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Full Length Research Paper

Cloning and expression of a *Trichoderma longibrachiatum* β-mannanase gene in *Pichia pastoris*

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Trichoderma **species are among the primary producers of β-mannanase, an enzyme that catalyses the hydrolysis of β-1, 4-glycosidic linkages in mannans and heteromannans. In this study, a** ***Trichoderma*** **species producing high mannanase activity was identified as** ***Trichoderma longibrachiatum*** **based on sequence analysis of its rDNA internal transcribed spacer region. The open reading frame of the gene encoding for β-mannanase of** ***T. longibrachiatum*****,** ***man1*** **is 1,441 bp and is separated by two introns. The MAN1 amino acid sequence showed 95% identity to** ***Trichoderma reesei*** **β-mannanase. Domain analysis classified MAN1 as a member of glycosyl hydrolase family 5 and detected the presence of a carbohydrate-binding domain family 1 at its C-terminus. The recombinant mannanase, rMAN1, was successfully expressed as a ~60 kDa extracellular recombinant protein in** ***Pichia pastoris*** **and was verified via western blotting analyses. The specific activity of the purified rMAN1 was 1416.18 U/mg. The optimal rMAN1 activity was recorded at 55°C and pH 5. The enzyme was stable with 30 min pre-incubation at temperatures ranging from 4 to 50°C. The enzyme was stable at pH 4 to 7 but became progressively unstable at pH values below 3 and above 8. rMAN1 had a high affinity towards locust bean gum as a substrate, with a K_m value of 0.95 mg/ml.**

Key words: *Trichoderma longibrachiatum*, mannanase, *Pichia pastoris*, expression, recombinant.

INTRODUCTION

β-Mannan-based polysaccharides including linear mannans, glucomannans, galactomannans and galactoglucomannans are a major component of the hemicellulose fraction in higher plant cell walls (Petkowicz et al., 2001). The degradation of the mannan backbone depends on the action of β-mannanases (EC 3.2.1.78), β-mannosidases (EC 3.2.1.25) and β-glucosidases (EC 3.2.1.21). β-Mannanases are endohydrolases that catalyze the random hydrolysis of β-1,4-linkages within the main chain of mannans (McCleary, 1988). The β-mannanase sequence analyzes the assigned members of this enzyme to either glycosyl hydrolase (GH) family 5 or 26. However, the mannanases in family 26 are of bacterial origin with the exception of a few anaerobic fungi. Both bacterial and eukaryotic mannanases have been annotated to family 5.

Mannanases have drawn scientific and commercial attention because of their possible application in pulp

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Table 1. Oligonucleotide primers used in the study.

Primer	DNA sequence (5' to 3')	Function
ITS1 (F) ITS4 (R)	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	To amplify the ITS region with a size between 600 to 800 bp
1 F 1 R	GGCTACTTYGCSGGVWCSAAC CARCTCCCACGCAAABAYSGC	To amplify the partial *man1* gene with a size of ~ 450 bp
TSP 1 TSP 2 TSP 3	TATCCCGCCGTAGTCGTT GCTCAGCGGACTTGACTACAT TTGACATCGTTGAAGCCCCATAC	To amplify the upstream sequence of the partial *man1* gene
TSP 4 TSP 5 TSP 6	ACCTTCAGCCACATCGCCT GAACGACTACGGCGGGATAAA CACCTGGTTCACTAACTCGGCG	To amplify the downstream sequence of the partial *man1* gene
man1F man1R	GGCTTACACCTTTGCATGAT GTGTCGTCTCCCTCTTTCTCAAT	To amplify the *man1* cDNA with a size of ~ 1.3 kb
PK-F PX-R	GGTACCGCCTCAAGCTTTGTGACAA TCTAGACCTGCAGACAGGCACTGCG	To amplify the *man1* cDNA for cloning into expression vector

(Montiel et al., 1999), coffee (Sachslehner et al., 2000), detergent (Aehle, 2007) and poultry feeds (Mehri et al., 2010) industries. Given the natural abundance of mannan, a wide variety of bacteria, actinomycetes, yeasts and fungi are known to be mannan degraders (Montiel et al., 1999; Puchart et al., 2004; Mudau and Setati, 2008; Bo et al., 2009). Mannanases are also produced in higher plants such as tomato (Marraccini et al., 2001) and are present in the gut of invertebrates such as the marine mollusk *Mytilus edulis* (Xu et al., 2002). Among the sources of mannanase, the production of β-mannanase by microorganisms is the most promising due to its low cost, high production rate and readily manipulated conditions. In practice, microorganisms utilized in producing mannanases are primarily fungi belonging to the genera *Aspergillus* (Chen et al., 2006), *Sclerotium* (Sachslehner et al., 2000) and *Trichoderma* (Ferreira and Filho, 2004).

In this work, a mannanase encoding gene from a fungus that produces mannanase with relatively high activity was isolated. The mannanase gene was cloned and expressed in *P. pastoris*, a methylotrophic yeast known for producing foreign proteins at high levels, providing proper protein folding and growing to high cell densities using inexpensive media. Subsequently, the enzyme was purified, and its biochemical properties were characterized.

MATERIALS AND METHODS

Strains and plasmids

Trichoderma sp. strain DSM 63060 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), Germany. The *Escherichia coli* strain DH5α was obtained from the Molecular Mycology Laboratory, Universiti Kebangsaan Malaysia, Malaysia, and the *P. pastoris* strain X-33 was purchased from Invitrogen, USA. pGEM-T® Easy Vector was purchased from Promega, USA, and the pPICZαA vector was obtained from Invitrogen, USA. The pGEM-T® Easy Vector was used for DNA cloning procedures, whereas pPICZαA was used for the heterologous expression of recombinant β-mannanase. The pPICZαA vector contains DNA sequences coding for an α-factor signal peptide at the N-terminus and a histidine tag (with six His residue) at the C-terminus.

Cloning of rDNA internal transcribed spacer (ITS)

Trichoderma sp. DSM 63060 was grown in potato dextrose yeast extract broth (10 peptone, 10 yeast extract and 4 g/L dextrose) at 30°C and 180 rpm on a rotary shaker. After 3 days of incubation, the mycelia were harvested in sterile conditions and grounded for genomic DNA extraction using polyvinylpyrrolidone (PVP) as described by Oh et al. (2009). The amplification of the ITS region from *Trichoderma* sp. DSM 63060 genomic DNA was carried out using universal fungal primers ITS1 and ITS4 (Table 1) (White et al., 1990). The PCR conditions were as follows: 94°C for 3 min, followed by 30 cycles at 94°C for 30 s, 62°C for 30 s, 72°C for 1 min and 72°C for 7 min. The PCR products were purified and cloned into pGEM-T® Easy Vector to facilitate DNA sequencing.

