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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