measuring the reducing sugar released, as described previously.[31,32] In both cases, 100 μL of reaction mixture contained 1.0 (%, w v^{-1}) substrate, 10 μL of enzyme (*Ct*Manf, 0.16 mg mL^{-1} and *Ct*ManT, 0.15 mg mL^{-1}). One-hundred microliter reaction mixtures for both the enzymes were incubated at 50 °C for 10 min separately with separate polysaccharides. In the case of Avicel, the reaction mixture was incubated for 60 min under shaking conditions. The resulting reducing sugars viz. mannose, xylose, glucose, and galactose concentrations were measured by the absorbance at A_{500nm}, using a spectrophotometer (Varian, Cary 100 Bio), and standard curves were prepared from standard mannose, xylose, glucose, and galactose (Sigma Chemical Company). The assays were carried out in triplicate.

A wide range of pH was chosen, ranging from pH 3.0 to 8.0 as β-mannanase displays optimum range within this range.[33] For studying the optimum pH profile of *Ct*Manf and *Ct*ManT, 50 mM sodium phosphate buffer, pH 3.0−8.0, was used for the enzyme assays that employed 1.0 (%, w v^{-1}) carob galactomannan at 50 °C. The optimum temperature study was carried out within the range from 10 to 100 °C at their respective optimum pH, using 1.0 (%, w v^{-1}) carob galactomannan.

After the verification of substrate specificity, the enzyme assays for *Ct*Manf and *Ct*ManT were performed using 50 mM sodium phosphate buffer at their optimum pH and temperature to analyze the kinetic parameters viz. K$_m$, V$_{max}$, k$_{cat}$, and k$_{cat}$/K$_m$. One unit of enzyme activity was determined as the release of 1 μmole of mannose per minute. The assays involving synthetic substrates *p*NP-β-D-mannopyranoside and *p*NP-α-D-mannopyranoside were performed as reported earlier by Bey et al. (2011).[34]

Zymogram Study and Activity Staining. Zymogram study of recombinant *Ct*Manf and *Ct*ManT were investigated by using 0.5% (w v^{-1}) carob galactomannan as the substrate incorporated in 12% (w v^{-1}) SDS−PAGE. Ten micrograms of each of purified *Ct*Manf and *Ct*ManT by IMAC was mixed with 1× the sample buffer (62.5 mM Tris-Cl, pH 6.8, 20% v v^{-1} glycerol, 2% w v^{-1} SDS, and 0.005% w v^{-1} bromophenol blue)[30] without β-mercaptoethanol[35] were loaded on

the gel. After the completion of electrophoresis, the gels were incubated in 2.5% (v v^{-1}) of TritonX 100 at 25 °C for 1 h followed by 1 h incubation in 50 mM sodium phosphate buffer, pH 7.0. Then the gels were incubated in preheated 50 mM sodium phosphate buffer (pH 6.5) at 55 °C for 30 min and then stained with 0.1% (w v^{-1}) congo red for 45 min, as described by Aboul-Enein et al. (2010).[35] After congo red staining, the gels were counter-stained with 1 N HCl, as described elsewhere.[36]

Effect of Metal Ions, Chaotropic Agents, And Detergent on Enzyme Activity. The effects of different metal cations, chaotropic agents, and detergent on the activity of *Ct*Manf and *Ct*ManT were determined. The enzyme activity of both *Ct*Manf and *Ct*ManT was determined in the presence of various metal salts, such as Ni^{2+} (NiSO$_4$·6H$_2$O), Zn^{2+} (ZnSO$_4$·7H$_2$O), Cu^{2+} (CuSO$_4$·5H$_2$O), Co^{2+} (CoCl$_2$·6H$_2$O), Mn^{2+} (MnCl$_2$·4H$_2$O), Al^{3+} (AlCl$_3$·6H$_2$O), or Ca^{2+} (CaCl$_2$·2H$_2$O), chaotropic agents like disodium EDTA, EGTA, urea, or guanidine hydrochloride and detergent such as SDS. The assays of *Ct*Manf and *Ct*ManT were performed at 60 and 50 °C, respectively, using 50 mM sodium phosphate buffer, of pH 6.9 and pH 6.5, respectively. One-hundred microliters of the reaction mixture containing carob galactomannan (1%, w v^{-1}) and metal salt at concentrations (up to 80 mM) or SDS (up to 20 mM) were incubated for 10 min, and a control sample in the absence of the additive was also run. The assays were performed in triplicates. Both enzymes were incubated with EDTA and urea for 1 h, before measuring the residual activity. The enzyme activity was determined, as described earlier.

Thin-Layer Chromatography of Hydrolyzed Products by *Ct*Manf. The qualitative analysis of hydrolyzed products by the reaction of *Ct*Manf on carob galactomannan was performed by thin-layer chromatography (TLC) on silica gel-coated aluminum foil (TLC Silica gel 60 F$_{254}$ 20 × 20 cm, Merck) for detecting sugars. The enzyme *Ct*Manf (10 μL and 0.16 mg mL^{-1}) with 1% (w v^{-1}) carob galactomannan in 100 μL reaction mixtures were incubated at optimized temperature 60 °C and optimized pH 6.9, for time intervals of 1, 4, 8, 16, and 24 h. The reaction products were boiled for 2 min to stop enzymatic hydrolysis and then centrifuged at 13000g for 5 min.[6] Then 0.2 μL of sample as well as of standard solutions (1.0 mg mL^{-1}) were loaded on the TLC plate and kept in the developing chamber saturated with the developing solution (mobile phase), which consisted of acetic acid−*n*-propanol−water−acetonitrile (4:10:11:14).[37] Mannose and oligosaccharides (mannobiose and mannotriose) were used as standards. At the end of the run, migrated sugars were visualized by immersing the TLC plate in a visualizing solution (sulphuric acid/methanol 5:95, v v^{-1}; α-napthol 5.0%, w v^{-1}). The TLC plates were then dried at 80 °C for 20 min. The migrated reaction products (sugars) appeared as spots on the TLC plate.

HPAEC Analysis of Polysaccharide Hydrolysis by *Ct*Manf. *Ct*Manf (10 μL and 0.16 mg mL^{-1}) with 1% (w v^{-1}) carob galactomannan in 100 μL of reaction mixtures were incubated, at optimal conditions of 60 °C, and pH 6.9 for 1, 4, 8, 16 and 24 h. These reaction mixtures were treated with 2 volumes (200 μL) of absolute ethanol to precipitate the remaining nonreacted polysaccharides (substrates) and then centrifuged at 13000g at 4 °C for 10 min. The supernatant containing the liberated sugar was transferred to another microcentrifuge tube, and the ethanol was removed by evaporation. The supernatant (50 μL) was diluted to 500 μL by adding ultrapure (Milli-Q, Millipore) water and filtered through a syringe filter using a 0.2 μm membrane. The liberated sugars were analyzed by high-pressure anion-exchange chromatography (HPAEC), using an ion chromatography system (Dionex, ICS-3000). From the filtered 500 μL, 25 μL of sample (liberated sugars) was run on CARBOPACK PA-200 column (150 × 3 mm, Dionex), attached with CarboPac PA200 guard column (30 × 3 mm, Dionex) with borate and amino trap columns which removed impurities and provided high resolution. The instrument (Dionex, ICS-3000) was kept at a constant temperature of 30 °C during the analysis, and the flow rate was maintained at 0.3 mL min^{-1}. The elution of liberated sugars released due to enzyme reaction was carried out with 100 mM sodium hydroxide using a pulsed amperometric detector (PAD). Ten micrograms per milliliter of D-mannose, mannobiose, and mannotriose

were used as standards. The solutions of standards were also filtered through a 0.2 μm membrane before loading onto the column. A standard curve was prepared by using a mixture of standards (mannose, mannobiose, and mannotriose) from 10 mg mL^{-1} stock solutions. Quantitative analysis enzyme-catalyzed hydrolysis products were determined from the peak intensity of the released products.