Isolation of the *man1* gene

The degenerate primers 1F and 1R (Table 1), used to amplify a partial β-mannanase gene, *man1*, from *Trichoderma* sp. DSM 63060 genomic DNA, were designed based on the conserved amino acid sequences of β-mannanase from *T. reesei* (accession no. AAA34208), *Aspergillus sulphureus* (accession no. ABC59553) and *Aspergillus aculeatus* (accession no. AAA67426) aligned using ClustalW version 2.0 (http://www.ebi.ac.uk/Tools/clustalw/; Larkin et

al., 2007). The amplification was carried out by an initial denaturation at 94°C for 3 min, 30 cycles at 94°C for 30 s, annealing temperatures for 30 s, and 72°C for 1 min, with the final extension cycle at 72°C for 7 min. The PCR products were subjected to DNA sequencing, and the sequences were analyzed using BLAST version 2.2.22 (http://blast.ncbi.nlm.nih.gov/; Altschul et al., 1997) to identify and confirm the identity of the amplified fragment. Based on the DNA sequences, three pairs of target-specific primers (TSP 1-6, Table 1) were designed for DNA walking PCR to isolate the remaining *man1* DNA sequence. DNA walking PCR was conducted using the DNA walking *SpeedUp*™ premix kit II (Seegen Inc., Korea) following the manufacturer's instructions. All PCR products were purified and sequenced.

Cloning of the *man1* cDNA

Trichoderma sp. DSM 63060 were grown in 0.5% (w/v) locust bean gum (LBG) broth (Sigma-Aldrich, USA) for 4 days at 30°C and 180 rpm. The mycelia were harvested using several pieces of muslin cloth and subjected to total RNA extraction using TRIzol® reagent (Invitrogen, USA) following the manufacturer's instructions.

The first-strand cDNA of *man1* was synthesized using the Omniscript® reverse transcription kit (Qiagen, USA) according to the manufacturer's instructions. The *man1* cDNA was amplified using total RNA as template and specific primer pair man1F/man1R (Table 1). The PCR conditions were as follows: 94°C for 3 min, 30 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 100 s and 72°C for 7 min. The purified PCR products were ligated into pGEM-T® Easy Vector to generate plasmid pGEMT-man.

Nucleotide and amino acids sequence analysis

Sequence analysis of the ITS region was performed using TrichOKEY version 2 (http://www.isth.info/tools/molkey/index.php; Druzhinina et al., 2005). This program was developed for the identification of *Hypocrea*/*Trichoderma* species. The putative motifs in the promoter region were predicted using the Neural Network Promoter Prediction version 2.2 (http://www.fruitfly.org/seq_tools/promoter.html; Reese, 2001). The SignalP server version 3.0 (http://www.cbs.dtu.dk/services/SignalP/; Bendtsen et al., 2004) and Phobius version 1.0 (http://phobius.sbc.su.se/; Käll et al., 2004) were used to predict the signal peptide of MAN1. ClustalW software (htpp://www.ch.embnet.org/software/ClustalW.html; Larkin et al., 2007) and BOXSHADE software (http://www.ch.embnet.org/software/BOX_form.html) were utilized to compare the MAN1 sequence with other fungal mannanases available in the public database. The MAN1 domain structure was examined using the Pfam database (http://pfam.sanger.ac.uk/search; Finn et al., 2008).

Construction of the expression plasmid

The *man1* cDNA was amplified using the primer pair PK-F and PX-R (Table 1). The PK-F primer was constructed such that it introduced a *Kpn*I restriction site at the N-terminus, whereas the PX-R primer was designed to add an *Xba*I restriction site with two additional nucleotides at the C-terminus so that the reading frame through the C-terminal $(His)_6$ tag was preserved. The amplification was performed using plasmid pGEMT-man as a template with the following conditions: 94°C for 3 min, 30 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 100 s and 72°C for 7 min. A PCR product with the size of ~1.3 kb was ligated into the pGEM-T® Easy Vector to generate pGEMT-man1. This plasmid was digested by *Kpn*I and *Xba*I, and the ~1.3 kb digestion product was ligated into the pPICZαA vector to generate plasmid pPICZαA-man1.

Recombinant protein expression

The plasmid pPICZαA-man1w was linearized using *Sac*I and electroporated into *P. pastoris* X-33 competent cells to generate clone X-33/MAN1. A positive transformant was selected on YPD (1.0 yeast extract, 2.0 peptone, 2.0 dextrose and 2.0% agar) plates supplemented with 2000 µg/ml Zeocin™. As a control, *P. pastoris* X-33 was transformed with pPICZα A vector without a DNA insert; the clone was designated as X-33/pPICZαA. Selected zeocin-resistant colonies were grown in Buffered glycerol-complex medium (BMGY: 1% yeast extract, 2% peptone, 100 mM potassium phosphate at pH 6, 1.34% yeast nitrogen base, 0.4 µg/ml biotin, 1% glycerol) containing 1% casamino acid overnight at 30°C and 250 rpm (Al-Rashed et al., 2010). The cells were harvested by centrifugation in a swing-out rotor at 4000 rpm (5415R centrifuge, Eppendorf, Germany) for 10 min at room temperature and thoroughly re-suspended in expression media, Buffered methanol-complex media (BMMY: contains 0.5% methanol instead of glycerol in BMGY), to an absorbance of ~1.0 at 600 nm. The culture was further grown at 30°C with shaking at 250 rpm. Sterile methanol was supplemented into the expression media to a final concentration of 0.5% every 24 h. The cultures were drawn at day 1, 2, 3 and 4 to detect the expression of the recombinant mannanase (rMAN1). Moreover, the expression levels were analyzed at different methanol induction concentrations ranging from 0.1 to 3.0%. Subsequently, the proteins in each culture supernatant were concentrated via ultrafiltration using the Vivaspin 20 ml centrifugal concentrator with a 10 kDa molecular weight cut-off (Sartorius Stedim Biotech, Germany). Recombinant proteins in the concentrated filtrates were detected by western blot, and the expression levels were assessed via SDS-PAGE and assayed for β-mannanase activity.

Western blotting

Separated proteins in the 12% resolving gel (Laemmli, 1970) were transferred to a nitrocellulose membrane using the Mini Trans-blot system (Bio-Rad Laboratories Inc., USA) and run at 200 mA for 2 h. After transfer, the membrane was probed sequentially with a 1/5000 (v/v) dilution of mouse anti-His-tag IgG antibody (Novagen, Germany) and 1/10000 (v/v) dilution of horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Invitrogen, USA). The reaction was visualized using the SuperSignal West Pico Chemiluminescent substrate kit (Pierce, USA).

