Bioresource Technology 107 (2012) 333–338

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Cloning, expression and applicability of thermo-alkali-stable xylanase of *Geobacillus thermoleovorans* in generating xylooligosaccharides from agro-residues

Digvijay Verma, T. Satyanarayana *

Department of Microbiology, University of Delhi South Campus, New Delhi 110 021, India

ARTICLE INFO

Article history:
Received 10 November 2011
Received in revised form 11 December 2011
Accepted 12 December 2011
Available online 21 December 2011

Keywords:
Thermo-alkali-stable xylanase
Recombinant *xyl*-gt
Geobacillus thermoleovorans
Xylooligosaccharides
Pre-bleaching of paper pulp

ABSTRACT

A xylanase gene (*xyl*-gt) of 1.224 kbp was cloned from the extremely thermophilic bacterium *Geobacillus thermoleovorans* that encodes a protein containing 408 amino acid residues. Eight conserved regions (signature sequences) of GH family 10 xylanases have been found in the xylanase. When the xylanase gene was cloned and expressed in *Escherichia coli* BL21 (DE3), the recombinant strain produced xylanase titer of 270 U mg^{-1} which is 27-fold higher than the wild strain. It is optimally active at 80 °C and pH 8.5 with a high thermostability over broad range of pH (6–12) and temperature (40–100 °C). The end products of the hydrolysis of birch wood xylan and agro-residues included xylobiose, xylotriose, xylotetraose and xylopentaose. The xylanase of *G. thermoleovorans* is one of the rare xylanases that exhibits thermo-alkali-stability, and thus, it is a suitable candidate for pre-bleaching of paper pulps and generating xylooligosaccharides from agro-residues for use as prebiotics.

1. Introduction

Xylans are hetero- and homo-polysaccharides which are main constituents of hemicellulosic biomass of terrestrial plant cell wall matrix and second most abundant polysaccharide on earth. Its core comprises β-1,4 linked xylosyl residues, and the attachment of this core chain with various groups (4-O-methyl-D-glucuronosyl, α-arabinofuranosylresidues and others) makes it a heteropolysaccharide. Consequently the complete hydrolysis of xylan requires a group of enzymes including endo-β-D-xylanase, β-xylosidase, arabinofuranosidase, glucuronidase, acetylxylan esterase, and ferulic acid and *p*-coumaric acid esterases. Among these, xylanases have attracted considerable attention due to their catalytic activity for releasing lower xylooligosaccharides, and their potential application in textile, feed and biofuel industries. Xylanases attracted attention further after Viikari et al. (1986) reported their applicability in bleaching of pulp in the paper industry. The inclusion of xylanase treatment step reduces the chlorine consumption up to 25–35% in bleaching kraft pulp to make the process environment friendly. Although several xylanases have been reported from a plethora of microorganisms, most of them do not tolerate the harsh conditions employed in pulp processing. This process requires thermo-alkali-stable and cellulase-free xylanase, because these processes are usually operated at higher temperature and alkaline conditions.

An alkali-thermo-stable and cellulase-free xylanase suitable for pre-bleaching of paper pulps has been reported from *Geobacillus thermoleovorans* (Sharma et al., 2007). The xylanase produced by the bacterium exhibits optimum activity at 80 °C and pH 8.5. The cloning and expression of xylanase encoding gene (*xyl*-gt) of *G. thermoleovorans* is crucial for enhancing the enzyme production in order to make its production cost effective. In this investigation, cloning and expression of xylanase gene (*xyl*-gt) from *G. thermoleovorans* and its applicability in saccharifying agro-residues to produce xylooligosaccharides (XOs) are presented.

2. Methods

2.1. Bacterial strains and plasmids

G. thermoleovorans was isolated from the pulp samples collected from Century Paper Mills, Lal Kuan, Uttaranchal (India) (Sharma et al., 2007). *Escherichia coli* DH5α and *E. coli* BL21 (DE3) were used as hosts for DNA manipulations. The vectors pGEM-T Easy (Promega) and pET 28a (+) (Novagen, Madison, USA) were used for sequencing of the amplified gene and construction of the expression vector, respectively.

