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High level expression of a novel β-mannanase from *Chaetomium* sp. exhibiting efficient mannan hydrolysis

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ABSTRACT

A novel β-mannanase gene (*CsMan5A*) was cloned from *Chaetomium* sp. CQ31 and expressed in *Pichia pastoris*. It had an open reading frame of 1251 bp encoding 416 amino acids and contained two introns. The deduced amino acid sequence shared the highest similarity (73%) with the β-mannanase from *Emericella nidulans* and belongs to glycosyl hydrolase family 5. The recombinant β-mannanase (CsMan5A) was secreted at extremely high levels of 50,030 U mL^{-1} and 6.1 mg mL^{-1} in high cell density fermentor. The purified enzyme was optimally active at pH 5.0 and 65 °C and displayed broad pH stability (pH 5.0–11.0) and exhibited specificity towards locust bean gum (K_m = 3.1 mg mL^{-1}), guar gum (K_m = 9.3 mg mL^{-1}) and konjac powder (K_m = 10.5 mg mL^{-1}). It efficiently degraded mannan polysaccharides into mannose and mannooligosacccharides, and also hydrolyzed mannotriose and mannotetraose. These properties make CsMan5A highly useful in food, feed and paper/pulp industries.

1. Introduction

Hemicelluloses are found in the plant cell wall, and are the second most abundant natural hetero-polysaccharides in lignocellulosic biomass. Mannans are complex biopolymers that are commonly found in plant cell walls where they are closely associated with cellulose and lignin (Girio et al., 2010). Together with xylans, they form the major component of the hemicellulose fraction and can be classified into linear mannan, glucomannan, galactomannan, and galactoglucomanan (Petkowicz, Reicher, Chanzy, Taravel, & Vuong, 2001). They consist of a backbone of a β-1,4-linked mannose and glucose residues substituted with side chains of α-1,6-linked galactosyl side groups (Liepman et al., 2007). Due to the complex nature of these polysaccharides, a combination of endo and exo acting enzymes such as β-1,4-mannanase, β-mannosidase and α-galactosidases are required for their complete degradation (Ademark et al., 1998; Wang et al., 2010). There has been growing interest over the years in the industrial potential of mannan degrading enzymes, especially β-mannanase.

Endo-β-1,4-D-mannanase (β-mannanase; EC 3.2.1.78) catalyses the random hydrolysis of β-1,4-mannosidic linkages in the main chain of mannan polymers thereby releasing linear and branched manno-oligosaccharides of various lengths (De Vries, 2003). β-1,4-Mannanases have a wide range of potential industrial applications. They are used in combination with xylanases in the paper and pulp industries for increasing the brightness of pulps and in the detergent industry (Benech et al., 2007). They are widely applied in poultry feed to reduce the anti-nutritional factor of mannan polymers found in corn-soy based feeds (Wu, Bryant, Voitle, & Roland, 2005). Furthermore, β-mannanases have potential applications in recycling of copra and coffee wastes and in processing of instant coffee (Jiang, Wei, Li, Chai, & Kusakabe, 2006). Due to an ever-increasing demand towards renewable resource utilization, these enzymes are gaining a great deal of interest for industrial applications, and there is scope for novel β-mannanases with better characteristics.

β-Mannanases have been isolated from a wide range of microorganisms, including bacteria and fungi (Dhawan & Kaur, 2007; Jiang et al., 2006; Zyl, Rosea, Trollopeb, & Gorgens, 2010). Fungal β-mannanases have been isolated from a number of species such as *Trichoderma reesei*, *Sclerotium rolfsii*, *Aspergillus oryzae*, *Aspergillus niger*, *Trichoderma harzianum*, etc. (Ferreira & Filho, 2004; Haltrich, Laussamayer, Steiner, Nidetzky, & Kulbe, 1994; Regalado et al., 2000; Stålbrand, Siika-aho, & Viikari, 1993). The production of fungal β-mannanases by submerged culture of wild-type strains

Abbreviations: AOX, alcohol oxidase; CAPS, (cyclohexylamino)-1-propanesulphonic acid; CHES, 2-(cyclohexylamino) ethanesulfonic acid; DTT, dithiothreitol; GH, glycosyl hydrolase; MES, 2-(N-morpholino)ethane sulfonic acid; MOPS, 4-(N-morpholino)-propane sulphonic acid; ORF, open reading frame; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; TLC, thin-layer chromatography.

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has resulted in lower expression levels as well as purity, making it uneconomical for industrial production. Cost-effective production methods are needed in order to meet the increasing demands of industrially important enzymes such as β-1,4-mannanases. Cloning of novel fungal β-mannanases genes and over-expressing the enzymes heterologously would help meet the high demand for these enzymes in industry. Few reports have been published describing the isolation and cloning of fungal and bacterial β-mannanase genes expressed in different heterologous systems such as yeast, fungi and bacteria with the aim of enzyme overproduction (Hatada et al., 2005; Stålbrand, Saloheimo, Vehmaanpera, Henrissat, & Penttila, 1995; Roth, Moodley, and van Zyl (2009); Zyl et al., 2010). The methylotrophic yeast *Pichia pastoris* has been increasingly exploited for protein production due to the various advantages it offers over bacterial and mammalian systems (Sue, Mariana, Brian, & Linda, 2005). Previously, β-mannanase genes have been cloned and expressed in *P. pastoris*, however only a few genes have been successfully expressed at amounts high enough for commercial production (Do et al., 2009; Duruksu, Ozturk, Biely, Bakir, & Ogel, 2009; Luo et al., 2009).

A moderately thermophilic fungus, *Chaetomium* sp. CQ31 was recently isolated from composting soil samples of Shandong province (China) in our lab. This species of fungus was shown to be a producer of xylanase and β-mannanase (Jiang, Cong, Yan, Kumar, & Du, 2010). In the present study, we have cloned and sequenced a novel β-mannanase gene termed *CsMan5A* from *Chaetomium* sp. CQ31 and expressed it in *P. pastoris*. To the best of our knowledge, the β-mannanase expression we have achieved in high cell density fermentation is the highest reported so far. The biochemical and hydrolytic properties of CsMan5A make it a highly useful candidate in various applications such as in the food and feed and paper and pulp industries.

2. Experimental

2.1. Reagents

Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA, USA). DNA polymerase *Pfu* was obtained from Promega (Madison, WI, USA). Locust bean gum (LBG), guar gum, cellulose, birchwood xylan, and carboxymethylcellulose (CMC, sodium salt, low viscosity) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Avicel was obtained from Merck Ltd. (Darmstadt, Germany). Q Sepharose Fast Flow was purchased from Pharmacia (Pharmacia, Uppsala, Sweden). *P. pastoris* expression kit was obtained from Invitrogen (San Diego, CA, USA). *Escherichia coli* strain JM109 (Stratagene) was used for propagation of plasmids, and *P. pastoris* GS115 (his4) strain (Invitrogen) was used for protein expression. The pMD-18T simple vector system was purchased from TaKaRa Corporation (Japan). All other chemicals were of analytical grade, unless otherwise stated.

2.2. Strain and media

Chaetomium sp. CQ31 used in the study (deposited at China General Microbiological Culture Collection Center (web-site: http://www.cgmcc.net/) under CGMCC No. 3341) was isolated from composting soils in Weihai city of Shandong province (Jiang et al., 2010). The medium for β-mannanase production consisted of the following ingredients (w/v) in distilled water: 0.5% NaH_2PO_4, 0.06% $MgSO_4{\cdot}7H_2O$, 0.4% yeast extract, 0.8% beef peptone and 2.0% konjac powder.