DNS assay for the measurement of recombinant β-mannanase activity

β-Mannanase activity was determined by monitoring the amount of reducing sugars liberated by the recombinant enzymes acting on galactomannan, such as LBG. The assay mixture for β-mannanase activity consisted of 0.625% (w/v) LBG dissolved in 0.8 ml of 50 mM

citrate sodium buffer at pH 5 and 0.2 ml of appropriately cell-free culture supernatant. After the mixture was incubated at 55°C for 5 min, the reaction was stopped by the addition of 1 ml of 3, 5-dinitrosalicylic acid, as described by Miller (1959). The reaction was boiled for 10 min and cooled to room temperature before the A_{540} was measured. The activity can be calculated based on the A_{540} value, where one unit of β-mannanase activity is defined as the amount of enzyme required to produce one µmol of reducing sugar per min.

Recombinant protein purification

The 5 ml His trap column was equilibrated with binding buffer (20 mM NaH_2PO_4, 0.5 M NaCl, pH 7.4) until the UV absorbance established a stable baseline. The concentrated culture supernatant was loaded into a 5 ml His trap column at a flow rate of 1 ml/min. The column was washed with 50 ml of binding buffer at a flow rate of 1 ml/min to remove the unbound proteins, and the bound protein was eluted with 50 ml of elution buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 200 mM imidazole, pH 7.4) at the same flow rate. Both unbound and bound proteins were analyzed with SDS-PAGE and western blotting.

Effect of temperature and pH on enzymatic activity

The optimal temperature for rMAN1 activity was determined by incubating 0.3 µg of purified enzyme with 0.5% LBG dissolved in 50 mM sodium citrate buffer at temperatures ranging from 4 to 90°C for 5 min. To estimate the thermal stability, 0.3 µg of purified enzyme was pre-incubated in 100 µl of 50 mM sodium citrate buffer pH 5 at various temperatures ranging from 4 to 90°C for 30 min. Subsequently, the rMAN1 activity was assayed under standard conditions (pH 5, 55°C for 5 min). The optimum pH for rMAN1 activity was measured by incubating 0.3 µg of purified enzyme with 0.5% LBG dissolved in the following 50 mM buffers: glycine-HCl (pH 2 to 3), sodium citrate (pH 3 to 6), sodium phosphate (pH 6 to 8) and Tris-HCl (pH 8 to 9). The pH stability of the purified recombinant enzyme was determined by measuring the residual enzyme activity under standard conditions after incubation of 0.3 µg of enzyme in the same buffer at 55°C for 30 min.

Substrate specificity and kinetic parameters

Assays for β-mannanase activity were performed by incubating the 0.3 µg of purified enzyme with LBG as well as guar gum at concentrations ranging from 0.5 to 2 mg/ml in 50 mM sodium citrate buffer, pH 5, at 55°C for 5 min. The released oligosaccharides were measured by the DNS assay. The Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) values were obtained from the Lineweaver-Burk plot.

RESULTS AND DISCUSSION

Species identification

The ITS DNA sequence data were collected, assembled and deposited into the NCBI nucleotide databases with the accession number HM192931. Sequence analysis of the ITS region using the TrichOKEY version 2 software showed that the isolated ITS DNA sequence belonged to *T. longibrachiatum* and *Hypocrea orientalis*. *H. orientalis* has been proposed as a teleomorph of *T. longibrachiatum*, and both are members of the *Trichoderma* section *longibrachiatum* (Samuels, 2006).

Analysis of the nucleotide and deduced amino acid sequence of the *T. longibrachiatum* β-mannanase

The full open reading frame of *T. longibrachiatum* β-mannanase gene, including DNA sequence 120 bp upstream, is given in Figure 1. The presence of two introns in the β-mannanase coding sequence was evident by aligning genomic DNA with the cDNA sequence. Both introns were confirmed and contained the invariant sequence of GT at the 5' end and AG at the 3' end. Analysis of the partial promoter region identified a "TATA box-like" sequence (Breathnach and Chambon, 1981), 5'-TATATAA-3', within the promoter at nucleotide -91 to -85. An additional motif with the sequence 5'-GTCAAT-3', which was homologous to the "CAAT box" consensus sequence (Breathnach and Chambon, 1981), was found at nucleotide -104 to -99. This DNA sequence was submitted to NCBI nucleotide databases with the accession number: HM230064. The gene was named *man1* and contained a 1441-bp open reading frame encoding a 437 amino acid primary protein with a calculated molecular mass of 44.43 kDa.

The deduced amino acid sequence was subjected to signal peptide prediction. Analysis using both SignalP and Phobius software suggested that a putative signal peptide cleavage site was situated between Ala19 and 20, which appeared to be a typical signal peptidase I Ala-X-Ala processing site. Additionally, an eight amino acid putative propeptide was located after the cleavage site, and it may have been cleaved by a proline-directed arginyl protease (Schwartz, 1986). Four putative N-glycosylation sites Asn-X-Ser/Thr, in which X represents any amino acids other than proline (Cereghino et al., 2002) were identified in MAN1.

T. longibrachiatum MAN1 showed 95, 58, 57 and 55% identity to glycosyl hydrolase (GH) family 5 β-mannanase of *T. reesei* MAN1, *A. aculeatus* MAN1, *A. sulphureus* MAN1 and *A. fumigatus* MANI, respectively. The high similarity of β-mannanase from *T. longibrachiatum* and *T. reesei* was due to the close relationship of the two species within the *Trichoderma* section *longibrachiatum*. In contrast, BLAST analysis revealed extremely weak similarities between the *T. longibrachiatum* MAN1 and the β-mannanases belonging to GH family 26. Domain