Production of CtManf and CtManT in Different Media. Media screening was performed in order to obtain higher production of recombinant CtManf and CtManT. Four different media were screened, as listed in Table 4. All four media LB, 5xLB, Terrific Broth (TB), and TY media were prepared, as previously described by Tripathi et al. (2009).[38] LB medium (100 mL) was prepared in a 250 mL flask by weighing constituents in (%, w/v) were tryptone, 1.0; yeast extract, 0.5; and NaCl, 1.0.[38] A 100 mL 5xLB medium was prepared by using (%, w/v) tryptone, 5.0; yeast extract, 2.5; NaCl, 2.5; glycerol, 1.0.[38] Terrific broth (TB) 100 mL was composed of constituents in (%, w/v) pancreatic digest of casein, 1.2; yeast extract, 2.4; dipotassium phosphate, 0.94; monopotassium phosphate, 0.22; and glycerol, 0.4.[38] The components of 100 mL TY medium used in (%, w/v) were tryptone, 2.68; yeast extract, 2.14; monopotassium phosphate, 0.54; diammonium hydrogen phosphate, 0.16; magnesium sulfate, 0.12; NaCl, 0.85; and glycerol, 0.1.[38] Initially, the seed culture was prepared by inoculating respective E. coli BL21 cells, harboring recombinant plasmids CtManf and CtManT in 5 mL LB medium supplemented with 50 μg mL^{-1} kanamycin and incubated at 37 °C and 180 rpm for overnight. Each 250 mL culture flasks of four media containing 100 mL medium supplemented with 50 μg mL^{-1} kanamycin were inoculated with 1 mL of seed culture. The cells were grown at 37 °C, 180 rpm up to the mid exponential phase ($A_{600\,nm} \approx 0.6$) followed by induction with 1.0 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for hyper-expression of recombinant proteins at 24 °C with 200 rpm for 24 h. Dry cell weight of the bacterial cell was measured, as described by Black (1996).[39] The 10 mL culture broth was centrifuged at 9000g for 15 min and the supernatant discarded. The resulting pellet washed with distilled water 3 times followed by centrifugation at 9000g for 15 min in each wash. The cell pellet was dried at 60 °C for 16 h, and the dry cell weight was measured by weighing.[39] The cells were harvested by centrifuging at 9000g at 4 °C for 20 min, and the resulting cell pellet was resuspended in 50 mM sodium phosphate buffer pH 7.0 containing 1 mM phenylmethanesulfonyl fluoride (PMSF). The cell suspensions were sonicated (Vibra cell, Sonics) on ice for 16 min (9 s on/9 s off pulse, 30% amplitude) and then centrifuged at 19000g at 4 °C for 20 min. The recombinant proteins were purified by a single step, using immobilized metal ion affinity chromatography (IMAC) on HiTrap chelating columns (GE Healthcare), as mentioned earlier.[29] The concentration of IMAC-purified recombinant proteins were determined by the Bradford method.[40]

Thermostability study and protein melting analysis of CtManf and CtManT. The ability of CtManf and CtManT to retain its enzymatic activity at a higher temperature was studied. Both CtManf and CtManT (30 μL each from the stock of 0.16 mg mL^{-1} and 0.15 mg mL^{-1}, respectively) were incubated at temperatures from 10 to 110 °C for 1 h. After the incubation, the enzyme activity was determined by taking 10 μL of CtManf and CtManT separately in a 100 μL reaction mixture containing 1% (w v^{-1}) carob galactomannan in 50 mM sodium phosphate buffer of pH 6.9 and pH 6.5, respectively. The protein melting curves were generated by subjecting CtManf and CtManT to various temperatures and measuring the change in the absorbance at 280 nm by a UV–visible spectrophotometer (Varian, Cary 100-Bio), following the method of Dvortsov et al.[18] The purified CtManf and CtManT at protein concentration of 0.3 mg mL^{-1} in 50 mM MES [2-(N-morpholino) ethanesulfonic acid] buffer, pH 7.0, were used. The absorbance at 280 nm was measured at different temperatures, varying from 40 to 100 °C using a peltier temperature controller. The protein solutions (1 mL, 0.3 mg mL^{-1}) of CtManf and CtManT were kept at the particular temperature for 10 min to attain the equilibrium. A similar experiment was carried out with the addition of 10 mM CaCl$_2$ in the 1 mL enzyme (0.3 mg mL^{-1}) solution, and the temperature was then varied. The experiment was repeated with the

addition of CaCl$_2$ and EDTA to 1 mL of enzyme solution (0.3 mg mL^{-1}) containing equimolar concentrations of 10 mM, and finally the change in absorbance at 280 nm was measured. A curve of relative derivative absorption coefficient (first derivative coefficient) versus temperature was plotted, as described earlier by Dvortsov et al. (2009).[18]

RESULTS

Sequence Analysis of CtManf. The molecular architecture of full length derivative of mannanase CtManf displayed an appended family 35 carbohydrate binding module (CtCBM35) at its N-terminal end and catalytic CtManT (Man26B) at its C terminal end (Figure S1 of the Supporting Information). Amino acid sequence analysis using InterProScan revealed that CtManf has two distinct modules: from regions 1 to 134, a noncatalytic carbohydrate binding module family 35 (CBM35) and from 135 to 478, a glycoside hydrolase family 26 (GH26). CtManT found the most similar 50% sequence homology, (UniProt id: Q9F1T9) to mannanase 26B of C. thermocellum strain F1 (CtF1Man26B), Bacillus licheniformis DSM13 (BlMan26B) (33.080%, UniProt id: Q65MP4), Paenibacillus sp. BME-14 (PsMan26B) (37%, UniProt id: C6KL35), and Cellvibrio japonicas (CjMan26B) (35%, UniProt id: Q840B9). Sequence homology was found less pronounced, while comparing with mannanase 26A of Cellulomonas fimi (CfMan26A) (28%, UniProt id: Q9XCV5), and Bacillus sp. JAMB750 (BsMan26A) (26%, UniProt id: Q2ACI1). These results were similarly analyzed from the phylogenetic tree where CtManT was most closely related with Man26B of the C. thermocellum strain F1 (Figure 1). Multiple sequence alignment of CtManT displayed
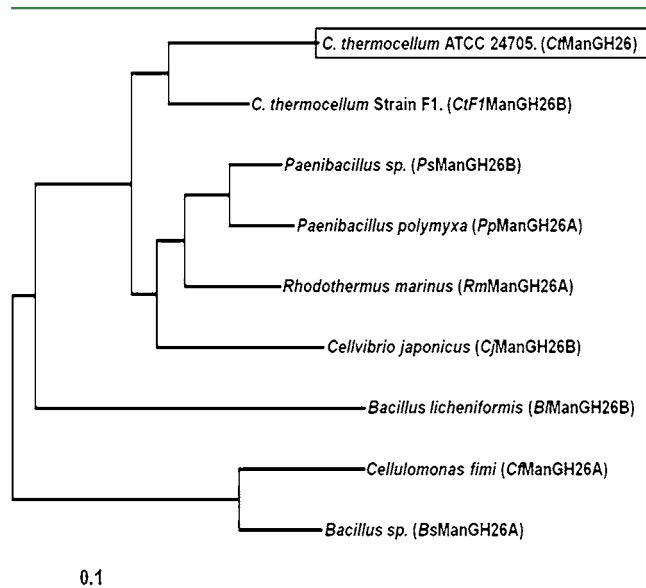
Figure 1. Phylogenetic tree showing the comparative study of our query CtManT (highlighted in red box) with two different types of GH26 mannanase (Man26A and Man26B) of representative members and their appearance during evolution based on sequence similarity.

a unique identifier sequence that was a highly conserved aromatic amino-acid-rich region with the consensus sequence WFWWG within all the ManGH26 family (Figure S2 of the Supporting Information, highlighted in the box), as also similarly stated by Xiaoyu et al. (2010).[21]