Table 1
Primers used in this study.

Primers	Primer sequence (5′–3′)[a]	Bases
DP1	CCTGCGCGTCtggggnttyaa	21
DP2	CGTTGGCCAGCtcccangcraa	22
5′GSP	CTAACTCCCACGCGAAGATGGTG	23
5′NGSP	GTAGCGGGTCACCATCTCTTTGAC	24
3′GSP	TGGTGACCCGCTACAAGGATTCTC	24
3′NGSP	ATCTTCGCGTGGGAGTTAGCCAAC	24
ManF	ATGGTGTTCTCGGCCCCGC	19
ManR	TTAGTCACTTCTCGCCTCGC	20
CsMan5A*Eco*RIF[b]	CCGGAATTCCCAAGCCGAGCTGTGCAGGCTCGA	33
CsMan5A*Not*IR[b]	ATAAGAATGCGGCCGCTTAGTCACTTCTCGCCTCGC	36

[a] D = A/G/T, M = A/C, N = A/T/C/G, R = A/G, Y = C/T.
[b] Restriction enzyme sites incorporated into primers are underlined.

2.3. Cloning and sequence analysis of a β-mannanase gene from Chaetomium sp. CQ31

Recombinant DNA techniques as described by Sambrook and Russell (2001) were employed to perform DNA manipulations. For isolation of genomic DNA, *Chaetomium* sp. CQ31 was grown at 37 °C for 4 days in media consisting of (g L^{-1}): locust bean gum, 5; tryptone, 10; yeast extract, 10; $MgSO_4{\cdot}7H_2O$, 0.3; $FeSO_4$, 0.3; $CaCl_2$, 0.3. Fungal mycelia were collected by centrifugation (5000 × *g*, 10 min) and washed twice with water at 4 °C. Genomic DNA was isolated with a Fungal DNA Midi Kit (Omega Biotek, Doraville, GA, USA). For isolation of RNA, cells were grown and collected as described above. The mycelia were frozen and ground to fine powder in liquid nitrogen. The total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA), and mRNAs were purified using the Oligotex mRNA Midi Kit (Qiagen, Germany). Genomic DNA of *Chaetomium* sp. CQ31 was used as template for subsequent polymerase chain reaction (PCR) amplification. To clone the β-1,4-mannanase gene, degenerate primers DP1 and DP2 (Table 1) were designed based on the conserved sequences (LRVWGF and FAWELANE) of known fungal β-mannanases using the CODEHOP algorithm (Rose, Henikoff, & Henikoff, 2003).

PCR conditions were as follows: a hot start at 94 °C for 5 min, 10 cycles of 94 °C for 30 s, 61–55 °C for 30 s and 72 °C for 1 min, followed by 20 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The PCR product was purified, ligated to pMD18-T vector, and sequenced. The full-length cDNA sequence of the β-1,4-mannanase was obtained by 5′ and 3′ RACE (rapid amplification of cDNA ends) using a SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) in accordance with the manufacturer's instructions. The PCR condition for RACE was: 10 cycles of 30 s at 94 °C and 3 min at 72 °C, followed by 20 cycles of 30 s at 94 °C, 30 s at 68 °C, and 3 min at 72 °C, and finally 10 min at 72 °C. PCR was performed with the primer pairs, 5′GSP and Universal Primer A Mix for the first PCR, followed by a nested PCR with the primers, 5′NGSP and Nested Universal Primer A (BD Biosciences) for 5′ RACE. Similarly, 3′ RACE was performed with 3′GSP and Universal Primer A Mix, followed by nested PCR with 3′NGSP and Nested Universal Primer A. The obtained PCR product was purified, cloned, and sequenced. The β-1,4-mannanase cDNA sequences from *Chaetomium* sp. CQ31 were deposited in the GenBank nucleotide sequence database with accession no. HQ718590. To amplify this region from the genomic DNA of CQ31, the same PCR conditions were performed using the specific primers, ManF (ATGGTGTTCTCGGCCCCGC) and ManR (TTAGTCACTTCTCGCCTCGC) (Table 1).

The amplified PCR product of the DNA was purified and cloned into the pMD18-T vector, and transformed into *E. coli* JM109 for sequencing, and subjected to BLAST analysis. Nucleotide and deduced amino acid sequences were analyzed with the ExPASy Proteomics tools (http://www.expasy.ch/tools/). Database homology searches of nucleotide sequences obtained

were carried out using BLAST in GenBank at the NCBI. The amino acid sequences were aligned using the ClustalW program (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalW/). The signal peptide was analyzed by Signal P 3.0 server (http://www.cbs.dtu.dk/services/SignalP). Search analysis of conserved domain and signature sequences was carried out using ScanProsite (http://www.expasy.ch/tools/ScanProsite). N-Glycosylation sites were predicted using NetNGlyc1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/).

2.4. Transformation of P. pastoris and expression in shake-flask

The cDNA encoding the mature β-mannanase gene (without the intron) was PCR amplified using the specific primers CsMan5A*Eco*RIF (5′-CCGGAATTCCCAAGCCGAGCTG-TGCAGGCTCGA-3′) and CsMan5A*Not*IR (5′-ATAAGAATGCGG-CCGCTTAGTCACTTCTCGCCTCGC-3′) (Table 1). PCR amplification was carried out as follows: 94 °C for 5 min, 30 cycles of 30 s at 94 °C, 30 s at 60 °C, and 2 min at 72 °C, and finally 10 min at 72 °C. Primer CsMan5A*Eco*RIF introduced an *Eco*RI site, and primer CsMan5A*Not*IR contained an *Not*I site (underlined). After digestion with *Eco*RI and *Not*I, the PCR product was purified and inserted into the vector pPIC9K (Invitrogen, USA), yielding the recombinant plasmid pPIC9K-CsMan5A. The recombinant plasmid was linearized with *Sal*1 and then transformed by electroporation into *P. pastoris* GS115 strain according to the manufacturer's instructions (Invitrogen). The transformants were plated on MD (Minimal Dextrose) plates (1.34% yeast nitrogen base, 4×10^{-5}% biotin, 2% dextrose, and 1.5% agar) and incubated at 30 °C for 2–3 days until colonies appeared. The colonies from MD plates were suspended in sterile water, pooled, and then plated on YPD-geneticin (G418) plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar containing 8 mg mL^{-1} G418) and grown at 30 °C until colonies appeared (3 days). Several colonies were selected from YPD-geneticin plates and grown in 5 mL BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 1% glycerol) in a rotary shaker at 30 °C at 220 rpm till the OD_{600} of the culture was between 2 and 6. The *P. pastoris* cells were then harvested by centrifugation at 5000 *g* for 5 min and resuspended in 10 mL of BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 0.5% methanol). The cells were further grown in a rotary shaker at 30 °C at 220 rpm for 5 days during which the concentration of methanol was maintained constant at 0.5% by adding methanol (100%) to the growing culture every 24 h. The cells were harvested by centrifugation (10,000 × *g* for 20 min) and the β-1,4-mannanase activity as well as the expression in the culture supernatant was analyzed by the 3,5-dinitrosalicylic acid (DNS) method and by SDS-PAGE respectively as given below.