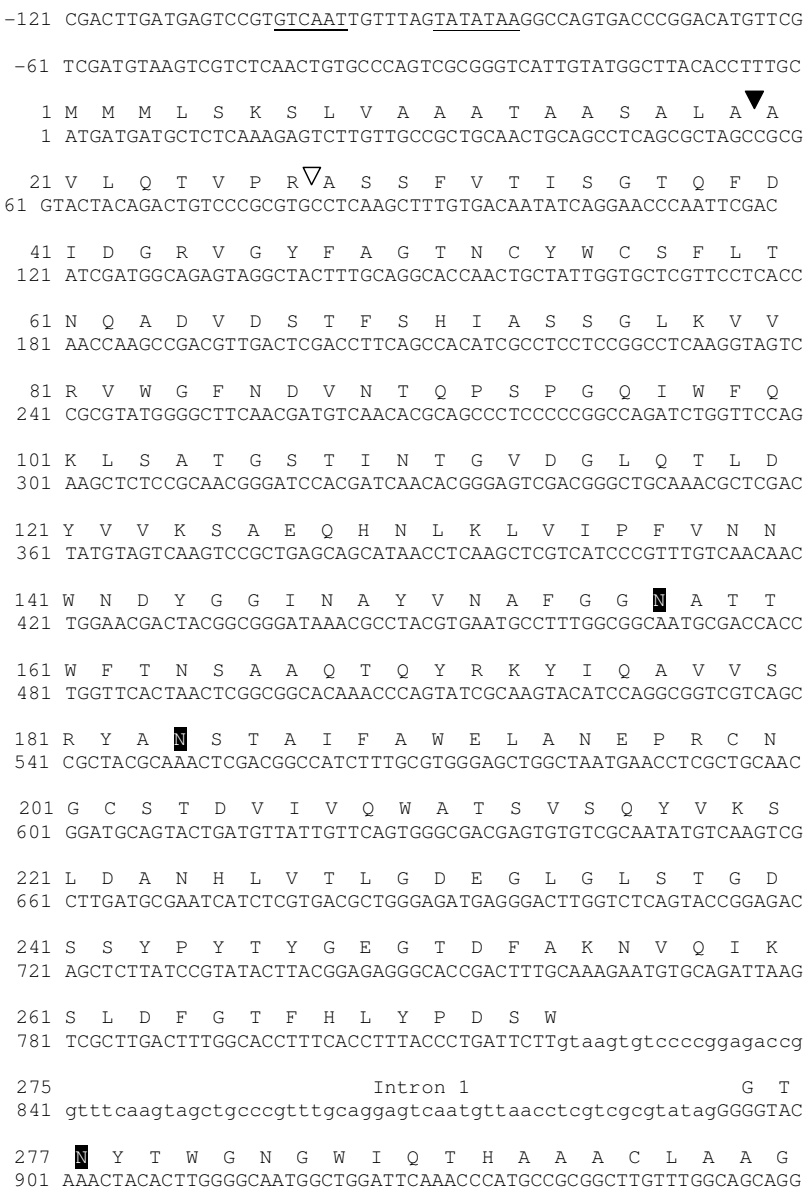

Figure 1. The nucleotide sequence of *T. longibrachiatum man1* and the deduced amino acid sequence of MAN1. The DNA sequence is presented from the 5' to 3' end. The intron sequences are in lowercase. A CAAT box-like sequence is underlined with a single line, whereas a TATA box-like sequence is underlined with two lines. The standard one-letter amino acid code is also used. The presumed signal peptide and propeptide cleavage sites are indicated by a solid and open arrowhead, respectively. Potential N-glycosylation sites are shaded in black. The stop codon is indicated by an asterisk (*).

analysis of MAN1 using Pfam database demonstrated that the protein structure at residues 14 to 279 had a high homology with that of GH family 5 catalytic domain. A carbohydrate-binding module of family 1 (CBM1) was also detected by Pfam at the C terminus of MAN1. In addition to the two domains, two active sites, Glu169 and Glu276, were predicted on MAN1. In *T. reesei* MAN1, eight identified active site residues were Arg54, His102, Asn168, Glu169, His241, Tyr243, Glu276 and Trp306 (Sabini et al., 2000). These residues were also present in

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 297  K  P  C  L  F  E  E  Y                Intron 2
 961 CAAGCCTTGCTTGTTCGAAGAATgtgagtgattatcctccgctttaccgctgagccaagc

 305                   G  A  Q  Q  N  P  C  A  N  E  A  P  W  Q
1021 taataaacgaccaccagACGGCGCGCAGCAAAATCCCTGCGCCAACGAGGCCCCCTGGCA

 319  T  T  S  L  T  T  R  G  M  G  G  D  L  F  W  Q  W  G  D  T
1081 AACAACCTCCCTCACGACTCGCGGCATGGGCGGCGACTTGTTTTGGCAGTGGGGAGACAC

 339  F  A  N  G  A  Q  S  N  S  D  P  Y  T  V  W  Y  N  S  S  N
1141 GTTTGCCAACGGTGCCCAGTCGAACAGTGACCCATACACCGTCTGGTACAATTCATCGAA

 359  W  Q  C  L  V  K  N  H  V  D  A  I  N  G  G  T  T  T  P  P
1201 CTGGCAATGCTTGGTTAAGAACCATGTCGATGCCATTAACGGCGGTACAACCACACCTCC

 379  P  V  S  S  T  T  T  T  S  S  R  T  S  S  T  P  P  P  P  G
1261 TCCCGTATCATCGACGACGACAACCTCGTCTAGGACCTCGTCAACGCCTCCACCGCCTGG

 399  G  S  C  T  Q  L  Y  G  Q  C  G  G  Y  G  Y  T  G  P  T  C
1321 AGGCAGCTGTACGCAATTGTATGGCCAGTGTGGAGGTTATGGATACACTGGTCCTACGTG

 419  C  A  Q  G  T  C  T  Y  L  N  Y  W  Y  S  Q  C  L  S  A  *
1381 CTGTGCGCAGGGAACTTGCACATATCTTAACTACTGGTACTCGCAGTGCCTGTCTGCATG

1441 AATGTCCGGGAAGACCCGGGGTGCGGATTGAGAAAGAGGGAGACGACACACTTATGTAGGA
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Figure 1: Continue

T. longibrachiatum MAN1 and conserved in several fungal mannanase (Figure 2), indicating the importance of these residues for the activity of the enzyme.

Expression of β-mannanase in *P. pastoris*

SDS-PAGE showed that an intact band with a molecular weight of ~60 ± 5 kDa was observed in culture filtrates of X-33/MAN1 with methanol induction (Figure 3a). As negative controls, culture filtrates of *P. pastoris* X-33 and X-33/pPICZαA did not show any expressed protein or develop any signal via western blotting. Western blot analysis revealed that the protein was recognized specifically by anti-His IgG antibody, demonstrating that the expressed heterologous proteins were rMAN1 (Figure 3b). The cell-free culture filtrates of X-33/MAN1 without methanol induction detected weak signals of MAN1 via western blotting. This result may be due to the basal expression of the protein in *P. pastoris* X-33. As compared to the non-induction group, the presence of methanol was significantly important as it helped in the induction of rMAN1 production.

The rMAN1 activity of each clone was assayed using 0.5% LBG in 50 mM citrate sodium buffer. These results show that the concentrated culture supernatant of X-33/MAN1 with methanol induction had higher activity (343.56 U/ml) than the control (Figure 4). However, culture filtrates of X-33/MAN1 without methanol induction had only slightly higher β-mannanase activity than the control (*P. pastoris* X-33 and X-33/pPICZαA), confirming the importance of methanol as an inducer of the strong *AOX1* promoter.

Determination of optimal post-induction time to harvest and methanol concentration for induction

To examine the expression of rMAN1, the optimal conditions necessary for recombinant protein expression in *P. pastoris* were optimized. The first parameter investigated was to determine the optimal post-induction time to harvest rMAN1. The SDS-PAGE analysis showed that the protein band of rMAN1 was the most intense band in the sample that was induced for 48 h (Figure 5a). This analysis was correlated with the result obtained for β-mannanase activity, because the highest β-mannanase activity was observed in concentrated culture supernatant that was harvested after 48 h of induction (Figure 5a). These results demonstrated that two days of induction were sufficient for the highest production of rMAN1. The decreased production of rMAN1 on day 3 and 4 may be due to cell death and it resulted in higher host proteases activity in the culture media (Zhang et al., 2007). The second parameter investigated was the optimal methanol concentration for induction.