2.2. Genomic DNA extraction, amplification and sequencing

The genomic DNA of *G. thermoleovorans* was extracted according to Bazzicalupo and Fani (1995) and processed for amplifying

* Corresponding author. Address: Department of Microbiology, University of Delhi South Campus, Bentio Juarez Road, New Delhi 110 021, India. Tel.: +91 11 24112008; fax: +91 11 24115270.

E-mail address: tsnarayana@gmail.com (T. Satyanarayana).

0960-8524/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2011.12.055

the xylanase gene. One set of internal degenerate primer (Geo-xyl-int1F1/R1) was designed from the highly conserved regions obtained from multiple sequence alignment (MSA) of the amino acid sequences of xylanase protein of various *Geobacillus* and *Bacillus* spp. PCR amplification was carried out in a Thermocycler (Biorad, USA), and the amplicon was cloned into pGEM-T Easy vector for sequencing of the cloned fragment. The resulted sequence revealed strong homology with xylanases of *Geobacillus* sp. WB1 and *Geobacillus stearothermophilus*. Therefore, new set of primers (Geo-xyl F/R) was designed from the end regions of xylanase encoding genes of *Geobacillus* WB1 in order to obtain the complete ORF. The full-length xylanase gene was amplified under defined PCR conditions (initial denaturation at 95 °C for 5 min followed by 29 cycles of 94 °C for 50 s, 58 °C for 30 s and 72 °C for 1 min 20 s) in 50 μL reaction with final extension of 10 min at 72 °C using Geo-xyl F/R primers in a Thermocyler (Biorad, USA). The primers used in this study are listed in Table 1.

2.3. Construction and sequencing of the expression vector

To construct the recombinant vector, *xyl*-gt of *G. thermoleovorans* was amplified with the primers Geo-BamF and Geo-SacR primers (Geo-xyl F/R primers with the added compatible restriction sites of Bam HI and Sac I, respectively). The digested PCR product was purified by Qiagen PCR purification kit and ligated into Bam HI and Sac I digested pET 28a (+) vector. The ligation mixture was transformed into *E. coli* DH5α competent cells. The clones were screened for the positive recombinants using colony PCR followed by double digestion of the recombinant vectors with Bam HI and Sac I. Five positive clones were sequenced at the Nucleic Acid Sequencing Facility, University of Delhi South Campus, New Delhi for obtaining the error free clone of xylanase gene.

2.4. Expression of the recombinant xylanase

One of the three clones having exactly the same gene sequence was selected for expressing the xylanase protein. Recombinant plasmid was isolated and transformed into *E. coli* BL21 (DE3). A 16–18 h old seed culture was prepared by growing the clone in Luria–Bertani (LB) broth containing kanamycin (50 μg/mL). Fifty milliliter of LB medium containing kanamycin (50 μg/mL) was inoculated with 1% (v/v) seed culture followed by further cultivation at 37 °C to achieve an optical density of 0.5–0.7 at 600 nm. Thereafter the expression of xylanase was induced by adding 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). The cell biomass was harvested periodically to study the growth profile as well as xylanase synthesis.

2.5. Bioinformatics analysis

Homology analysis of the *xyl*-gt nucleotide sequence and its predicted amino acid sequence with other known xylanases was carried out by using the database BLASTn (Basic Local Alignment Search Tool for Nucleotides) and BLASTp (Basic Local Alignment Search Tool for Protein), respectively. The CLUSTALW program (http://www.ebi.ac.uk/clustalW) and MEGA 4 (with minimum evolution) were used for the multiple sequence alignment (MSA) of amino acid sequences and preparing the dendrogram, respectively.

Table 1
Primers used in this investigation.