2.5. High cell density fermentation cultivation

The transformed strain showing the highest β-mannanase activity in shake-flask culture was cultivated in high cell density fermentor. Methods used are described in the *Pichia* Fermentation Guidelines (Version B, 053002, Invitrogen Inc.). All cultivations were carried out in a 5.0 L fermentor with a 1.5 L working volume at 30 °C. The fermentor was inoculated with 150 mL of a shake flask culture grown at 30 °C and 250 rpm overnight (18 h) to an optical density (OD_{600}) of more than 10 in BMGY medium. The fermentor medium was composed of FBS (Fermentation basal salt) medium with 4.35 mL of PTM_1 trace salts solution (per liter). The cultivation was maintained at constant pH with the aid of ammonium hydroxide which was provided during the cultivation if needed. The agitation rate was maintained at 950 rpm. Initially, the dissolved oxygen levels were maintained at >20% air-saturation. Upon depletion of initial glycerol, fed-batch fermentation was initiated with a fed-batch medium at a rate of 18.4 mL h^{-1} L^{-1} of initial volume. After a 6 h glycerol fed-batch phase, the methanol induction phase was initiated with a feed containing 12 mL PTM_1 trace salts (per liter) at pH 6.0. The concentration of methanol was kept stable by monitoring the dissolved oxygen (DO) content and maintaining it at greater than 20% air-saturation. Changes in the DO concentrations (DO spikes) ensure that the methanol feed does not exceed the rate of consumption since excess methanol (>1–2%, v/v) may be toxic. If dissolved oxygen falls below 20%, the methanol feed is stopped until the dissolved oxygen spikes. Samples were withdrawn at various time-intervals during the methanol induction phase and the OD_{600}, wet weight of the cells, β-mannanase activity and the protein content were determined.

2.6. Enzyme activity and protein content

The activity was determined by the DNS method of Miller (1959). 100 μL of purified enzyme suitably diluted with 50 mM citrate buffer (pH 5.0) was added to 900 μL of 0.5% Locust bean gum (LBG) in 50 mM citrate buffer (pH 5.0) and incubated at 55 °C for 10 min. The reaction mixture was boiled at 100 °C for 15 min and 1 mL of 40% sodium–potassium tartarate was added to terminate the reaction. The release of reducing sugars was determined colorimetrically by measuring the absorbance at 540 nm using mannose (Sigma) as the standard. One unit of β-mannanase activity was defined as the amount of enzyme liberating 1 μmol of reducing sugars per minute under the conditions described. Protein concentration was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as the standard.

2.7. Purification of recombinant β-mannanase

The fermentation broth was centrifuged (10,000 × *g* at 4 °C for 20 min) to obtain the cell-free crude supernatant fraction. 10 mL of the supernatant was dialyzed against buffer A (20 mM sodium phosphate, pH 6.5) for 16 h at 4 °C. The dialyzed sample was loaded onto a Q-Sepharose column (10 cm × 1 cm) which was pre-equilibrated with 3 column volumes (CV) of buffer A. The column was washed with 5 CV of buffer A at a flow rate of 1 mL min^{-1} and the bound protein was then eluted with a linear gradient from 0 to 100% buffer B (20 mM sodium phosphate buffer (pH 6.5) containing 0.5 M NaCl). The elution fractions showing β-mannanase activity were pooled and dialyzed against 20 mM sodium citrate buffer (pH 5.0) and the purity was analyzed by SDS-PAGE.

2.8. SDS-PAGE analysis and determination of molecular mass

The homogeneity and molecular weight of CsMan5A was determined by 12.5% SDS-PAGE as described by Laemmli (1970). Protein bands were visualized by staining with Coomassie brilliant blue R-250. The molecular weight standard used was the low molecular weight calibration kit for SDS electrophoresis (Amersham): phosphorylase b (97.0 kDa), albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa). The native molecular mass of CsMan5A was determined by size-exclusion chromatography using a Superdex-75 gel filtration column (1 cm × 40 cm) which was equilibrated with buffer A containing 150 mM NaCl at a flow-rate of 0.3 mL min^{-1}. Standard proteins of known molecular weight (fetuin from fetal calf serum, 68 kDa; chicken egg white albumin, 45 kDa; α-chymotrypsinogen A from bovine pancreas, 25.7 kDa; cytochrome c, 13 kDa) were run under the same conditions to generate a calibration curve for calculation.

2.9. Deglycosylation of recombinant β-mannanase

10 μg of CsMan5A was denatured at 100 °C for 10 min and treated with 250 U of endo-β-N-acetylglucosaminidase H (Endo H) for 2 h at 37 °C according to the supplier's instructions (New England Biolabs, Ipswich, MA, USA). The untreated (control) and above treated samples were analyzed by SDS-PAGE.

2.10. pH and temperature profile of recombinant β-mannanase

The optimum pH for β-mannanase activity was determined in different buffers such as sodium citrate (pH 3.5–6.0), sodium acetate (pH 4.0–5.5), MOPS (pH 6.0–8.0), sodium phosphate (pH 6.0–8.0), CHES (pH 8.0–10.0), CAPS (pH 9.0–11.0) and Glycine-NaOH (pH 9.0–12.0) at 50 °C using 0.5% locust bean gum (LBG) as the substrate by the DNS method. For determining the pH stability, the enzyme was incubated with the buffers at 50 °C for 30 min, cooled on ice for 30 min and the residual activity was assayed under the standard conditions. Furthermore, the stability of the enzyme was also checked at pH 5.0 for 24 h by incubating the enzyme in 50 mM sodium citrate buffer (pH 5.0) at 50 °C. Aliquots were removed after 0.5 h, 2 h, 4 h, 6 h, 12 h and 24 h, cooled on ice and the activity assayed according to the standard method. The initial enzyme activity (without incubation) was regarded as 100%. The optimum temperature for enzyme activity was determined in 50 mM sodium citrate buffer (pH 5.0) in the temperature range of 30–90 °C. In order to study the thermostability, the enzyme was incubated in 50 mM sodium citrate buffer (pH 5.0) at different temperatures for 30 min, cooled on ice for 30 min and then the residual activity was analyzed as mentioned above.

2.11. The effect of various reagents on enzyme activity

The effect of various agents on CsMan5A activity was determined as follows. The purified enzyme was incubated with different metal ions such as K^+, Sr^{2+}, Ca^{2+}, Mg^{2+}, Ni^{2+}, Fe^{3+}, Ba^{2+}, Cu^{2+}, Co^{2+}, Na^+, Zn^{2+}, Li^+, Mn^{2+}, Ag^+, Hg^{2+} as well as the metal-chelating agent, EDTA, denaturant, SDS and reducing agent, DTT at a final concentration of 1 mM at 50 °C in 50 mM sodium citrate buffer (pH 5.0) for 30 min. After cooling on ice for 30 min, the residual activity was assayed according to the standard assay method.

2.12. Substrate specificity analysis and determination of kinetic parameters

The specificity of CsMan5A for various substrates such as LBG, guar gum, konjac powder, birchwood xylan, carboxymethyl cellulose (CMC), starch, and avicel was analyzed. The enzyme activity was assayed in the presence of 5 mg mL^{-1} of different substrates at 50 °C in 50 mM citrate buffer (pH 5.0) by the DNS method. The kinetic parameters, K_m and V_{max} were determined for the substrates, LBG, guar gum and konjac powder in the concentration range of 1.5–17.5 mg mL^{-1} at 50 °C in 50 mM citrate buffer (pH 5.0) for 5 min using DNS method. The Michaelis–Menten parameters K_m and k_{cat} were calculated using the software Grafit (Leatherbarrow, 1999).