The culture filtrates loaded onto SDS-PAGE (30 µg) showed that the protein bands of rMAN1 were the most

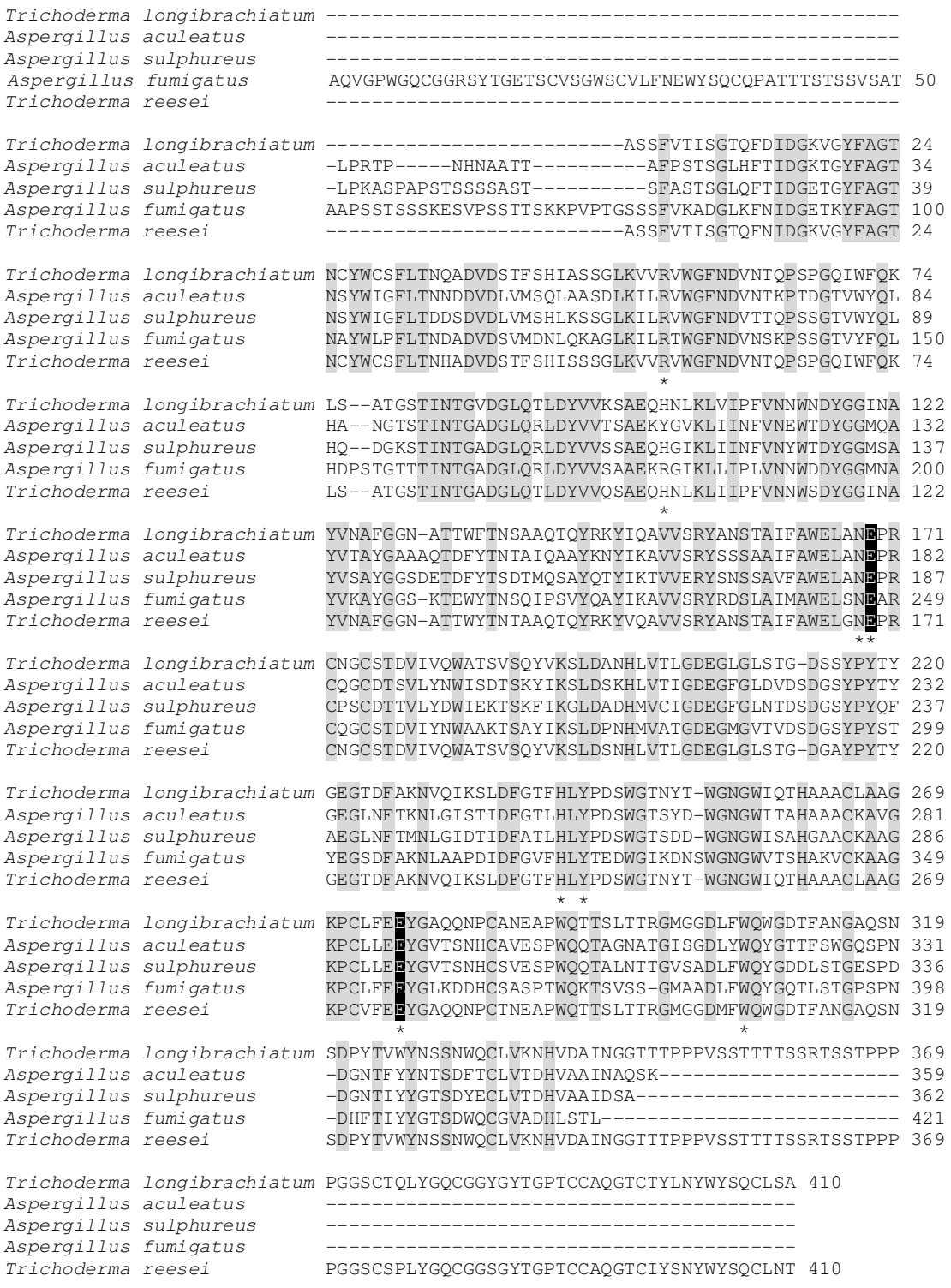

Figure 2. ClustalW alignment of *T. longibrachiatum* MAN1 with β-mannanases from *T. reesei*, *A. aculeatus*, *A. sulphureus* and *A. fumigatus*. Identical residues are indicated by a grey background, whereas putative catalytic residues are shown by a black background. Active site residues of *T. reesei* β-mannanase are indicated by an asterisk (*), whereas gaps in the sequence to facilitate the alignment are indicated by a hyphen(-).

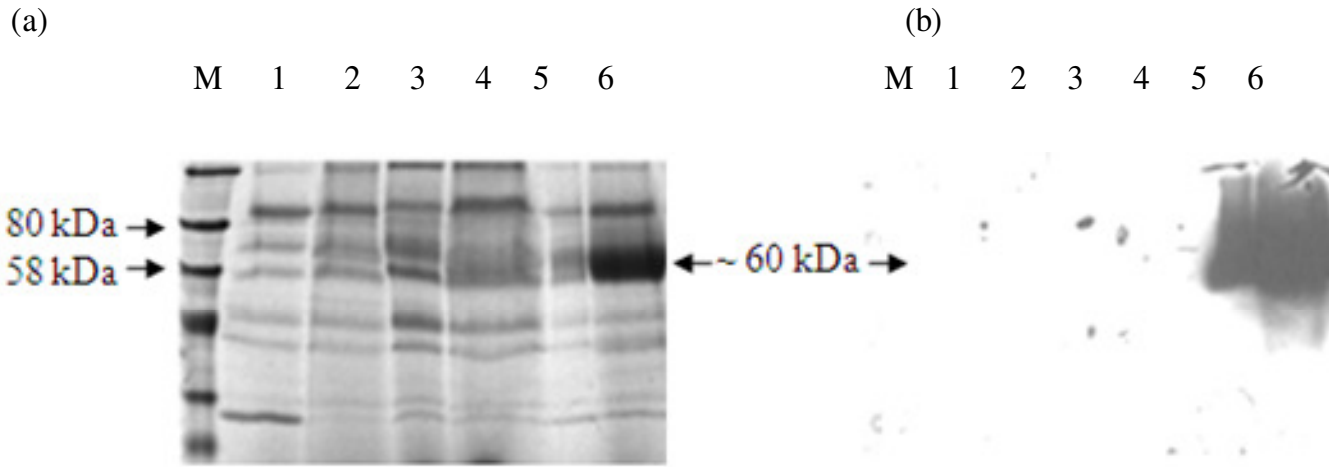

Figure 3. SDS-PAGE and western blot profiles of recombinant β-mannanase.(a) The SDS-PAGE (12%) profile shows overexpression of recombinant mannanase at ~ 60 kDa. Lane M: Pre-stained protein marker (New England BioLabs®, USA.); Lanes 1, 3 and 5: Proteins from culture filtrates of *P. pastoris* X-33, X-33/pPICZαA and X-33/MAN1W without methanol induction; Lanes 2, 4 and 6: Proteins from culture filtrates of *P. pastoris* X-33, X-33/pPICZαA and X-33/MAN1 with methanol induction. (b) Western blot analysis of SDS-PAGE from Figure 3a.