Cloning and Expression of CtManf and CtManT. The open-reading frames of CtManT and CtManf amplified by
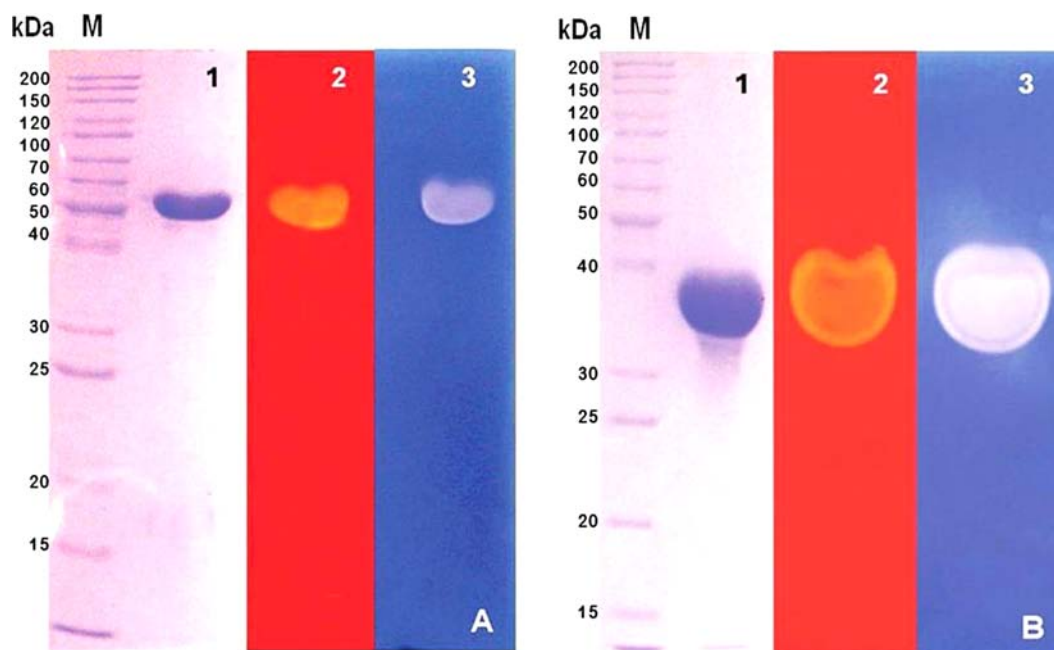
Figure 2. Zymogram study using 12% SDS−PAGE (A) CtManf (panel 1: purified protein, 2: congo red staining, and 3: 1 N HCl counter staining) and (B) CtManT (panel 1: purified protein, 2: congo red staining, and 3: 1 N HCl counter staining).

polymerase chain reaction resulted in 1449 bp and 1029 bp sequences, respectively. The amplified DNAs were cloned into the pET-28a (+) expression vector and then transformed the *E. coli* DH5α cells using the cloned plasmids. The positive clones of CtManf and CtManT were identified by digestion of resulted recombinant plasmids with *NheI-XhoI* restriction enzymes. The *E. coli* BL-21 (DE3) cells were transformed with recombinant plasmids, harboring CtManf and CtManT. The positive clones were screened by growing individual colonies in LB medium and after, inducing with 1 mM IPTG and further incubating at 24 °C for 24 h. The expressed proteins were analyzed by SDS−PAGE. The two *Clostridial* recombinant His$_6$-tagged proteins were purified by immobilized metal ion affinity chromatography (IMAC) from the cell-free extracts to homogeneity. The SDS−PAGE analysis of purified CtManf and CtManT displayed molecular sizes of 53 kDa and 38 kDa, respectively (Figure 2, panels A and B). Both the recombinant proteins expressed as soluble proteins.

Specificity and Kinetic Parameters of CtManf and CtManT with Natural Substrates. The optimum pH and temperature for CtManf were 6.9 and 60 °C, respectively, and for CtManT were 6.5 and 50 °C, respectively. The substrate specificities of CtManf and CtManT with natural substrates were determined at optimized pH and temperature. The enzyme activities with natural substrates are displayed in Table 1. It is conspicuous from the Table 1 that both CtManf and CtManT have specificity for galactomannan, and the highest enzyme activity was achieved with carob galactomannan 97.0 ± 5.0 units mg^{-1} and 91.0 ± 4.0 units mg^{-1}, respectively. Both displayed activity in decreasing order with locust bean galactomannan, konjac glucomannan, and guar galactomannan. Both CtManf and CtManT with insoluble polysaccharide ivory nut mannan displayed a biphasic hydrolysis pattern (Figure S3 of the Supporting Information), where rapid hydrolysis of the substrate occurred up to 15 min of incubation followed by slower hydrolysis (Figure S3 of the Supporting Information). These enzymes perhaps acted on the amorphous sites

Table 1. Substrate Specificity of CtManf and CtManT from C. thermocellum

substrate (1%, w v^{-1})	specific activity CtManf (units mg^{-1})	specific activity CtManT (units mg^{-1})
carob galactomannan	97.0 ± 5.0	91.0 ± 4.0
locust bean galactomannan	85.4 ± 6.0	83.1 ± 5.0
konjac glucomannan	81.0 ± 3.0	79.8 ± 4.0
guar galactomannan	47.6 ± 3.0	38.7 ± 4.0
ivory nut mannan	50.0 ± 2.0	26.5 ± 0.9
mannan	40.0 ± 1.0	21.2 ± 2.0
barley-β-glucan	2.94 ± 0.2	1.74 ± 0.1
lichnan	1.92 ± 0.8	1.22 ± 0.2
carboxymethyl cellulose	1.09 ± 0.5	0.9 ± 0.05
hydroxyethyl cellulose	0.87 ± 0.03	0.47 ± 0.03
Avicel	0.39 ± 0.02	0.26 ± 0.03
xyloglucan	1.5 ± 0.5	1.0 ± 0.3
rye arabinoxylan	NA	NA
glucuronoxylan	NA	NA
arabinogalactan	NA	NA
polygalactouronic acid	NA	NA

Values are in mean ± SD (n = 3). NA = no activity was determined.

(hydrolyzable region) of the substrate during rapid hydrolysis in the first phase and then accessed the crystalline sites (tougher region) in the second phase. Similar results were reported by Mizutani et al. (2012).[41] The enzyme activities of both enzymes with insoluble ivory nut mannan and β-(1→4)-mannan from the first phase were calculated (Table 1). CtManf displayed approximately, two times higher activity than CtManT with both the substrates (Table 1). The specific activity of CtManf was 50.0 units mg^{-1}, whereas CtManT was 26.5 units mg^{-1} with ivory nut mannan and with β-(1→4)-

Table 2. Kinetic Properties and Catalytic Efficiencies of *Ct*Manf and *Ct*ManT from *C. thermocellum* ATCC 27405

	K_m (mg mL^{-1})		k_{cat} (min^{-1})		k_{cat}/K_m (min^{-1} mg^{-1} mL)	
substrate	*Ct*Manf	*Ct*Man T	*Ct*Manf	*Ct*Man T	*Ct*Manf	*Ct*Man T
natural substrates						
carob galactromannan	1.8 ± 0.2	1.6 ± 0.2	737	634	4.1×10^2	3.9×10^2
locust bean galactomannan	1.5 ± 0.1	1.4 ± 0.4	590	520	3.9×10^2	3.7×10^2
konjac glucomannan	1.5 ± 0.3	1.4 ± 0.2	510	462	3.4×10^2	3.3×10^2
guar galactomannan	1.2 ± 0.2	1.1 ± 0.2	320	283	2.6×10^2	2.5×10^2
ivory nut mannan	0.9 ± 0.1	0.8 ± 0.2	310	199	3.4×10^2	2.4×10^2
mannanan	0.9 ± 0.2	0.7 ± 0.1	283	159	3.1×10^2	2.2×10^2
synthetic substrates						
*p*NP-*β*-D-manno- pyranoside	ND	ND	ND	ND	ND	ND
*p*NP-*α*-D-manno- pyranoside	ND	ND	ND	ND	ND	ND

Values are in mean ± SD (n = 3). ND = not detected.

mannan the enzyme activities were 40.0 units mg^{-1} and 21.2 units mg^{-1}, respectively (Table 1). Therefore, the above results indicated that *Ct*CBM35 plays a role in potentiating the enzyme activity of the full length enzyme *Ct*Manf in hydrolyzing the insoluble substrates. A similar comment was stated earlier by Mizutani et al. (2012) for CBM32 appended to GH5 mannanase from *C. thermocellum*.[41]

Both *Ct*Manf and *Ct*ManT displayed low but significant activity against barley *β*-glucan, lichenan, carboxymethyl cellulose, hydroxyethyl cellulose, Avicel and xyloglucan, whereas no activity was observed with arabinogalactan, rye arabinoxylan, glucuronoxylan, and polygalactouronic acid (Table 1). The kinetic properties and catalytic efficiency of both the enzymes were determined with the natural substrates (Table 2). *Ct*Manf and *Ct*ManT displayed turnover numbers (k_{cat}) of 698 and 684 min^{-1}, respectively, and catalytic efficiencies (k_{cat}/K_m) of 4.1×10^2 and 3.9×10^2 min^{-1} mg^{-1} mL, respectively, with carob galactomannan. Both the enzymes *Ct*Manf and *Ct*ManT efficiently acted on insoluble ivory nut mannan, showing catalytic efficiencies (k_{cat}/K_m) of 3.4×10^2 and 2.4×10^2 min^{-1} mg^{-1} mL and with *β*-(1→4)-mannan, 3.1×10^2 and 2.2×10^2 min^{-1} mg^{-1} mL, respectively (Table 2).