2.13. Hydrolysis of mannan polymers and mannooligosaccharides

The degradation of mannan polysaccharides by CsMan5A was performed as given below. 5 U of purified β-mannanase was incubated in a reaction volume of 1 mL with 1% of different mannan polysaccharides such as locust bean gum and konjac powder at 50 °C in 50 mM sodium citrate (pH 5.0) for 24 h. Aliquots were withdrawn at different time-points, boiled for 10 min and analyzed by TLC. The reaction mixtures were deionized by mixing with cation-exchange and anion-exchange resins (Shantou Xilong Chemicals, China) for about 30 min and then spotted on to a silica gel plate (Merck Silica Gel 60F 254, Germany), and developed twice in a solvent system containing n-propanol:ethanol:water (7:1:2, v/v/v). Saccharides were detected by heating in an oven after spraying the plates with a mixture of methanol: sulfuric acid (95:5, v/v). A mixture of mannooligosaccharides consisting of mannose (M1), mannobiose (M2), mannotriose (M3), mannotetraose (M4) and mannopentaose (M5) was used as the standard.

Furthermore, the ability of CsMan5A to hydrolyze various manno-oligosaccharides was also assessed. The reaction mixture consisting of 5 U of enzyme and 1% of different mannooligosaccharides such as mannobiose, mannotriose and mannotetraose in 50 mM sodium citrate buffer (pH 5.0) was incubated at 50 °C for 24 h. The hydrolysis products were analyzed by TLC as described above.

3. Results and discussion

3.1. Cloning and sequence analysis of a β-mannanase gene from Chaetomium sp. CQ31

A partial gene for a β-1,4-mannanase of *Chaetomium sp.* was amplified by PCR using degenerate primers (Table 1). Sequence analysis of the 445 bp amplified fragment showed the highest nucleotide sequence identity (76%) to endo-β-1,4-mannanase from *Emericella nidulans* (DQ490502). Thus, this obtained partial sequence was used to design gene-specific primers for 5′ and 3′ RACE to obtain the sequence of the full-length gene. The 5′ and 3′ RACE yielded 619-bp and 861-bp DNA fragments, respectively. After sequence assembling, the putative full-length β-1,4-mannanase cDNA of 1495 bp of the gene was obtained with an ORF of 1251 bp. Comparison with a 1397 bp genomic sequence indicated the existence of two introns of 81 bp and 65 bp, respectively, in the coding region. The full-length ORF contains a sequence for a predicted signal peptide of 21 residues. The deduced product of 416 amino acids has a predicted molecular mass of 46,648 Da and a deduced pI of 4.5.

Based on the sequence similarity results, the deduced amino acid sequence of CsMan5A shares highest similarity with the β-mannanase from *E. nidulans* (74%, ABF50878) (Bauer, Vasu, Persson, Mort, & Somerville, 2006) followed by *Aspergillus terreus* NIH2624 (72%, EAU29440), *Verticillium albo-atrum* VaMs.102 (63%, EEY21635), *Phanerochaete chrysosporium* (44%, ABG79371) (Fig. 2). Comparison of the protein sequence of CsMan5A to other β-mannanases in the GenBank database revealed that the enzyme belonged to glycosyl hydrolase (GH) family 5. Mannan-degrading enzymes have been grouped and classified in families on the basis of their amino acid sequence similarities and β-mannanases belong to GH families 5 and 26 (Henrissat & Davies, 1997). The GH family 5 members represent mannan-degrading enzymes from bacterial and eukaryotic organisms, such as *P. chrysosporium, T. reesei,* and *Aspergillus fumigatus* (Benech et al., 2007; Duruksu et al., 2009; Stålbrand et al., 1993). Four potential *N*-glycosylation sites were identified in the amino acid sequence of the mature protein (Fig. 1). The sequence shows the presence of two glutamic acid residues, E188 and E305 which are conserved in all members of GH family 5 and have been postulated to participate in catalysis. Comparison of the CsMan5A amino acid sequence with those of *Me*Man5A and other GH family 5 β-mannanases suggest that E188 could be the catalytic acid/base and E305, the catalytic nucleophile (Larsson et al., 2006). Besides, two Cys residues, C212 and C226 are present in the β-mannanase from *Chaetomium* sp. CQ31 and conserved in the other β-mannanases as shown in Fig. 2. They might have a role in