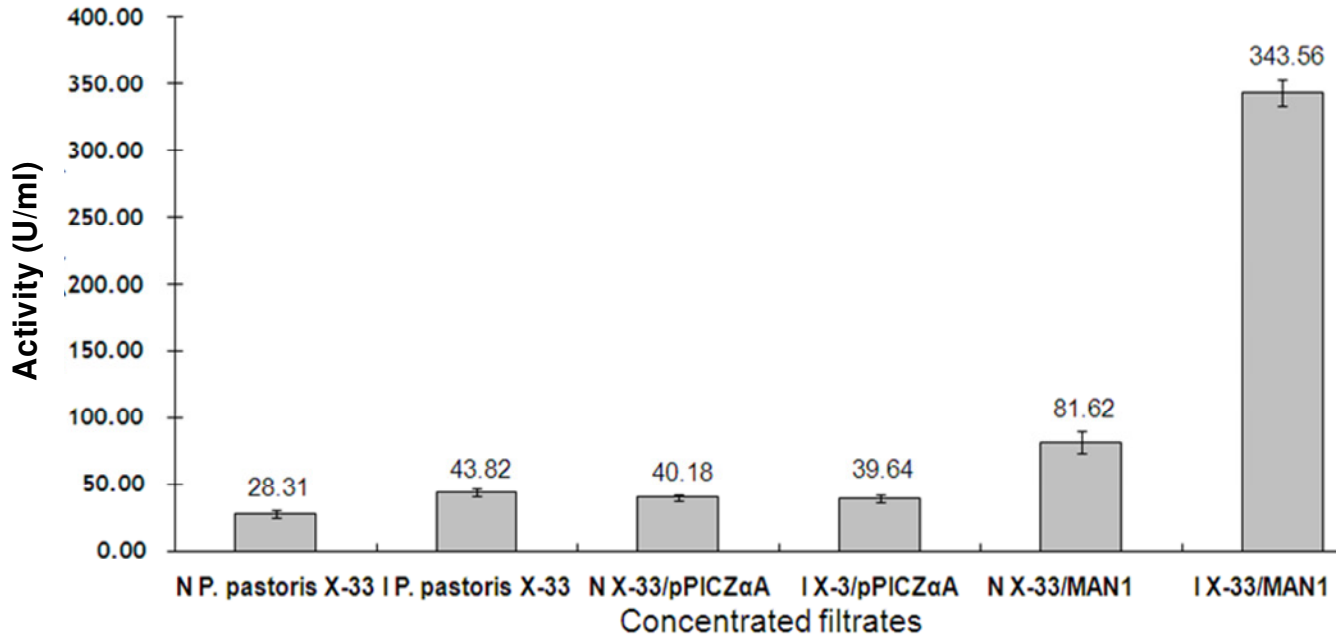

Figure 4. Comparison of enzymatic activity between partially purified proteins, which were grown with and without methanol, produced by *P. pastoris* X-33, X-33/pPICZαA and X-33/MAN1. The letter N in the graph represents non-induction, whereas the letter I represents induction. The standard deviations for triplicate samples are indicated with an error bar.

intense when X-33/MAN1 was induced with methanol at a final concentrations of 1.5% (Figure 5b). This observation is in agreement with the result obtained for the relative β-mannanase activity, and the highest activity was observed from the culture filtrates of X-33/MAN1 induced with 1.5% methanol (Figure 5b).

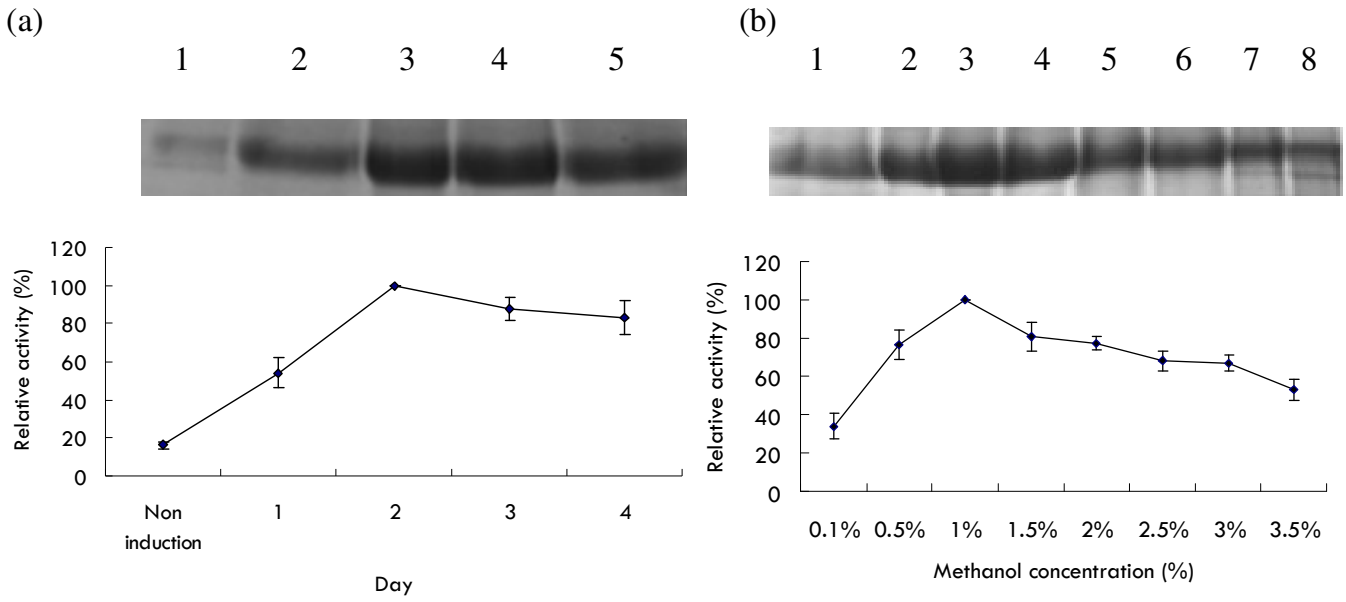

Figure 5. SDS-PAGE and relative recombinant β-mannanase activity profiles of culture filtrates harvested at different time points and concentrations of methanol. (a) Effect of induction times on MAN1 expression. Lane M: Pre-stained protein marker (New England BioLabs®, USA); Lane 1: Protein produced by X-33/MAN1 with no methanol induction for 96 h; Lanes 2 to 5: Proteins from X-33/MAN1 that were induced for 24, 48, 72 and 96 h. (b) Effect of methanol concentration on MAN1 expression. Lanes 1 to 8: 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5%.