The present results showed that *Ct*Manf gave approximately 1.1-fold higher activity against carob galactomannan and approximately 2-fold higher activity against insoluble ivory nut mannan and *β*-(1→4)-mannan than the catalytic *Ct*ManT. Similar results were reported earlier, where enhanced activity in the presence of a carbohydrate binding domain (CBD) in Man26A (mannanase A from family GH26) was observed by Halstead et al. (1999).[8] The presence of CBD at the N-terminal of Man26A enhanced turnover of carob galactomannan by 1.1-fold and 2-fold against insoluble ivory nut mannan.[8]

Specificity and Kinetic Parameters *Ct*Manf and *Ct*ManT with Synthetic Substrates. Both *Ct*Manf and *Ct*ManT did not show any activity against *p*NP-*β*-D-mannopyranoside and with *p*NP-*α*-D-mannopyranoside. On the basis of the enzyme activity of *Ct*Manf and *Ct*ManT against natural as well as synthetic substrates, it was evident that both these enzymes are predominantly endo-*β*-D-mannanase. The enzymes specifically cleaved the *β*-(1→4)-glycosidic linkages between mannopyranosyl residues.

Zymogram Study of *Ct*Manf and *Ct*ManT. Separate SDS−PAGE gels were used in the zymogram study to show the active bands of *Ct*Manf and *Ct*ManT against carob galactomannan (Figure 2, panels A and B). *Ct*Manf displayed an active band around 53 kDa and *Ct*ManT around 38 kDa, congo red staining, and counter staining with 1 N HCl (Figure

2, panels A and B). Both the enzymes displayed homogeneous bands and a clear zone of activity with carob galactomannan. Mannan endo-*β*-(1→4)-mannanase activity was detected as clear zones against red (after staining with Congo red) and blue background (after counter stained with 1 N HCl). The results clearly indicated that both of these enzymes have manno-configured substrate specificity.

Effects of Metal Ions and Chemical Agents on *Ct*Manf and *Ct*ManT. The enzymatic activity of *Ct*Manf and *Ct*ManT significantly increased by 1.5-fold in the presence of low concentrations of Ca^{2+} (10 mM) and Mg^{2+} (15 mM) (Table 3).

Table 3. Effects of Metal Ions and Other Agents on *Ct*Manf and *Ct*ManT from *C. thermocellum* ATCC 27405

|		relative activity (%)	
ions/reagents	concentration (mm)	*Ct*Manf	*Ct*ManT
control[a]	−	100	100
Ca^{2+}	10	150	150
Mg^{2+}	15	150	150
Mn^{2+}	10	80	60
Ni^{2+}	8	80	80
Co$^+$	30	70	70
Zn^{2+}	10	70	60
Cu^{2+}	5	20	10
Al^{3+}	6	20	10
EDTA	8	20	20
EGTA	10	20	20
SDS	8	6	2
urea	4×10^3 (4 M)	10	5
GnHCl	100	2	1

[a]No additives were added in the control, and the activity was taken as 100%.

Both *Ct*Manf and *Ct*ManT retained moderate activities in the presence of 10 mM Mn^{2+} (80% and 60%), 8 mM Ni^{2+} (80% and 80%), 30 mM Co^{2+} (70% and 70%), and 10 mM Zn^{2+} (70% and 60%) salts, respectively. The enzyme activities were adversely affected by low concentrations of Cu^{2+} (5 mM) or Al^{3+} (6 mM) salts, and *Ct*Manf lost 80% and *Ct*ManT lost 90% of the activity at the mentioned concentrations of Cu^{2+} and Al^{3+} salts (Table 3). The enzyme activity of both the catalytic modules decreased to more than 80% in the presence of EDTA (8 mM) or 10 mM EGTA (Table 3). The presence of SDS (10 mM) *Ct*Manf lost 94% enzyme activity, while *Ct*ManT almost completely lost the activity. The decrease in activity in the presence of EDTA indicated that Ca^{2+} ions may be essential for

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enzyme activity as EDTA and specifically binds and chelates the calcium ions in a 1:1 molar ratio.[36] The enzyme activity drastically reduced with chaotropic agents such as guanidine hydrochloride (GnHCl) and urea at higher concentrations. The enzymatic activity of *Ct*Manf decreased by 90% at 100 mM GnHCl, whereas *Ct*ManT lost 95% activity at the same concentration of GnHCl. In contrast, much higher concentration of urea (4 M) was required for complete diminution (98%−99%) of enzyme activities of *Ct*Manf and *Ct*ManT.

Analysis of Polysaccharide Hydrolysis Products by TLC. The analysis of recombinant *Ct*Manf hydrolyzed products of carob galactomannan by TLC is displayed in Figure 3A.
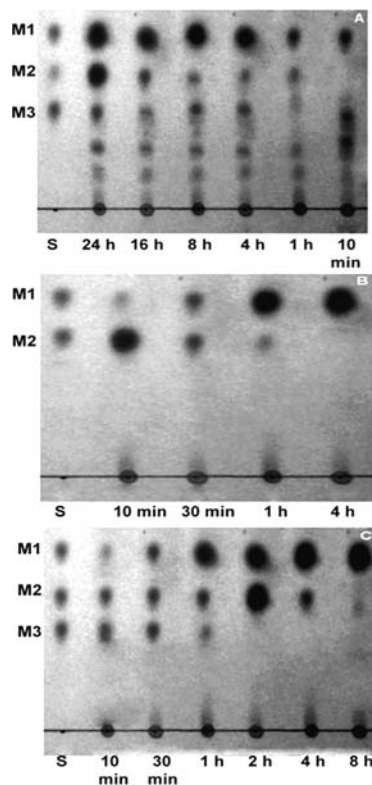
Figure 3. Thin layer chromatography analysis of hydrolysis products from (A) carob galactomannan, (B) mannobiose, and (C) mannotriose by *Ct*Manf. (A) Carob galactoniannan (1%, w v^{-1}) was incubated with *Ct*Manf for 10 min to 24 h, (B) mannobiose (1 mg mL^{-1}) was incubated with *Ct*Manf for 10 min to 4 h, and (C) mannotriose (1 mg mL^{-1}) was incubated with *Ct*Manf for 10 min to 8 h. Samples were taken in intervals, and hydrolysates were analyzed by TLC (standards used M1: mannose, M2: mannobiose, and M3: mannotriose).
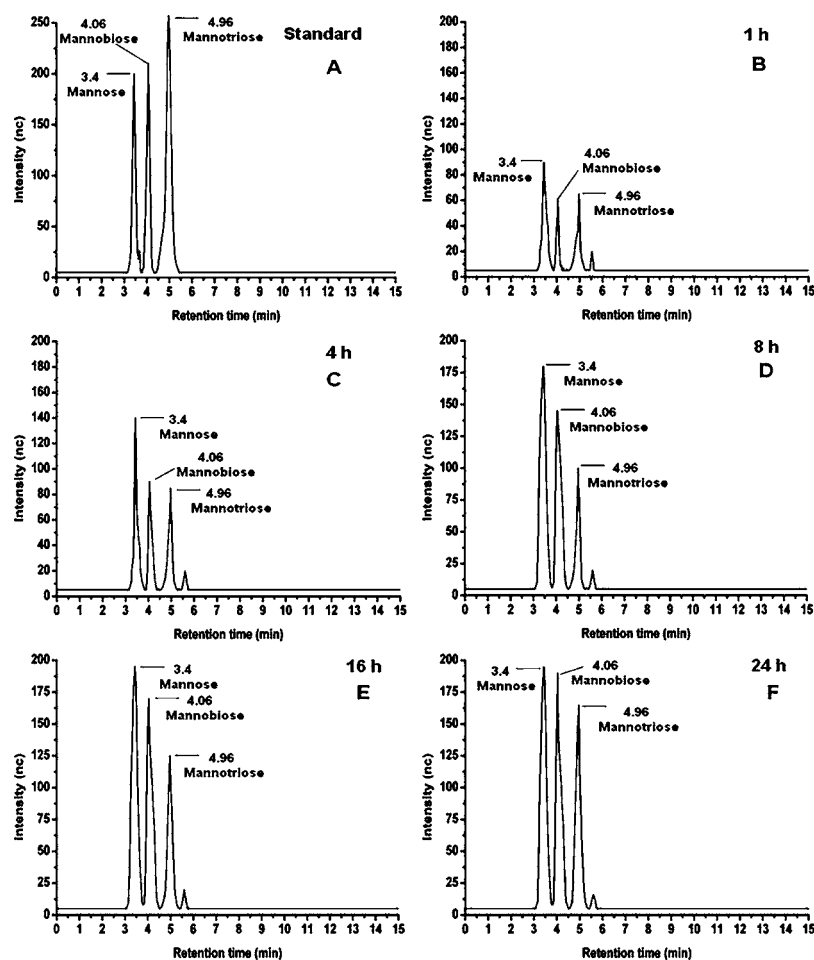
Time-dependent hydrolysis of carob galactomannan after 10 min of reaction displayed the release of mannose (dp 1), but after 1 h of consecutive hydrolysis there was a little release of mannobiose (dp 2) and mannotriose (dp 3) (Figure 3 A). Standards of mono and oligosaccharides (M1: mannose, M2: mannobiose, and M3: mannotriose) were run in parallel to compare the released sugars (Figure 3A). Up to 8 h of incubation with *Ct*Manf, carob galactomannan was not completely hydrolyzed, but after 16 h, a large amount of mannose and comparable amounts of mannobiose and mannotriose were released (Figure 3A). Complete hydrolysis of carob galactomannan was achieved after 24 h of incubation, where the maximum amount of mannose, mannobiose, and