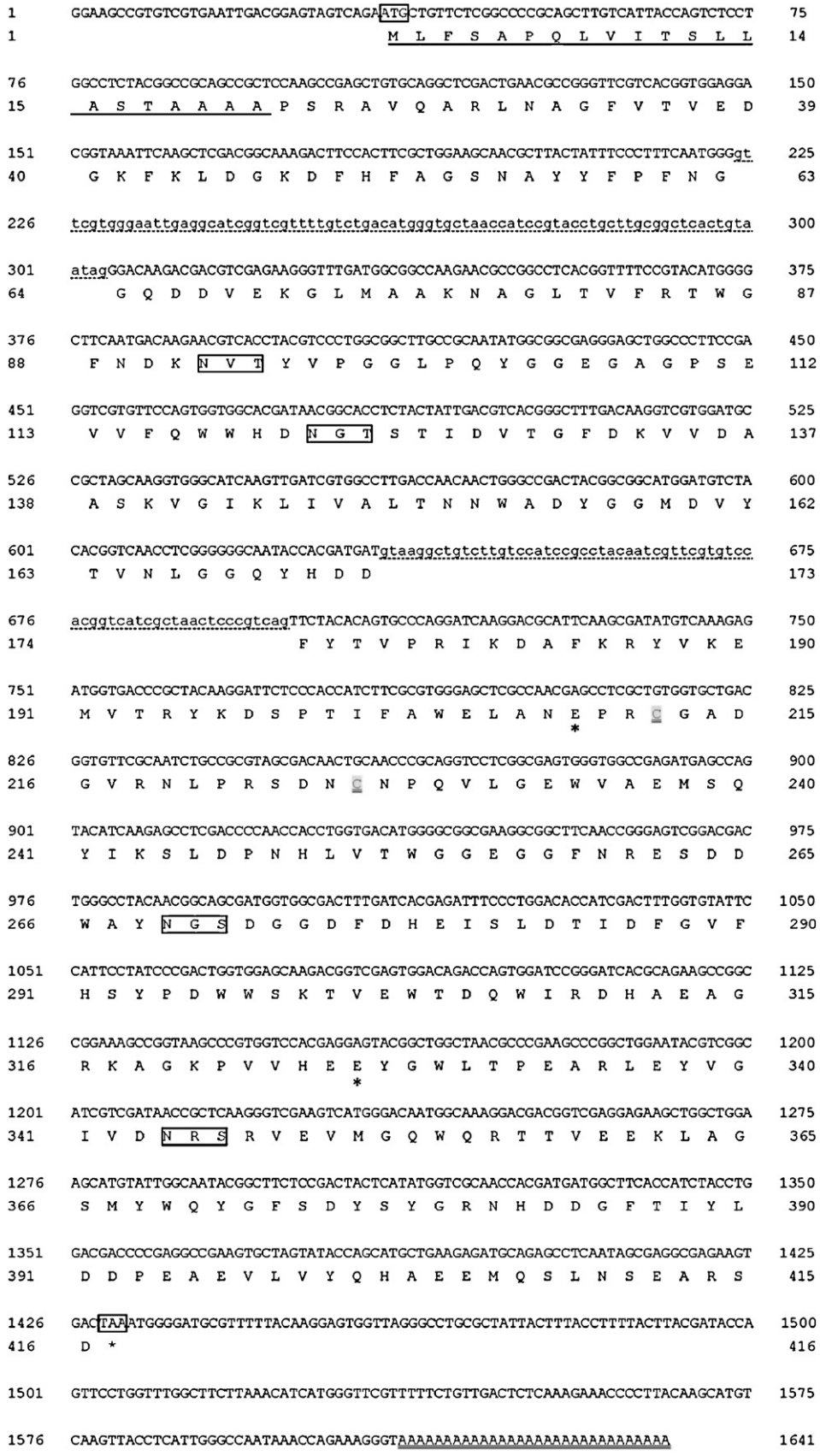

Fig. 1. Nucleotide and deduced amino-acid sequences of the cDNA, flanking regions and genomic DNA of *Chaetomium* sp. CQ31 β-mannanase. The translational initiation codon ATG, and termination codon TAA are boxed. Two intron sequences are shown in lowercase letters and indicated by a dotted underline. A poly (A+) tail is double lined. Conceptual translation of the ORF to the 416 amino acids is shown in a one-letter code below the respective codon. A putative signal peptide is indicated by underline. The asterisk indicates the stop codon. Four putative *N*-glycosylation sites are boxed. Two Cys residues are highlighted in grey and indicated by double underlined. Residues participating in catalytic action as nucleophile or proton donor E188 and E305 are marked by asterisk (*). The nucleotide sequence reported here has been submitted to GenBank and has been given accession number HQ718590.

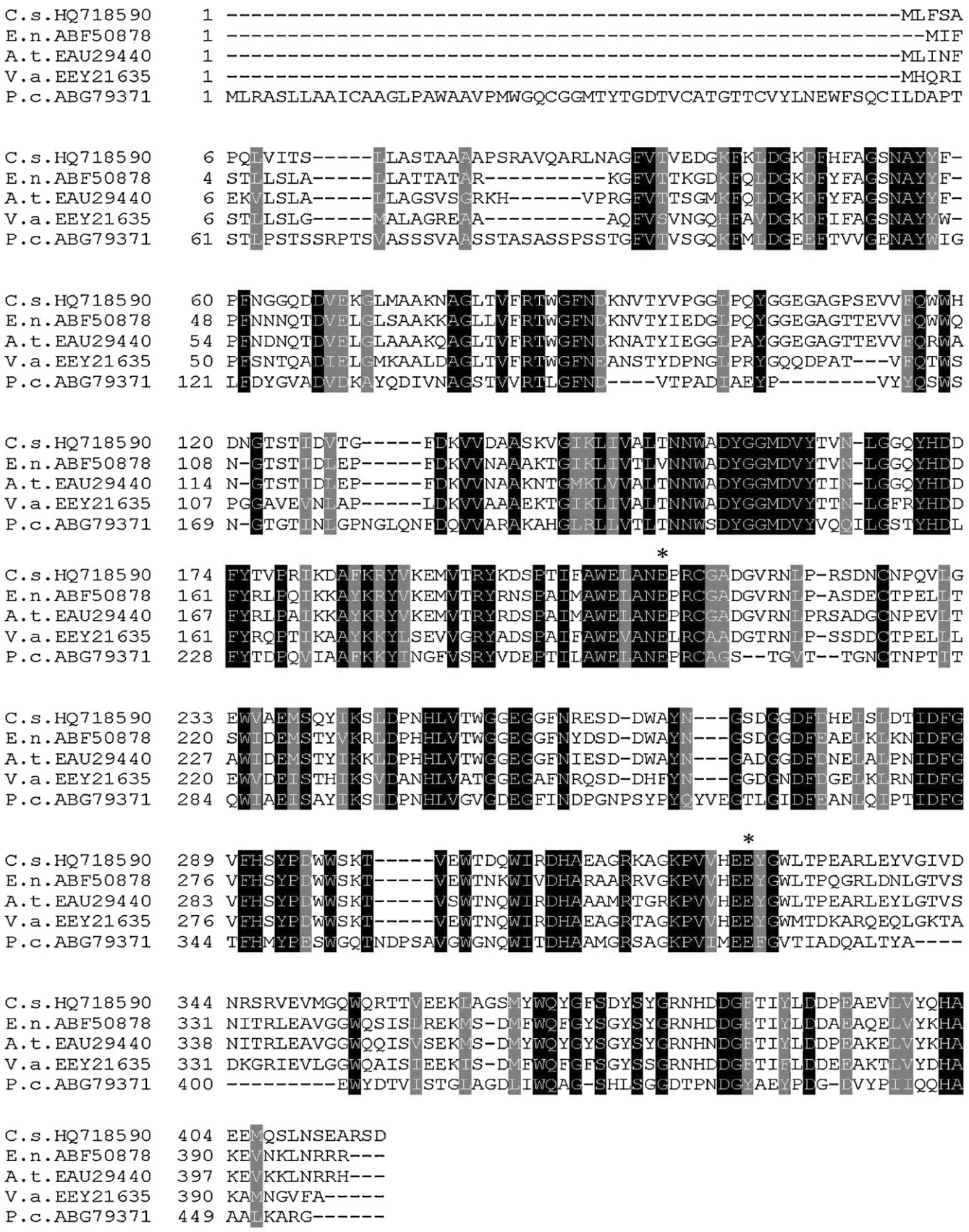

Fig. 2. Multiple alignment of amino acid sequences for GH family 5 β-mannanases. Numbers on the left are the residue number of the first amino acid in each line. Sequences listed include those of β-mannases form *Chaetomium* sp. CQ31 in this study (*C. s.* HQ718590), *Emericella nidulans* (*E. c.* ABF50878), *Aspergillus terreus* NIH2624 (*A. t.* EAU29440), *Verticillium albo-atrum* VaMs.102 (*V. a.* EEY21635), *Phanerochaete chrysosporium* (*P. c.* ABG79371). Single-letter amino acid code is used. Identical residues are shaded in black, and conserved residues are shaded in grey. Residues participating in catalytic action as nucleophiles or proton donors in GH family 5 enzymes are marked by asterisk (*).

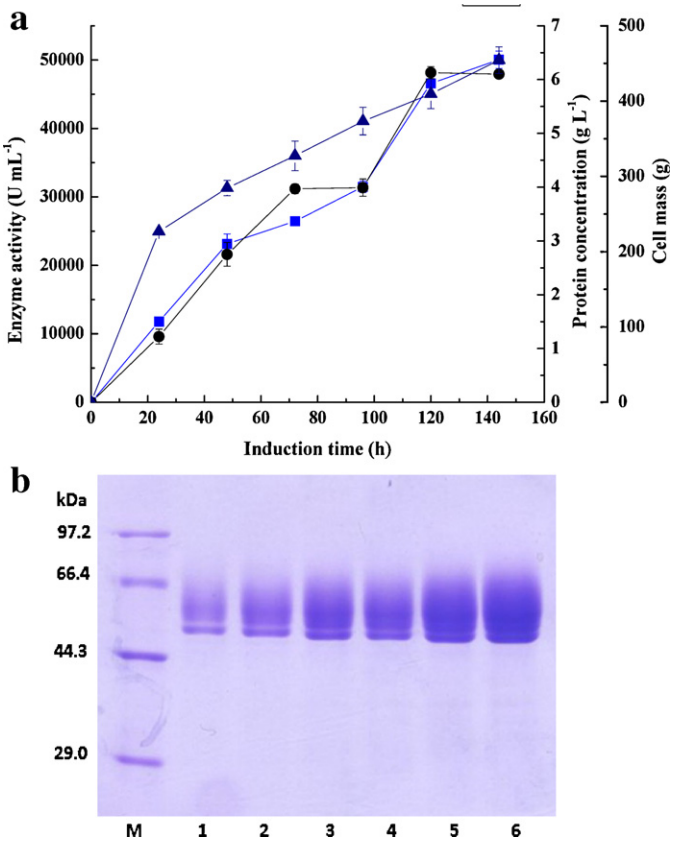

Fig. 3. Analysis of time-course of recombinant β-mannanase expression in 5 L fermentor (a) and expression on SDS-PAGE (b). The enzyme activity (■), protein concentration (•) and cell mass (▲) were monitored during high cell density cultivation. The β-mannanase activity was determined by DNS method at 55 °C. Lane M, low MW marker; lanes 1–6, culture supernatant after 24 h, 48 h, 72 h, 96 h, 120 h and 144 h.