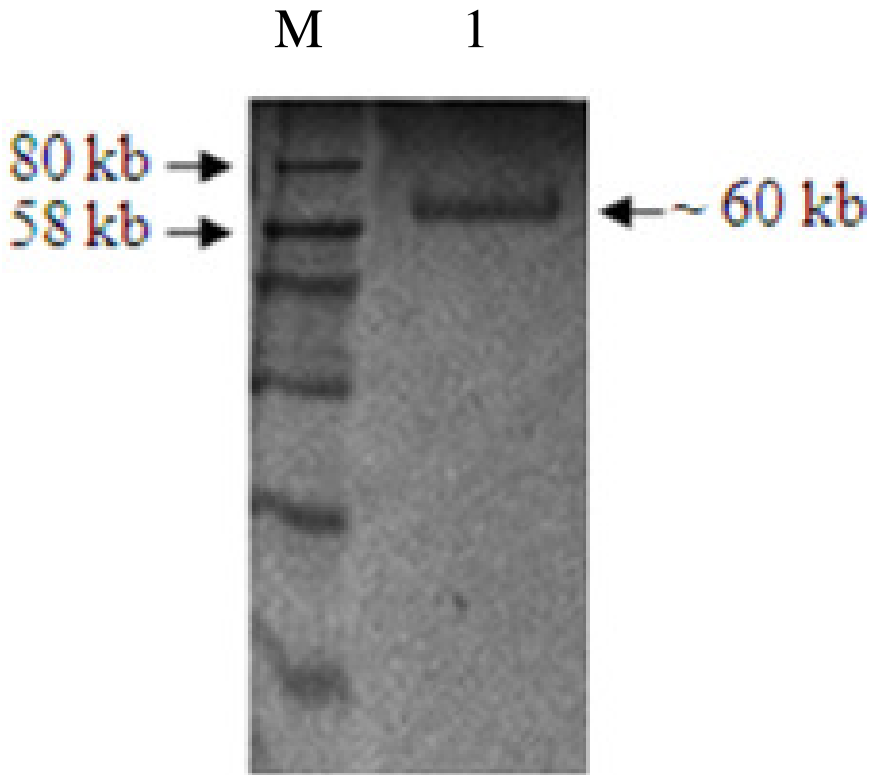

Figure 6. SDS-PAGE (12%) profile of purified MAN1 recombinant proteins.

Table 2. Summary of recombinant MAN1 purification.

Purification step	Volume (ml)	Amount of protein (mg/ml)	Amount of total protein (mg)	Activity (U/ml)	Total Activity(U)	Specific activity (U/mg)	Purification factor(Fold)	Recovery (%)
Crude supernatant	1000	0.20	201.6	54.58	54579.44	270.73	1	100
Ultra filtration	20	6.30	126.08	2063.26	41265.28	327.29	1.21	75.61
IMAC	0.9	21.82	19.64	30896.35	27806.71	1416.18	5.23	50.95

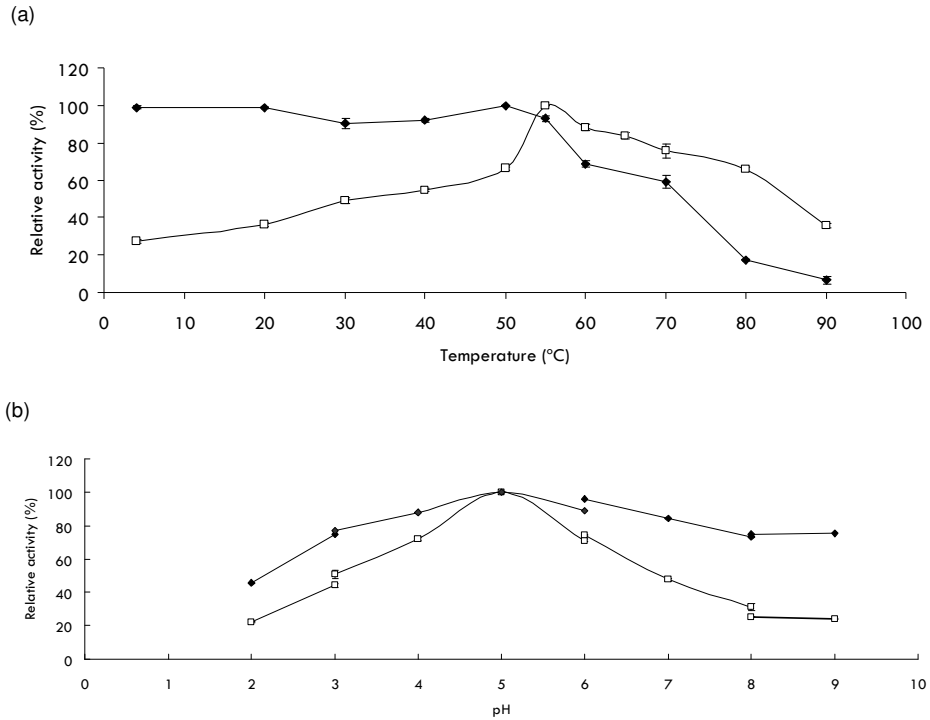

Figure 7. Biochemical characterization of *T. longibrachiatum* rMAN1. (a) Effect of temperature (□) and thermal stability (◆) of the purified rMAN1. (b) Effect of pH (□) and pH stability (◆) of the purified rMAN1.

Purification of recombinant MAN1

The purification steps for MAN1 are summarized in Table 2. High level expression of recombinant protein in *P. pastoris* X-33 was obtained. From the shake flask cultivation, 201.6 mg/L of protein was obtained for X-33/MAN1. The production of rMAN1 in this experiment was higher than that of *Mytilus edulis* β-mannanase expressed in *P. pastoris* (100 mg/L) (Xu et al., 2002) and *A. aculeatus* β-mannanase in *S. cerevisiae* (86 to 150 mg/L) (Setati et al., 2001). However, the amount of β-mannanase produced here was lower than that of Chen et al. (2006), who showed a production level of 262 mg/L *A. sulphureus* β-mannanase in *P. pastoris*. The purification steps presented yielded an enzyme preparation that appeared to be homogeneous and pure, as observed by a Coomassie-stained single band detected for rMAN1 in SDS-PAGE (Figure 6). The molecular mass of mature MAN1 deduced from the amino acid sequence was ~49 kDa; however, the expressed rMAN1 from *P. pastoris* X-33 transformant was ~60 kDa. The higher molecular mass of rMAN1 might be ascribed to glycosylation as four glycosylation sites were predicted in the deduced amino acid sequence.