mannotriose were produced (Figure 3A). Apart from the appearance of dp 1, dp 2, and dp 3, there were two other higher oligosaccharide (dp 4 and dp 5) spots observed and displayed similar appearance throughout the hydrolysis process (Figure 3A). *Ct*Manf displayed a typical endoacting bond cleavage during mannobiose and mannotriose hydrolysis, releasing principally mannose and mannobiose (Figure 3, panels B and C, respectively). *Ct*Manf was unable to hydrolyze mannobiose up to 1 h, but after 4 h, complete hydrolysis of mannobiose occurred, leaving only a mannose spot on the TLC plate (Figure 3B). Whereas, the hydrolysis mannotriose by *Ct*Manf after 4 h released predominantly mannose and a trace amount of mannobiose. The mannobiose spot disappeared completely after 8 h, leaving only the mannose spot (Figure 3C). Thus *Ct*Manf cleaved β-(1→4) bonds of these manno-oligosaccharides elegantly. Therefore, based on the significant role in specifically cleaving the β-(1→4) bond, this enzyme was classified and named as endo-β-(1→4)-mannanase.

HPAEC Analysis of Enzyme Reaction Products. Qualitative and quantitative analysis of *Ct*Manf hydrolyzed products of carob galactomannan were monitored by HPAEC-PAD. Time-dependent hydrolysis of carob galactomannan by *Ct*Manf is displayed in Figure 4. The peak intensities of standards are displayed in Figure 4A. After 1 h of *Ct*Manf treatment of carob galactomannan, the prominent peaks of mannose at 3.4 min, mannobiose at 4.06 min, and mannotriose at 4.96 min were observed with concentrations 2.12, 0.73, and 0.76 mg mL^{-1}, respectively (Figure 4B). Mannobiose peak was more prominent than mannotriose after 4 h of carob galactomannan hydrolysis, while the mannose intensity increased continuously (Figure 4C). The concentrations were determined as 2.23 mg mL^{-1} mannose, 1.12 mg mL^{-1} mannobiose, and 0.80 mg mL^{-1} mannotriose after 4 h of incubation. *Ct*Manf was able to hydrolyze carob galactomannan to a greater extent after 8 h, and the products obtained were mannose (3.16 mg mL^{-1}), mannobiose (1.81 mg mL^{-1}), and mannotriose (0.90 mg mL^{-1}), with much higher peak intensities (Figure 4D). After a 16 h incubation, the mannobiose and mannotriose concentrations increased to 2.1 mg mL^{-1} and 1.1 mg mL^{-1}, respectively. This increase was approximately 1.17 fold and 1.3 fold, respectively, for mannobiose and mannotriose (Figure 4E), as compared with that obtained after 8 h hydrolysis of carob galactomannan. The complete hydrolysis of carob galactomannan by *Ct*Manf was observed after 24 h of enzymatic reaction, where mannose, mannobiose, and mannotriose concentrations of 3.6 mg mL^{-1}, 2.3 mg mL^{-1}, and 1.4 mg mL^{-1}, respectively, were obtained (Figure 4F). All the concentrations were determined from the regression equation of mannose, mannobiose, and mannotriose standard curves. Therefore, *Ct*Manf quite effectively hydrolyzed 10 mg mL^{-1} (1%, w v^{-1}) carob galactomannan and released a maximum after 24 h, yielding 36% mannose, 23% mannobiose, and 14% mannotriose. The overall results quite interestingly described the performance of *Ct*Manf in the releasing of manno-oligosaccharides from carob galactomannan, which can be scaled up for commercial production.

Production of Enzymes (*Ct*Manf and *Ct*ManT) in Different Media. The highest concentration of recombinant proteins were obtained from a 100 mL flask containing TY and TB media, followed by LB and 5xLB media after 24 h of incubation. TY medium achieved the highest cell densities of 31 and 30 g L^{-1} dry cell weight, respectively, for *Ct*Manf and *Ct*ManT. The protein concentration of *Ct*Manf and *Ct*ManT

Figure 4. HPAEC-PAD analysis of hydrolyzed products of carob galactomannan by CtManf. (A) Elution patterns of standards used mannose (3.4 min), mannobiose (4.06 min), and mannotriose (4.96 min). Elution pattern of mannose, mannobiose, and mannotriose from carob galactomannan (1%, w v^{-1}) treated by CtManf after (B) 1, (C) 4, (D) 8, (E) 16, and (F) 24 h.

after sonication and purification by IMAC obtained was 910 and 880 mg L^{-1}, respectively. Similar results were reported by Tripathi et al. (2009),[38] where TY medium gave the highest cell density of E. coli cells achieved, 1.12 g L^{-1}, and the recombinant dengue protein was expressed at a concentration of 10.37 mg L^{-1}.[38] CtManf and CtManT gave less DCW with TB medium 28 g L^{-1} and 26.9 g L^{-1}, respectively, as compared with TY medium. The purified protein concentrations from TB medium obtained were 500 mg L^{-1} and 410 mg L^{-1}, respectively, for CtManf and CtManT. The dry cell weight (DCW) from 100 mL LB medium obtained 21 and 20 g L^{-1} for CtManf and CtManT, respectively. The concentrations of purified protein obtained from LB medium were 160 mg L^{-1} for CtManf and 150 mg L^{-1} for CtManT. These results are similar to those of the earlier report of Tripathi et al. (2009).[37] The lowest growth of cell mass of E. coli BL21 (DE3) cells were observed for both enzymes in 5xLB medium. The cell densities obtained were 12.0 and 10 g L^{-1}, respectively, for CtManf and CtManT. The recombinant CtManf and CtManT proteins after purification obtained were 300 mg L^{-1} and 280 mg L^{-1} in 5xLB medium, respectively. Therefore, it can be suggested that productivity of recombinant mannanase and cell mass can be improved by altering and optimizing media components.

Thermostability Study and Protein Melting Analysis of CtManf and CtManT. Thermostability study displayed stability of CtManf and CtManT at higher temperatures (Figure

5 A). CtManf remained stable up to 60 °C, retaining 100% activity for 1 h. The enzyme activity of CtManf decreased after 60 °C and left with 20% at 100 °C. CtManf and CtManT retained more than 55% and 30% enzyme activity at 80 °C. CtManT was stable up to 50 °C and lost 90% at 100 °C. Therefore, CtManf was more thermostable than CtManT. The results indicated that the higher thermostability of CtManf could be due to the presence of the carbohydrate binding module, CtCBM35. Protein stability was also observed with protein melting curve analysis. The full-length CtManf showed two separate melting peaks at 50 and 80 °C (Figure 5 B), whereas CtManT displayed a single melting peak at around 80 °C (Figure 5C). This suggested that the peak at 50 °C corresponded to noncatalytic CtCBM35 and the peak at 80 °C to the catalytic module CtManT and that the two modules are melting independently (Figure 5B). The presence of Ca^{2+} ions (10 mM) caused significant changes in CtManf, as well as in CtManT protein-melting profiles. The peak for CtManT shifted toward higher a temperature (i.e., from 80 to 100 °C), but the peak corresponding to CtCBM35 in CtManf was masked in the presence of Ca^{2+} ions (Figure 5, panels B and C). On addition of EDTA (10 mM) to the enzyme−substrate reaction mixture containing Ca^{2+} (10 mM), the melting peaks shifted back to the original temperature of 80 °C of catalytic CtManT and CtCBM35 (Figure 5, panels B and C, small dotted lines). Therefore, from both thermostability and protein melting study
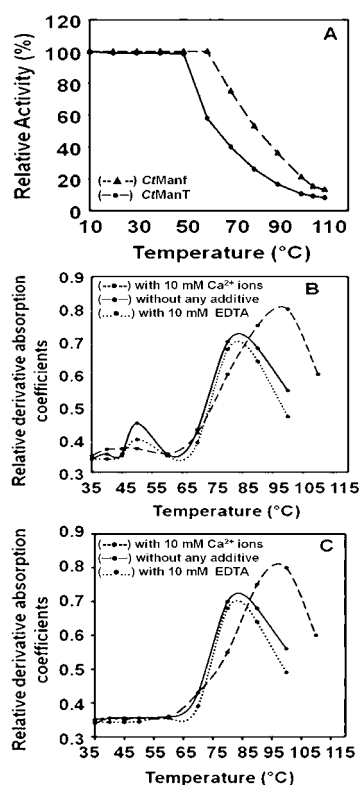
Figure 5. Thermal stability analysis of (A) *Ct*Manf and *Ct*ManT from 10 to 110 °C. Protein-melting analysis of displaying normal melting curve without any additives, in the presence of 10 mM Ca^{2+} ions and in the presence of 10 mM Ca^{2+} ions and 10 mM EDTA of (B) *Ct*Manf and (C) *Ct*ManT.