maintaining the stability of β-mannanase by forming a S–S bridge in the molecule. The endo-β-1,4-mannanase gene reported in the present study is the first gene cloned from the *Chaetomium* species.

3.2. Expression of the β-mannanase gene in P. pastoris by high cell density fermentation

CsMan5A was expressed in *P. pastoris* under the *AOX1* promoter. Recombinants carrying multiple copies of the integrated gene were selected from high concentrations (8 mg mL^{-1}) of G418. G418 resistance levels roughly depend on the copy number of kanamycin resistance genes integrated and insertion of multiple plasmid copies can thus easily be detected by highest G418 resistance (Scorer, Clare, McCombie, Romanos, & Sreekrishna, 1994). Furthermore, integration of high copy number of the gene of interest often leads to high levels of protein expression. A single *P. pastoris* transformant showing the highest β-mannanase activity in shake-flask (567.8 U mL^{-1}) was cultivated in a 5 L fermentor. During cultivation in high cell density fermentor, the OD_{600} increased with time reaching a maximum of 543 with a wet cell mass of 454 g after 144 h (Fig. 3a). The secreted mannanase showed an activity of 50,030 U mL^{-1} and protein content of 6.1 mg mL^{-1}. The expression level of CsMan5A is the highest β-mannanase expression ever reported. The β-1,4-mannanase genes from fungi and other organisms such as *Bacillus subtilis* (1800 mg L^{-1}; 1102 U mL^{-1}), *A. niger* (243 mg L^{-1}; 669 U mL^{-1}), *Aspergillus fumigates* (173 mg L^{-1}; 61 U mL^{-1}), *Aspergillus sulphureus* Mann (262 mg L^{-1}; 96 U mL^{-1}); *Bispora* sp. (148 mg L^{-1}; 500 U mL^{-1}) which have been cloned and expressed in *P. pastoris* were comparatively much lower (Chen, Cao, Ding, Lu, & Li, 2007; Do et al., 2009; Duruksu et al., 2009; Luo et al., 2009). In addition, the high purity of the crude supernatant as well as the high specific activity (8202 U mg^{-1}) makes this enzyme highly cost-effective for commercial applications.

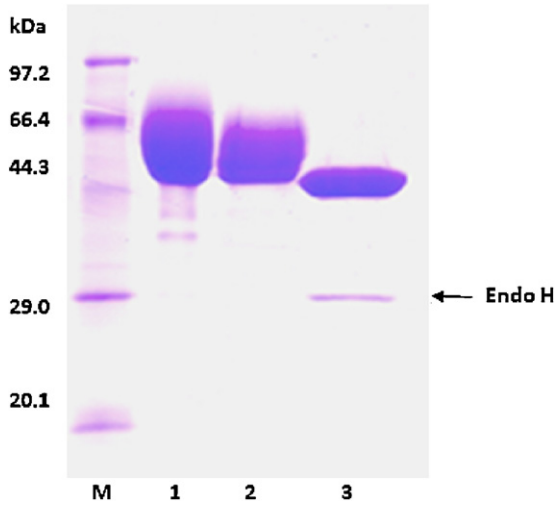

Fig. 4. Analysis of purification of β-mannanase on 12.5% SDS-PAGE. Lane M, molecular weight standard; lane 1, crude secreted protein; lane 2, after purification on Q-Sepharose; lane 3, after deglycosylation by Endo H. The gel was stained with Coomassie brilliant blue R-250.

3.3. Purification and deglycosylation of the recombinant β-mannanase

CsMan5A could be purified to apparent homogeneity by SDS-PAGE (Fig. 4, lane 2) in a single ion-exchange chromatography step using Q-Sepharose. This resulted in a 1.2-fold purification, giving a yield of 93% from 5 mL of the extracellular supernatant obtained after centrifugation of the fermentation culture. CsMan5A showed a high specific activity of 9699 U mg^{-1} (data not shown) when assayed with 0.5% LBG as the substrate. The purified enzyme showed a single, homogenous band on SDS-PAGE at around 50 kDa which is higher than the predicted molecular mass of 44.591 kDa. Also, the β-mannanase bands in the crude and purified fractions appeared as smeared bands on SDS-PAGE. The amino acid sequence contains four potential *N*-glycosylation sites at Asn-X-Ser/Thr residues suggesting that the enzyme is possibly glycosylated. To test this, the purified enzyme was deglycosylated with Endo H. After treatment with Endo H, a single, homogenous band at 45 kDa was observed, indicating that CsMan5A was indeed glycosylated in *P. pastoris* (Fig. 4, lane 3). The oligomerization state of native (glycosylated) CsMan5A was determined using gel filtration chromatography (data not shown), and it was shown to elute as a monomer.

3.4. Biochemical properties of the purified recombinant β-mannanase

CsMan5A exhibited maximal activity at pH 5.0 (Fig. 5a). The enzyme was found to be stable in a broad pH range from pH 5.0–11.0 for 30 min where it retained more than 70% of its activity (Fig. 5b). As compared to acidic pH, the enzyme exhibited better stability in the neutral-alkaline pH range. The enzyme was ~50% active at pH 11.5 but lost its activity at pH 12.0. The stability of CsMan5A at pH 5.0 at various time-intervals up to 24 h was also assayed. Our results indicate that the purified β-mannanase retained 98%, 90%, 86%, 81%, 78% and 75% of its initial activity after 0.5 h, 2 h, 4 h, 6 h, 12 h and 24 h respectively. CsMan5A displayed a temperature optimum of 65 °C (Fig. 5c). The enzyme was fully active up to 55 °C for