Biochemical characterization of rMAN1

Effect of temperature and pH

rMAN1 was active between 4 and 90°C, with an optimum activity at 55°C (Figure 7). The optimum temperature for *T. longibrachiatum* β-mannanase activity was similar to the β-mannanases of other mesophilic microorganisms such as *A. sulphureus* (Chen et al., 2006), *Aspergillus fumigatus* IMI 385708 (Puchart et al., 2004), *Bacillus licheniformis* THCM 3.1 (Kanjanavas et al., 2009) and *Trichoderma harzianum* T4 (Ferreira and Filho, 2004), which were in the range of 45 to 55°C (Table 3). However, the optimum temperature was lower when compared to β-mannanases of thermophiles, *T. neapolitana* 5068, displayed an optimum activity at 90°C (Table 3). Temperatures above 80°C resulted in the rapid inactivation of rMAN1, defining an upper limit to its thermotolerance. It is noted that the enzyme was stable up to 55°C, with a half-life of 30 min at 70°C (Figure 7a). This result showed that MAN1 is more thermostable than *M. edulis* β-mannanase, which loses its activity within 1 min at 60°C, and *Clostridium tertium* KT-5A, which only retains stability up to 45°C for 10 min (Table 3).

Purified rMAN1 exhibited enzymatic activity over a pH range of 2 to 9 with an optimal pH at 5 (Figure 7b). The optimum pH for rMAN1 was similar to those of fungal β-mannanases that usually lie between 3.0 and 5.5 (Table 3). However, it is lower than those of bacterial β-mannanases, which have an optimum pH close to neutral (Table 3). The relative activity of the recombinant MAN1 was less than 50% at pH levels lower than pH 3 and higher than pH 6. The enzyme was stable at 55°C for 30 min from pH 4 to 7 but became progressively unstable at pH values below 3 (Figure 7b).

Determination of kinetic parameters

When LBG, guar gum and CMC were used as the substrates, larger quantities of reducing sugars were liberated during the hydrolysis of LBG. A decline in reducing sugars of ~75% was observed when guar gum was used. However, no detectable reducing sugars were released during incubation with 1% CMC. These results indicate that the hydrolytic ability of rMAN1 depended on the nature of the substitution sugar in the mannan backbone; it could not hydrolyze the β-1, 4-cellulosidic linkages of CMC but could only hydrolyze the β-1,4-mannosidic linkages of mannan.

The K_m and V_{max} values were determined directly from Lineweaver-Burk double reciprocal plots. From the analysis, MAN1 had lower K_m in LBG (0.95 mg/ml) as compared to guar gum (2.65 mg/ml). Therefore, MAN1 exhibited low specificity with guar gum (mannose/galactose ratio, 2:1) but high specificity with LBG (mannose/galactose ratio, 4:1). This result indicated that the hydrolysis yield depended on the degree of galactose present as the side chain. MAN1 was found to exhibit decreased specificity with an increase in galactose substitution in galactomannans. It has been suggested that *T. reesei* β-mannanase contains at least four substrate binding subsites (Harjunpää et al., 1995). Therefore, binding to at least four subsites is required for efficient hydrolysis. Substitution of the substrate monomers at one of the subsite positions restricted hydrolysis, likely by inhibiting binding (McCleary, 1988). The K_m value of MAN1 for LBG was considerably lower than β-mannanases from *T. harzianum* T4, *A. fumigatus* IMI 385708, *B. stearothermophilus*, *P. occitanis* Pol6 and *M. edulis*, indicating higher substrate specificity (Table 3). Furthermore, rMAN1 exhibited higher catalytic efficiency towards LBG, and the k_{cat}/K_m was 2.39-fold higher than the catalytic efficiency towards guar gum.

Conclusion

A fungus producing β-mannanase was identified as *T. longibrachiatum*, and its β-mannanase encoding gene was isolated. The recombinant MAN1 was successfully expressed and validated by western blotting. The consi-

Table 3. Comparison of characteristics of *T. longibrachiatum* MAN1 and other β-mannanases.

Organism	Enzyme	M_w (kDa)	Optimum		Stability		Substrate	K_m (mg/ml)
			pH	Temperature (ºC)	pH	Temperature (°C)		
T. longibrachiatum (Current study)	MAN1	60	5	55	4-6 (30 min)	55 (30 min)	LBG	0.95
A. sulphureus[c] (Chen et al., 2006)	MANN	48	2.4	50	2.4-3.4 (1 h)	40 (6 h)	LBG	0.93
A. fumigatus IMI 385708 (Puchart et al., 2004)	MAN I	60	4.5	65	4-8.5 (5 h)	55 (6 h)	LBG	3.07
	MAN II	63	4.5	65	4.5-9 (5 h)	55 (6 h)	LBG	3.12
B. licheniformis THCM 3.1[a] (Kanjanavas et al., 2009)	ManBL3.1	40	9	45	7-9 (48 h)	45 (48 h)	LBG	2.52
B. stearothermophilus[a] (Ethier et al., 1998)	ManF	73	6.5	70	ND[g]	ND[g]	LBG	2.4
C. tertium KT-5A (Kataoka and Takiwa, 1998)	β -mannanase	ND[h]	6.5	55	6-7 (3.5 h)	45 (10 min)	LBG, Spino gum, Guar gum, Konjac flour,Waxy starch, CMC[i]	ND[h]
M. edulis (Xu et al., 2002)	ManA	39	5.2	50	4-9.5 (24 h)	40 (ND[g])	LBG	3.95
	ManB	39	5.2	50	4-9.5 (24 h)	40 (ND[g])	LBG	ND[g]
Penicillium occitanis Pol6 (Blibech et al., 2010)	ManIII	18	4	40	2-12 (24h)	70 (30 min)	LBG	17.94
Thermotoga neapolitana 5068 (Duffaud et al., 1997)	β -mannanase	65	7	90	ND[g]	ND[g]	LBG Azo-carob galactomannan	0.55 0.23
T. harzanum T4 (Ferreira and Filho, 2004)	Man I	32.5	3	55	7.5-8 (ND[g])	50 (ND[g])	LBG	1.3
T. reesei RutC30[b] (Stålbrand et al., 1995)	Man1 Pi 4.6	51	5.3	70	4-7 (24 h)	80 (30 min)	LBG	ND[g]
	Man1 Pi 5.4	53	5.3	70	3.5-6 (24 h)	80 (30 min)	LBG	

[a]Expressed in *E. coli,* [b]Expressed in *S. cerevisiae,* [c]Expressed in *P. pastoris,* [d]*N*-bromosuccinimide, [e]5,5-dithio-bis(2-nitrobenzoic acid), [f]Dithiothreitol, [g]Not determined.[h] Dimethyl sulfoxide[i] Carboxymethyl cellulose

derable high specific activity (1416 U/mg) and high level of expression in shake flask cultivation (0.2 mg/ml) made rMAN1 potentially cost-effective. Moreover,subsequent research can be focused on how to increase the recombinant protein yield using a bioreactor.

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