of *Ct*Manf and *Ct*ManT, it could be concluded that both these enzymes are thermostable and Ca^{2+} ions provide significant thermal stability.

■ DISCUSSION

The molecular architecture of full length *Ct*Manf from *C. thermocellum* ATCC 27405 displayed a modular structure. Sequence homology of the catalytic module *Ct*ManT identified as Man26B, showed highest similarity with that of the *C. thermocellum* F1 strain.[21] The Man26B enzymes have subtle differences with Man26A enzymes in topology of substrate binding and functional property of substrate hydrolysis as previously described by Hogg et al. (2003) in a study of Man26A and Man26B from *C. japonicas*.[22] Both *Ct*Manf and *Ct*ManT preferred higher degrees of catalysis against carob galactomannan than locust bean galactomannan and konjac glucomannan, while similar observations were stated earlier by Xiaoyu et al. (2010) and Kurokawa et al. (2001) for Man26B isolated from *Paenibacillus* sp. BME-14 and *C. thermocellum* strain F1, respectively.[21,23] It is worth mentioning here about the wide range of substrate specificity of both *Ct*Manf and *Ct*ManT. Both these enzymes were able to hydrolyze β-(1→4)-gluco based substrates viz. barley β-glucan, lichenan, carboxymethyl cellulose, hydroxyethyl cellulose, Avicel, and xyloglucan. ManA from *Thermoanaerobacterium polysaccharolyticum* similarly showed both mannanase and endoglucanase activities.[42] Low activities of *Ct*Manf and *Ct*ManT against ivory nut mannan and β-(1→4) may be attributed to the crystalline nature of the substrate, which greatly inhibits the access of enzymes for catalysis. Interestingly, *Ct*Manf exhibited 2-fold

higher activities against insoluble ivory nut mannan and β-(1→4)-mannan, as compared to *Ct*ManT. By comparing the hydrolyzing capacity of *Ct*Manf and *Ct*ManT against different mannans, it was concluded that the *Ct*CBM35 of *C. thermocellum* ATCC 27405 GH26 mannanase played an important role in the degradation of insoluble ivory nut mannan and β-(1→4) (Table 2). The apparent biphasic action of *Ct*Manf and *Ct*ManT against ivory nut mannan and β-(1→4)-mannan was quite distinguishable and thus suggested that both these enzymes preferred to attack the amorphous region of the substrate in the early reaction stage and the crystalline region later. This might give a lucid idea about the substrates that consist of two distinct regions, which was more resistant toward *Ct*ManT action. Similar, types of observations were reported by Mizutani et al. (2012),[41] while comparing the role of Man26A from *C. thermocellum* against insoluble substrate hydrolysis. Higher degree of hydrolyzing capacity of *Ct*Manf than *Ct*ManT was attributed to the appended N-terminal *Ct*CBM35 domain which facilitated the increased catalysis by concentrating the catalytic module in the vicinity of the substrate. *Ct*CBM35 helped in prolonged binding of the substrate and decreased its resistance to the catalytic attack by *Ct*ManT. As an instance CBM32 from *C. thermocellum* ATCC 27405 has recently been shown to improve hydrolysis of insoluble substrates by mannanase of GH5.[38] Moreover, higher activity of *Ct*Manf at a higher temperature was attributed to the presence of N-terminal carbohydrate binding domain (*Ct*CBM35) than catalytic *Ct*ManT. Similarly in an earlier report, it was suggested by Xiaoyu et al. (2009),[21] the appended carbohydrate binding domain in Man26B of *Paenibacillus* sp. BME-14 potentiated in higher activity against locust bean gum than the lone catalytic module.[21] The presence of *Ct*CBM35 in *Ct*Manf played a unique role in higher hydrolysis of soluble carob galactomannan as compared with the catalytic *Ct*ManT. Both enzymes exhibited significant turn over against other soluble as well as insoluble substrates. It was reported previously that carob galactomannan composed of 78% mannose formed the β-(1→4)-mannan backbone, while galactose contributes 22%.[1] Each β-(1→4)-mannan is substituted by the α-(1→6)-galactose side chain. The enzymes randomly hydrolyze β-(1→4)-linkages in diverse substrates, such as galactomannans and glucomannan. Both *Ct*Manf and *Ct*ManT did not show any activity against synthetic substrates *p*NP-β-mannopyranoside and *p*NP-α-mannopyranoside. Therefore, it is evident that both *Ct*Manf and *Ct*ManT are endo-acting enzymes and are endo-β-(1→4)-mannanases (endo-Man26B) from *C. thermocellum* ATCC 27405. The performances of *Ct*Manf and *Ct*ManT were investigated under the influence of salts, chaotropic agents, and detergent. Both *Ct*Manf and *Ct*ManT are metalloenzymes, and Ca^{2+} and Mg^{2+} ions act as cofactors for these enzymes. The enzyme activities of *Ct*Manf and *Ct*ManT increased significantly by 1.5-fold in the presence of Ca^{2+} and Mg^{2+} salts, which suggested that these ions are required as cofactors. However, the enzyme activity was unaffected by lower concentrations of Mn^{2+}, Ni^{2+}, Co^+, and Zn^{2+} and were able to retain their moderate activities. The enzyme activities of *Ct*Manf and *Ct*ManT were completely inhibited by lower concentrations of Cu^{2+} and Al^{3+}. Similar observation was reported earlier by Yoshikawa et al. (2009), where a noncompetitive type of inhibition imposed by Cu^{2+} ions by binding at different sites other than the α-glucosidase active center or enzyme−substrate complex.[43] In the presence of a low concentration of chelating agents such as EDTA or

EGTA, the enzyme activity sharply decreased. The enzyme activity of CtManf and CtManT was adversely affected at the low concentration of SDS. The higher concentration urea and lower concentration of guanidine hydrochloride was required to inactivate the enzymes.

CtManf-catalyzed substrate hydrolysis products were analyzed by TLC and HPAEC. It was apparent from TLC that CtManf released mannotriose, mannobiose, and mannose in prolonged hydrolysis of carob galactomannan. But, in the earlier stages, the amount of mannobiose was less, as compared to mannotriose and mannose. After complete hydrolysis of carob galactomannan, CtManf was able to release mannose, mannobiose, and mannotriose. The salient feature of CtManf catalysis involved only β-(1→4)-bond cleavage, when mannobiose and mannotriose were used as substrates and liberated mainly mannose as the main product. The release of large amounts of mannose at early stages of enzymatic reaction by CtManf was commonly seen against carob galactomannan and manno-oligosaccharides. In an earlier report by Hogg et al. (2003), the release of mannose at the early stage of hydrolysis of manno-configured substrates and oligosaccharides is characteristic of a typical Man26B mannanase.[22] HPAEC analysis corroborated the results of TLC analysis of hydrolysis of carob galactomannan products released by CtManf. The results of HPAEC showed that CtManf exclusively cleaves carob galactomannan into mannotriose, mannobiose, and mannose. It was apparent from TLC and HPAEC analyses that CtManf was able to hydrolyze only the β-(1→4) bond cleavage and had potential to produce manno-oligosaccharides from carob galactomannan. Thus effective β-(1→4)-mannanase from C. thermocellum ATCC 27405 may be exploited for higher production of manno-oligosaccharides, especially for the controlled synthesis of mannobiose and mannotriose. Kurakake et al. (2006) reported the synthesis of manno-oligosaccharides from guar gum by utilizing of β-mannanase from Penicillium oxalicum SO.[44]