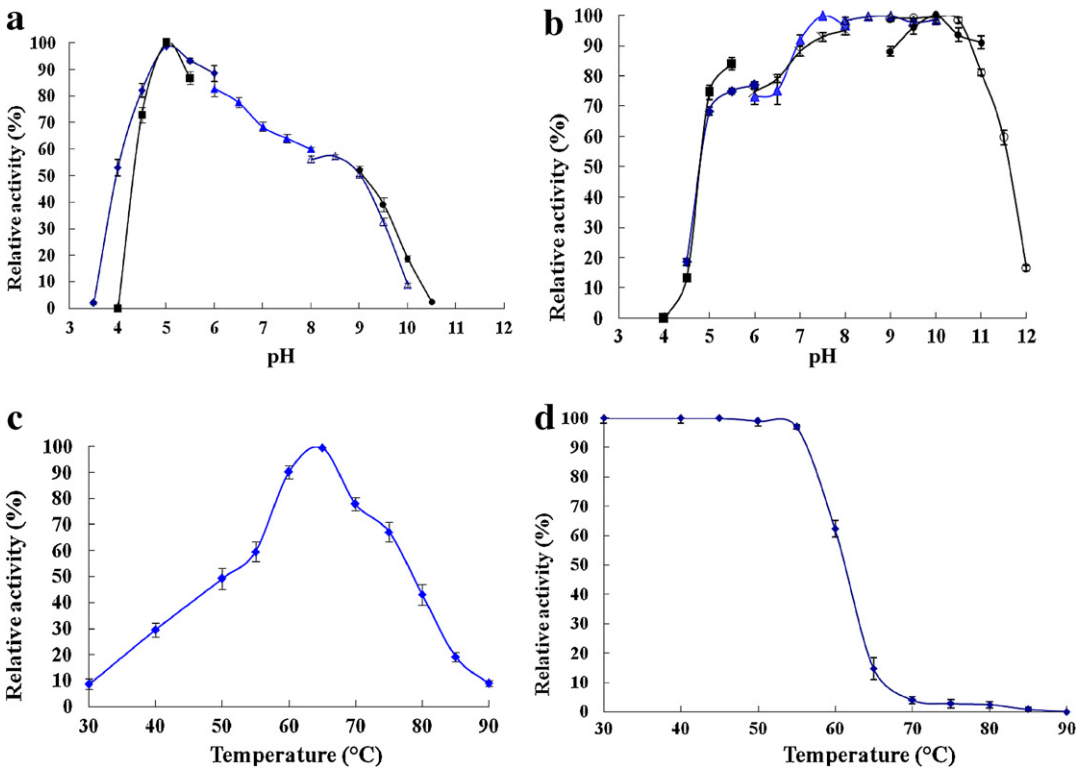

Fig. 5. Determination of optimum pH (a), pH stability (b), optimum temperature (c) and thermostability (d) of the purified recombinant β-mannanase. The optimal pH was determined in different buffers such as sodium citrate (♦) (pH 3.5–6.0); sodium acetate (■) (pH 4.0–5.5); MOPS (▲) (pH 6.0–8.0); sodium phosphate (×) (pH 6.0–8.0); CHES (△) (pH 8.0–10.0); CAPS (●) (pH 9.0–11.0); Glycine-NaOH (○) (pH 9.0–12.0) at 50 °C. Assays were performed as described in the methods. For pH stability analysis, the enzyme was incubated with the above buffers for 30 min at 50 °C. The optimal temperature was determined at temperatures ranging from 30 to 90 °C in 50 mM sodium citrate buffer (pH 5.0). For analysis of thermostability, the enzyme was incubated in 50 mM sodium citrate (pH 5.0) for 30 min at 30–90 °C. All the experiments were done in triplicates and the error bars indicate standard deviations.

30 min at which it retained more than 90% of its activity (Fig. 5d). The resistance of β-mannanase to some metal ions was determined. The enzyme was enhanced by some metal ions such as K^+ (112%), Sr^{2+} (138%), Ca^{2+} (119%), Mg^{2+} (116%), Ni^{2+} (130%), Fe^{3+} (143%), Ba^{2+} (142%), Cu^{2+} (180%), Co^{2+} (219%), Na^+ (110%) and Zn^{2+} (110%) after incubation for 30 min. EDTA enhanced the enzyme activity to 116% indicating that CsMan5A is not a metal-dependent enzyme. The reducing agent DTT enhanced the enzyme activity to 123% (data not shown). The activity was inhibited by Mn^{2+}, Ag^+, SDS (relative activities, 50%, 20%, 20% respectively) and completely inactivated by Hg^{2+}.

The optimal pH (pH 5.0) of CsMan5A compares well with other fungal β-mannanases (Duruksu et al., 2009; Kurakake, Sumida, Masuda, Oonishi, & Komaki, 2006; Naganagouda, Salimath, & Mulimani, 2009). Generally, fungal β-mannanases are stable in the acidic to neutral pH range while a few have been shown to be stable up to pH 8.5 (Zyl et al., 2010). This is a drawback in the paper and pulp industries which require enzymes able to function efficiently at high pH (Zyl et al., 2010). Bacterial β-mannanases, on the other hand have been shown to exhibit alkaline-pH stability (Hatada et al., 2005; Takeda et al., 2004). Thus, CsMan5A exhibited better stability at alkaline pH range as compared to other fungal β-mannanases. So far, high stability over a wide range of pH (pH 0.5–11.0) was observed only in the case of r-MAN5A from an acidophilic fungus, *Bispora* (Luo et al., 2009). A xylanase from the same *Chaetomium* sp. CQ31 was also found to be alkaline pH stable (Jiang et al., 2010). CsMan5A may thus prove valuable in paper and pulp industries and detergent industries where the use of fungal β-mannanases have been limited so far. Fungal β-mannanases from thermophilic fungi exhibit variability in their thermostability. The enzyme from *A. niger* is thermostable at 70 °C while the others are stable in the temperature range of 40–60 °C (Benech et al., 2007; Do et al., 2009). CsMan5A is more thermally and pH stable than β-mannanases from *T. reesei*, *A. niger*, *A. sulphureus*, and *A. fumigatus* (Ademark et al., 1998; Chen et al., 2007; Duruksu et al., 2009; Stålbrand et al., 1993). The β-mannanase displays better resistance to most of the metal ions as compared to some other β-mannanases (Chen et al., 2007; Do et al., 2009; Naganagouda et al., 2009).

3.5. Substrate specificity and kinetic parameters

The relative activity of CsMan5A on various galacto and gluco mannan substrates was determined since β-mannanases from different organisms are known to display great variation in their substrate specificities. The enzyme showed highest activity for LBG galactomannan (100%) followed by guar gum galactomannan (71.3%) and konjac glucomannan (46.1%). The enzyme did not show any activity with carboxymethyl cellulose (CMC), starch, xylan and avicel. The kinetic parameters for various substrates were determined. The results revealed that CsMan5A had a higher affinity towards LBG (K_m = 3.1 mg mL^{-1}) than that of other mannans like guar gum (K_m = 9.3 mg mL^{-1}) and konjac powder (K_m = 10.5 mg mL^{-1}) (Table 2).

CsMan5A was found to be specific only for mannan polymers, exhibiting preference in the order of galactomannan > glucomannan. The absence of activity towards cellulose & xylan suggests that the β-mannanase can be used in the paper and pulp industries which require the enzyme to be cellulase free (Ferreira & Filho, 2004). Since the enzyme has strong affinity towards natural mannans, it can also be applied in the food and feed industries.

Table 2
Kinetic parameters for the purified recombinant β-mannanase.[a]

Substrate	V_{max} (μmol min^{-1} mg^{-1})	K_m (mg mL^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (mg^{-1} s^{-1} mL)
LBG	5974.3 ± 134	3.1 ± 0.16	99.6	32.1
Guar gum	5693.1 ± 170	9.3 ± 0.62	94.9	10.2
Konjac powder	3167.3 ± 57	10.5 ± 0.43	52.8	5.0

[a] Enzymatic reactions were carried out for 5 min at 50 °C in 50 mM citrate buffer (pH 5.0).