Primers[a]	Oligomeric sequences (5′–3′)
Geo-xyl-intF1	CACACACTCGTTTGGCACARCCAA
Geo-xyl-intR1	NATGCTCACATCAAGCTCAGTNADNTG
Geo-xylF	CCC<u>GGATCC</u>ATGCGGAACGTCGT
Geo-xylR	CCC<u>GAGCTC</u>CTATTTGTGGTCGATAATAGC

N: A/C/G/T; R: A/G; Y: C/T.
[a] Internal degenerate primer (Geo-xyl-int1F1/R1) was used to obtain partial region of xylanase gene from *G. thermoleovorans*. This region showed strong homology with *Geobacillus* sp. WB1 and *Geobacillus stearoethermophilus*. Therefore, new set of primer (Geo-xylF/R) was picked from the end regions of xylanase encoding genes of *Geobacillus* WB1 in order to obtain the full length gene.

2.6. Analysis and localization of recombinant xylanase (xyl-gt)

Localization of recombinant xylanase was done for confirming the cytoplasmic, periplasmic or extracellular nature of xylanase. The induced culture was harvested by centrifugation after 5 h of induction. The supernatant was collected as extracellular fraction while the pellet was suspended in 20 mM TE buffer (pH 8.0) with 25% (w/v) sucrose and 1 mM EDTA. The cell suspension was shaken in an incubation shaker at 200 rpm for 15 min and cells were sedimented by centrifugation at 4 °C, and subjected to osmotic treatment for 10 min by suspending the pellet into 5 mM chilled $MgSO_4$ solution to release the periplasmic fraction of the cells which was collected as supernatant on centrifugation. Finally the intracellular fraction was collected on homogenizing the cells in chilled sonication buffer (25 mM Tris–HCl, 100 mM NaCl, 10 mM $MgCl_2$, 6 M urea and 1 mg/mL lysozyme). The intracellular protein was released on sonication with 10 cycles of 1 min pulse (2 s on/off). The supernatant was separated from the cell debris after centrifugation and stored at −20 °C for assaying the enzyme in the intracellular fraction.

2.7. Xylanase assay

The activity of the recombinant xylanase was determined according to Archana and Satyanarayana (1997). The reaction mixture consisting of 500 μL of 1% (w/v) birchwood xylan (Sigma, St. Louis, MO) prepared 0.1 M glycine–NaOH buffer (pH 8.5) and 500 μL of appropriately diluted xylanase enzyme sample was incubated at 80 °C for 10 min. The reducing sugars liberated by the action on xylan were determined using 3, 5-dinitrosalicylic acid reagent (DNSA) (Miller, 1959). One unit of xylanase is defined as the amount of enzyme that liberates 1 μmol of reducing sugars as xylose under the assay conditions.

2.8. Purification of recombinant xylanase and zymogram analysis

The recombinant xylanase was purified by affinity chromatography using Ni^{2+}-NTA agarose resins with minor alterations (Singh et al., 2004). Supernatant was collected after sonication of the induced cells in lysis buffer [6 M Urea, 100 mM NaCl, 25 mM Tris HCl (pH 8.0), 10 mM $MgCl_2$ and 1 mg/mL lysozyme]. The column having 5 mL of Ni^{2+}-NTA resin were washed with sterile MQ (Milli Q water) and equilibrated with five column volume of buffer A (6 M urea in 100 mM phosphate buffer, pH 8, containing 5 mM β-mercaptoethanol) and buffer B [buffer A containing 10 mM Tris HCl (pH 8.0)] followed by buffer C [buffer B containing 20 mM Tris HCl (pH 8.0)]. Thereafter, supernatant having recombinant xylanase was passed five times through the column to bind the histidine-tagged protein. The column was washed with buffer C–D (100 mM phosphate buffer containing 10 mM Tris HCl pH 8.0, 20% v/v glycerol and 5 mM β-mercaptoethanol) in gradient manner followed by buffer E (buffer D containing 300 mM NaCl) to remove the unbound, non-specific proteins as well as other impurities. Finally, the bounded protein was eluted from the column by passing buffer D having a range of 100–500 mM imidazole. The fractions were collected and analyzed on 15% SDS–PAGE for the purity of the recombinant protein. The activity of the pure protein was confirmed by zymogram analysis on native PAGE. The gel was overlaid onto a 0.3% (w/v) xylan containing agar plate and incubated at