Media composition plays a significant role in production of recombinant proteins.[38] Use of chemically defined medium is a common practice in producing recombinant proteins.[45−47] The recombinant CtManf and CtManT showed the highest cell density and concentration of protein in TY medium. In TB medium, moderate cell densities and protein production were observed. In LB medium, moderate cell density was achieved with low protein concentration. The 5xLB medium did not support the growth due to higher concentrations of yeast extract, tryptone, and sodium chloride, and as a result, the lowest protein concentration was achieved. Similar effects of media were stated earlier, while producing recombinant dengue protein in E. coli.[38] The rich source of tryptone, yeast extract, and phosphate salts facilitated to achieve highest cell densities in TY media as compared to other chemically defined media used. Yeast extract is a known source of trace components and can relieve cellular stress responses such as the production of proteases during synthesis of recombinant protein in E. coli.[38] Higher concentration of phosphate is important for attaining high cell densities of E. coli, as the lower concentrations of phosphate limits the growth.[37] The phosphate salts in the medium provided buffering capacity against pH fluctuations, which adversely affects the metabolic activity of cells.[38] The low cell densities and lower production of recombinant proteins in LB and 5xLB medium were due to a lack of buffering capacity.

Protein stability while functioning at higher temperature is a major concern in industry. A temperature stability study of CtManf and CtManT showed that after 1 h of incubation at 60 and 50 °C, respectively, 100% activity was retained. But they have retained around 10% of enzymatic activity at 100 °C. When compared with recombinant ManB from Bacillus licheniforms DSM13,[24] the recombinant Man26B from C. thermocellum ATCC 27405 was thermally more stable at higher temperatures. The protein melting phenomenon of recombinant CtManf and CtManT was analyzed to study their thermostability. Protein-melting curves of full length CtManf showed that the catalytic module CtManT and carbohydrate binding module CtCBM35 melt independently of each other. The protein-melting peaks of CtManT and CtCBM35 shifted to higher temperature in the presence of Ca^{2+} ions. However, on addition of equimolar concentration of EDTA to the solutions of CtManf and CtManT, the melting temperature peaks shifted back to the original positions. The shift of peak to a higher temperature in the presence of Ca^{2+} ions might be due to the reason that Ca^{2+} ions provide stability to the protein structure by inducing electrostatic interactions with amino acids, as reported by Noorbatcha et al. (2012).[48] The electrostatic interactions imparted by Ca^{2+} ions in bound protein resulted in less hydrogen bonds and higher number of salt bridges as compared to nonbonded proteins.[48] Because of the higher fluctuations in the backbone of protein at higher temperature, the number of hydrogen bonds will be destabilized, which allowed residues in close proximity to calcium ions to form more numbers of salt bridges in the Ca^{2+} ion-bound state as compared with the Ca^{2+} ion-free state.[48] Thus, the binding by Ca^{2+} ion makes protein more conformationally stable at higher temperature.[47] The shifting back of melting peaks in the presence of EDTA was due to chelation of calcium ions, making them unavailable for the enzyme. This is the first report of cloning and biochemical characterization of a thermostable Man26B form C. thermocellum ATCC 27405 and its potential role in manno-oligosaccharide production from manno-configured substrates.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

Molecular architecture of full length CtManf of C. thermocellum ATCC 27405; multiple sequence alignment of CtManT with Man26A from Cellulomonas fimi; and biphasic hydrolysis pattern of insoluble ivory nut mannan. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ REFERENCES

(1) Hirst, E. L.; Jones, J. K. N. The galactomannan of carob-seed gum (gum gatto). *J. Chem. Soc.* **1948**, 1278−1282.

(2) Katsuraya, K.; Okuyamab, K.; Hatanakab, K.; Oshimab, R.; Satoc, T.; Matsuzakic, K. Constitution of konjac glucomannan: Chemical analysis and ^{13}C NMR spectroscopy. *Carbohydr. Polym.* **2003**, *53*, 183−189.

(3) Dumitriu, S. In *Polysaccharides: Structural diversity and functional versatility*, 2nd ed.; CRC Press: Boca Raton, FL, 2010; pp 1020.

(4) Henrissat, B. A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* **1991**, *280*, 309−316.

(5) Bolam, D. N.; Hughes, N.; Virden, R.; Lakey, J. H.; Hazlewood, G. P.; Henrissat, B.; Braithwaite, K. L.; Gilbert, H. J. Mannanase A from *Pseudomonas fluorescens* ssp. *cellulosa* is a retaining glycosyl hydrolase in which E212 and E320 are the putative catalytic residues. *Biochemistry* **1996**, *35*, 16195−16204.

(6) Zhang, Y.; Ju, J.; Peng, H.; Gao, F.; Zhou, C.; Zeng, Y.; Xue, Y.; Li, Y.; Henrissat, B.; Gao, G. F.; Ma, Y. Biochemical and structural characterization of the intracellular mannanase AaManA of *Alicyclobacillus acidocaldarius* reveals a novel glycoside hydrolase family belonging to clan GH-A. *J. Biol. Chem.* **2008**, *14*, 31551−31558.

(7) Couturier, M.; Roussel, A.; Rosengren, A.; Leone, P.; Stålbrand, H.; Berrin, J. G. Structural and biochemical analyses of glycoside hydrolase families 5 and 26 beta-(1,4)-mannanases from *Podospora anserina* reveal differences upon manno-oligosaccharides catalysis. *J. Biol. Chem.* **2013**, DOI: 10.1074/jbc.M113.459438.

(8) Halstead, J. R.; Vercoe, P. E.; Gilbert, H. J.; Davidson, K.; Hazlewood, G. P. A family 26 mannanase produced by *C. thermocellum* as a component of the cellulosome contains a domain which is conserved in mannanases from anaerobic fungi. *Microbiology* **1999**, *145*, 3101−3108.

(9) Hogg, D.; Woo, E. J.; Bolam, D. N.; McKie, V. A.; Gilbert, H. J.; Pickersgill, R. W. Crystal structure of mannanase 26A from *Pseudomonas cellulosa* and analysis of residues involved in substrate binding. *J. Biol. Chem.* **2001**, *276*, 31186−31192.

(10) Cartmell, A.; Topakas, E.; Ducros, M. A. V.; Suits, M. D. L.; Davis, G. J.; Gilbert, H. J. The *Cellvibrio japonicus* mannanase CjMan26C displays a unique exo-mode of action that is conferred by subtle changes to the distal region of the active site. *J. Biol. Chem.* **2008**, *283*, 34403−34413.

(11) Fontes, C. M. G. A.; Gilbert, H. J. Cellulosomes: Highly efficient nanomachines designed to deconstruct plant cell wall complex carbohydrates. *Annu. Rev. Biochem.* **2010**, *79*, 655−681.

(12) Asano, I.; Hamaguchi, K.; Fujii, S.; Iino, H. In vitro digestibility and fermentation of mannooligosaccharides from coffee mannan. *Food. Sci. Technol. Res.* **2003**, *9* (1), 62−66.

(13) Asano, I.; Ikeda, Y.; Fujii, S.; Iino, H. Effects of mannooligosaccharides from coffee on microbiota and short chain fatty acids in rat cecum. *Food. Sci. Technol. Res.* **2004**, *10*, 273−277.

(14) Kim, Y. J.; Park, G. G. Identification and growth activity to *Bifidobacterium* spp. of locust bean gum hydrolysates by *Trichoderma harzianum* β-mannanase. *J. Korean Soc. Appl. Biol. Chem.* **2005**, *48*, 364−369.

(15) Hoshino-Takao, U.; Fujii, S.; Ishii, A.; Han, L. K.; Okuda, H.; Kumao, T. Effects of mannooligosaccharides from coffee mannan on blood pressure in dahl salt-sensitive rats. *J. Nutr. Sci. Vitaminol.* **2008**, *54*, 181−184.

(16) Fierobe, H. P.; Mingardon, F.; Mechaly, A.; Belaich, A.; Rincon, M. T; Pagès, S.; Lamed, R.; Tardif, C.; Bélaïch, J. P.; Bayer, E. A. Action of designer cellulosomes on homogeneous versus complex substrates: Controlled incorporation of three distinct enzymes into a defined trifunctional scaffoldin. *J. Biol. Chem.* **2005**, *280*, 1625−1634.