3.6. Hydrolysis of mannan polymers and manno-oligosachharides

In order to demonstrate the function and application of CsMan5A, the hydrolysis mechanism was studied. The degradation of various mannan polymers such as LBG and konjac powder by the recombinant enzyme was performed. The substrates used have different structures because of the different ratios of monomers found in the backbone and side chain substituents. As shown in Fig. 6, CsMan5A was able to efficiently degrade different types of mannan polymers such as galactomannan and glucomannan. The hydrolysis of LBG yielded predominantly mannobiose and mannose as the main products (Fig. 6a). The degradation of konjac powder resulted in formation of a mixture of mannose linked sugars such as mannotriose as well as mannooligosaccharides of different molecular sizes (Fig. 6b). The ability of the β-mannanase to hydrolyze various manno-oligosaccharides such as mannobiose, mannotriose and mannotetraose was assessed. CsMan5A was found to be highly efficient in hydrolysis of various manno-oligosaccharides. Mannotriose was completed hydrolyzed to mannobiose and mannose in 2 h (Fig. 6c) whereas mannotetraose was hydrolyzed to mannotriose and mannobiose in just 30 min which was further converted to mannose in 2 h (Fig. 6d). The β-mannanase did not hydrolyze mannobiose, thus the enzyme displayed an endo-type mode of action.

A few fungal β-mannanases such as those from *T. reesei* (Stålbrand et al., 1993) and *A. niger* (Ademark et al., 1998) produced

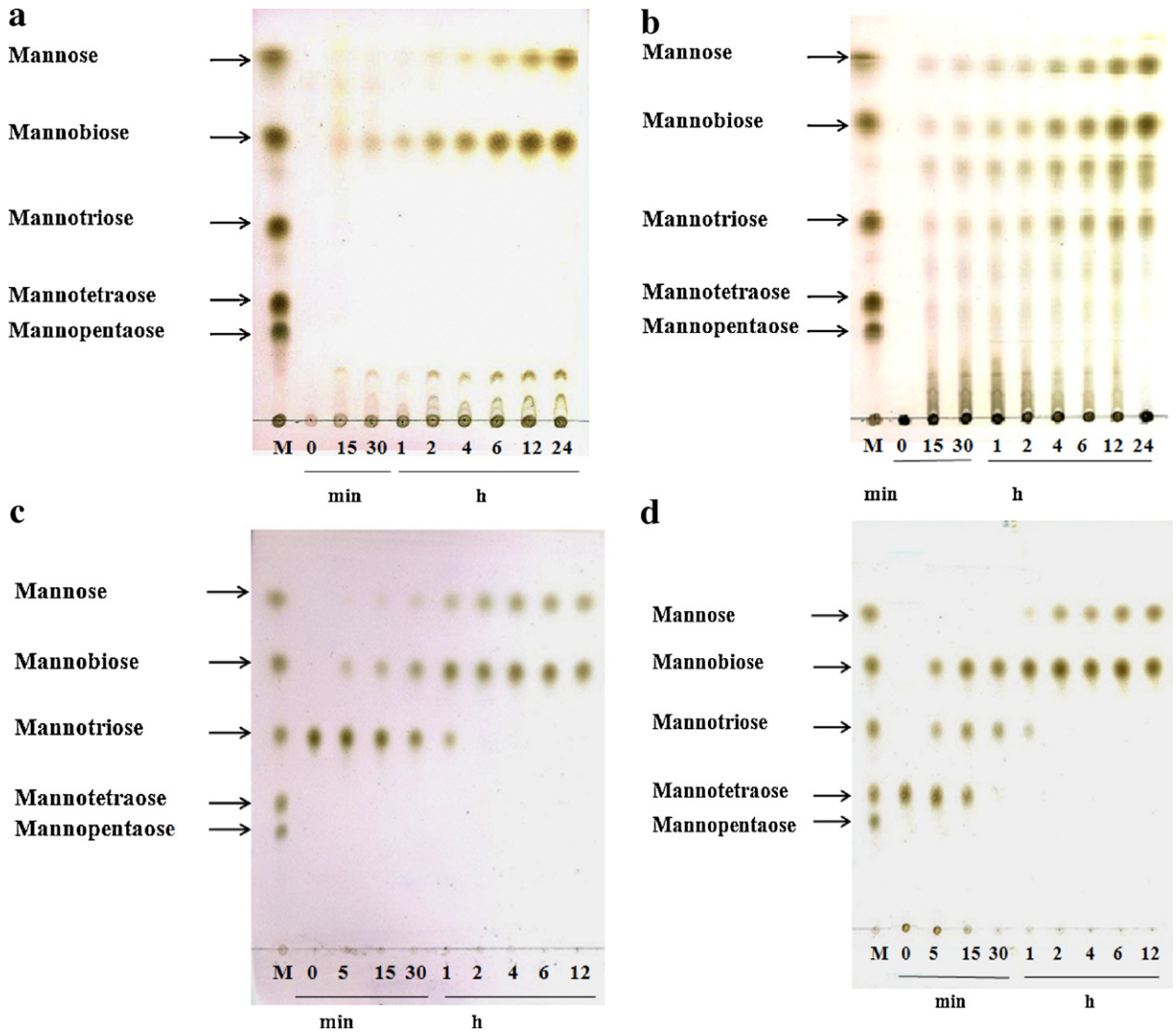

Fig. 6. Hydrolysis of various galacto and gluco mannans and manno-oligosaccharides by the recombinant β-mannanase. 5 U mL^{-1} of enzyme was incubated with different substrates such as 1% LBG (a), 1% konjac powder (b), 1% mannotriose (c) and 1% mannotetraose (d) in 50 mM sodium citrate buffer (pH 5.0) for 24 h at 50 °C. The samples of different time-intervals were analyzed by TLC. Incubation times (h or min) are indicated. Lane M, manno-oligosaccharides consisting of mannose, mannobiose, mannotriose, mannotetraose and mannopentaose.

mainly mannobiose and mannotriose and traces of higher oligosaccharides from hydrolysis of konjac powder. Some β-mannanases can also generate minor amounts of mannose and mannotetraose (Jiang et al., 2006; Luo et al., 2009). Konjac powder is widely available in Asia and has been used as a gelling and thickening agent in food and beverages but it has a low commercial value (Zyl et al., 2010). Hydrolysis of konjac powder using β-mannanase can result in formation of prebiotics such as mannooligosaccharides thereby increasing the commercial value of konjac powder. Although β-1,4-mannanases display an endo-type mechanism, they differ in the extent of degradation of different polysaccharide chains. A majority of the β-mannanases degrade mannooligosaccharides down to a DP4 (Stålbrand, 2002). Some fungal β-mannanases can convert mannotetraose to mannotriose and mannobiose through the transglycosylation reaction (Puchart et al., 2004). A recombinant β-mannanase (Man5D) from *P. chrysosporium* produced mannobiose as the major product when mannotetraose or mannopentaose was used as substrates (Benech et al., 2007). However, only some β-mannanases are active on mannotriose and at a much lower hydrolysis rate as compared to mannohexaose and mannotetraose (Harjunpaa, Teleman, Siika-aho, & Drakenberg, 1995). It is noteworthy that CsMan5A displayed efficient hydrolysis of mannoligosaccharides of DP4 as well as DP3.

4. Conclusions

A β-mannanase gene (*CsMan5A*) from the fungus, *Chaetomium* sp. was cloned and expressed in *P. pastoris* under the control of *AOX1* promoter for the first time. The enzyme expression levels are by far the highest obtained, thus making it highly cost-effective for commercial production. The enzyme exhibited high specific activity, good thermal and pH stability as well as resistance to many metal ions. Its efficiency in hydrolyzing various mannan polysaccharides makes it potentially useful in a wide variety of applications in the food and feed as well as paper and pulp industries.

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