(17) Boraston, A. B.; Bolam, D. N.; Gilbert, H. J.; Davies, G. J. Carbohydrate-binding modules: Fine-tuning polysaccharide recognition. *Biochem. J.* **2004**, *382*, 769−781.

(18) Dvortsov, I. A.; Lunina, N. A.; Chekanovskaya, L. A.; Schwarz, W. H.; Zverlov, V. V.; Velikodvorskaya, G. A. Carbohydrate-binding properties of a separately folding protein module from beta-1,3-glucanase Lic16A of *C. thermocellum. Microbiology* **2009**, *155*, 2442−2449.

(19) Shoseyov, O.; Shani, Z.; Levy, I. Carbohydrate binding modules: Biochemical properties and novel applications. *Microbiol. Mol. Biol. Rev.* **2006**, *70*, 283−295.

(20) Couturier, M.; Roussel, A.; Rosengren, A.; Leone, P.; Stålbrand, H.; Berrin, J. G. Glycoside hydrolase families 5 and 26 β-(1,4)-mannanases from *Podospora anserina* reveal differences upon manno-oligosaccharide catalysis. *J. Biol. Chem.* **2013**, *288*, 14624−14635.

(21) Xiaoyu, F.; Huang, X.; Liu, P.; Lin, L.; Wu, G.; Li, C.; Feng, C.; Hong, Y. Cloning and characterization of a novel mannanase from *Paenibacillus sp.* BME-14. *J. Microbiol. Biotechnol.* **2010**, *20*, 518−524.

(22) Hogg, D.; Pell, G.; Dupree, P.; Goubet, F.; Martin-Oru, S. M.; Armand, S.; Gilbert, H. J. The modular architecture of *Cellvibrio japonicus* mannanases in glycoside hydrolase families 5 and 26 points to differences in their role in mannan degradation. *Biochem. J.* **2003**, *371*, 1027−1043.

(23) Kurokawa, J.; Hemjinda, E.; Arai, T.; Karita, S.; Kimura, T.; Sakka, K.; Ohmiya, K. Sequence of the *C. thermocellum* mannanase gene man26B and characterization of the translated product. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 548−554.

(24) Songsiriritthigul, C.; Buranabanyat, B.; Haltrich, D.; Yamabhai, M. Efficient recombinant expression and secretion of a thermostable GH26 mannan endo-1,4-β-mannosidase from *Bacillus licheniformis* in *Escherichia coli. Microb. Cell Fact.* **2010**, *9* (20), 1−13.

(25) Stoll, D.; Boraston, A.; Stålbrand, H.; Warren, R. A. Mannan-degrading enzymes from *Cellulomonas fimi. Appl. Environ. Microbiol.* **1999**, *65*, 2598−2605.

(26) Politz, O.; Krah, M.; Thomsen, K. K.; Borriss, R. A highly thermostable endo-(1,4)-beta-mannanase from the marine bacterium *Rhodothermus marinus. Appl. Microbiol. Biotechnol.* **2000**, *53*, 715−721.

(27) Hatada, Y.; Takeda, N.; Hirasawa, K.; Ohta, Y.; Usami, R.; Yoshida, Y.; Grant, W. D.; Ito, S.; Horikoshi, K. Sequence of the gene for a high-alkaline mannanase from an alkaliphilic *Bacillus sp.* strain JAMB-750, its expression in *Bacillus subtilis* and characterization of the recombinant enzyme. *Extremophiles* **2005**, *9*, 497−500.

(28) Cho, K. M.; Hong, S. Y.; Lee, S. M.; Kim, Y. H.; Kahng, G. G; Kim, H.; Yun, H. D. A cel44C-man26A gene of endophytic *Paenibacillus polymyxa* GS01 has multi-glycosyl hydrolases in two catalytic domains. *Appl. Microbiol. Biotechnol.* **2006**, *73*, 618−630.

(29) Das, S. P.; Ravindran, R.; Ahmed, S.; Das, D.; Goyal, D.; Fontes, C. M. G. A.; Goyal, A. Bioethanol production involving recombinant *C. thermocellum* hydrolytic hemicellulase and fermentative microbes. *Appl. Biochem. Biotechnol.* **2012**, *127*, 1475−1488.

(30) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(31) Nelson, N. A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.* **1944**, *153*, 375−380.

(32) Somogyi, M. A new reagent for the determination of sugars. *J. Biol. Chem.* **1945**, *160*, 61−68.

(33) Huang, J. L.; Bao, L. X.; Zou, H. Y.; Che, S. G.; Wang, G. X. High-level production of a cold-active B-mannanase from Bacillus subtilis BS5 and its molecular cloning and expression. *Mol. Genet., Mikrobiol. Virusol.* **2012**, *4*, 14−27.

(34) Bey, M.; Berrin, J. G.; Poidevin, L.; Sigoillot, J. C. Heterologous expression of *Pycnoporus cinnabarinus* cellobiose dehydrogenase in *Pichia pastoris* and involvement in saccharification processes. *Microb. Cell Fact.* **2011**, *10*, 1−15.

(35) Aboul-Enein, A.; Abouelalla, F.; Serour, E.; Hussien, T. Purification and characterization of a novel thermoactive cellulase from thermophilic actinomycetes isolated from soil sample of Egypt. *J. Int. Acad. Res* **2010**, *2*, 81−86.

(36) Ruijssennars, H. J.; Hartmans, S. Plate screening method for the detection of polysaccharide- producing microorganisms. *Appl. Microbiol. Biotechnol.* **2001**, *55*, 143−149.

(37) Cote, G. L.; Leathers, T. D. A method for surveying and classifying *Leuconostocs* pp Glucansucrases according to strain-

dependent acceptor product patterns. *J. Ind. Microbiol. Biotechnol.* **2005**, *32*, 53−60.

(38) Tripathi, N. K.; Shrivastva, A.; Biswal, K. C.; Rao, P. V. L. Optimization of culture medium for production of recombinant dengue protein in *Escherichia coli*. *Ind. Biotechnol.* **2009**, *5*, 179−183.

(39) Black, J. G. *Microbiology Principles and Applications*, 3rd ed.; Prentice Hall: Upper Saddle River, NJ, 1996; pp. 140−144.

(40) Bradford, M. M. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(41) Mizutani, K.; Fernandes, V. O.; Karita, S.; Luís, A. S.; Sakka, M.; Kimura, T.; Jackson, A.; Zhang, X.; Fontes, C. M.; Gilbert, H. J.; Sakka, K. Influence of a mannan binding family 32 carbohydrate binding module on the activity of the appended mannanase. *Appl. Environ. Microbiol.* **2012**, *78*, 4781−4787.

(42) Cann, I. K. O.; Kocherginskaya, S.; King, M. R.; White, B. A.; Mackie, R. I. Molecular cloning, sequencing and expression of a novel multidomain mannanase gene from *Thermoanaerobacterium polysaccharolyticum*. *J. Bacteriol.* **1999**, *181*, 1643−1651.

(43) Yoshikawa, Y.; Hirata, R.; Yasui, H.; Hattorib, M.; Sakurai, H. Inhibitory effect of $CuSO_4$ on a-glucosidase activity in ddY mice. *Metalomics* **2009**, *2*, 67−73.

(44) Kurakake, M.; Sumida, T.; Masuda, D.; Oonishi, S.; Komaki, T. Production of galacto-manno-oligosaccharides from guar gum by α-mannanase from *Penicillium oxalicum* SO. *J. Agric. Food. Chem.* **2006**, *54*, 7885−7889.

(45) Lim, H. K.; Jung, K. H. Improvement of heterologous protein productivity by controlling post induction specific growth rate in recombinant *Escherichia coli* under control of the PL promoter. *Biotechnol. Prog.* **1998**, *14*, 548−553.

(46) Cserjan-Puschmann, M.; Kramer, W.; Duerrschmid, E.; Striedner, G.; Bayer, K. Metabolic approaches for the optimization of recombinant fermentation processes. *Appl. Microbiol. Biotechnol.* **1999**, *53*, 43−50.

(47) Zhang, J.; Greasham, R. Chemically defined media for commercial fermentations. *Appl. Microbiol. Biotechnol.* **1999**, *51*, 407−421.

(48) Noorbatcha, I. A.; Sultan, A. M.; Azura, A.; Salleh, H. M. Molecular dynamics study of the effect of calcium ions on the thermostability of *Bacillus Amyloliquefaciens* phytase. *Aust. J. Basic Appl. Sci.* **2012**, *6*, 109−116.