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Influence of transposition and insertion of additional binding domain on expression and characteristics of xylanase C of *Clostridium thermocellum*

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ABSTRACT

Clostridium thermocellum encodes a xylanase gene (*xyn*C) which is the major component of its cellulosome. XynC is a multidomain enzyme comprising of a substrate binding domain at the N-terminal followed by the catalytic domain and a dockerin domain. To study the influence of binding domain on activity, stability and expression of the enzyme the protein with the binding domain at C-terminal (XynC-CB), and the one with the binding domain at both N- and C-terminal (XynC-BCB) were expressed in *E. coli*. Recombinant plasmids, pXynC-CB and pXynC-BCB were constructed by inserting the corresponding gene in pET22b(+). XynC-CB and XynC-BCB were expressed at levels around 30% and 33% of the total *E. coli* cell proteins, respectively, while losing 40% and 20% of their activities at 70 °C for 120 min, respectively. The specific activities of XynC-CB, XynC-BCB were 76 and 98 U mg^{-1}, while the activities on equimolar basis were 4410 and 7450 U μM^{-1} against birchwood xylan, respectively. Their overall activities produced in the culture were 3660 and 5430 U L^{-1} $OD_{600}{}^{-1}$. Substrate binding studies showed that in case of XynC-C 51% of the activity remained unbound to birchwood xylan, whereas in the cases of XynC-BC, XynC-CB and XynC-BCB the activities left unbound were 33%, 32% and 12%, respectively, under the assay conditions used. Similar binding values were obtained in the case of oat spelt xylan. K_m values for XynC-CB and XynC-BCB against birchwood xylan were found to be 3.1 and 1.47 mg ml^{-1}, respectively. Thus addition of a second carbohydrate binding domain at the C-terminal of the catalytic domain enhances activity, substrate affinity as well as thermostability.

1. Introduction

Xylan, a major component of hemicelluloses, contains backbone of xylopyranose residues linked by β-1-4 glycosidic bond, which is mainly hydrolyzed by xylanases (E.C.3.2.1.8) (Shallom and Shoham, 2003). *Clostridium thermocellum* is an anaerobic thermophile, which produces its cellulolytic and xylanolytic enzymes in a multienzyme complex called cellulosome. The hydrolytic enzymes associated with cellulosome are usually multimodular having a distinct carbohydrate binding domain, a catalytic domain and a dockerin domain. The binding domains may be present at either terminus of catalytic domain and are also found in other plant cell wall hydrolases like β-mannanase, acetyl xylan esterase, arabinofuranosidases, and cellulases (Mangala et al., 2003).

Several xylanases like *Thermoanaerobacterium saccharolyticum* xylanase A (Lee et al., 1993), *Thermotoga maritima* xylanase A (Meissner et al., 2000), *Cellulomonas fimi* xylanase C (Clarke et al., 1996), and *C. thermocellum* xylanase X (Kim et al., 2000) have been reported to have thermostabilizing domains. Thermostabilizing domain of some multidomain enzymes also have a role of substrate binding as in the case of xylanase A of *Caldibacillus cellulovorans* (Sunna et al., 2000).

Xylanase C (XynC) of *C. thermocellum* is a major cellulosomal enzyme having a family 22 carbohydrate binding domain, a family 10 catalytic domain and a dockerin domain which are linked to each other with short linker peptides (Hayashi et al., 1997). In a previous study we reported enhanced expression of truncated derivatives of XynC and XynZ (Sajjad et al., 2010). The presence of family 22 carbohydrate binding domain was found to enhance thermostability of XynC. However, removal of the binding domain of XynZ did not show any effect on its thermostability. This study reports the effects of moving binding domain from N-terminal of the XynC catalytic domain to its C-terminal and also having binding domains at both the termini on their expression in *E. coli*, activity and thermal stability.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth media

Chromosomal DNA of *C. thermocellum* (ATCC 27405D), was used as a source of the xylanase genes (*xynC*: GenBank accession

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Table 1
Nucleotide sequence of PCR primers used for amplifications.

No.	Primer	Sequence	Restriction site[a]	ΔG (kcal mol^{-1})
1	*xynC-B*-F1	5′-CTGGATCCGCAGCTCTGATTTACGATG-3′	*Bam*HI	−1.5
2	*xynC-B*-R1	5′-GAGAATTCATGCAGGTGTTGCTGTGAAATC-3′	*Eco*RI	–
3	*xynC-C*-F2	5′-CACATATGAGCTTGAAAGACGTCTTTGC-3′	*Nde*I	−1.2
4	*xynC-BC*-F3	5′-CACATATGGCAGCTCTGATTTACGATGA-3′	*Nde*I	−1.5
5	*xynC-C*-R2	5′-CTGGATCCGGCGGTTCTGTCG-3′	*Bam*HI	–

[a] Restriction sites are underlined in the sequences.

no. D84188). pTZ57R/T vector obtained from Fermentas (Ontario, Canada) was used to clone PCR products. *E. coli* DH5α was used for vector propagation and transformation, while *E. coli* BL21 Codon-Plus (RIPL) and vector, pET22b(+) used for over-expression, were obtained from Novagen (Madison, USA). InsT/Aclone PCR product cloning kit was obtained from Fermentas (Ontario, Canada). QIAquick gel extraction kit was from QIAgen Inc. (USA). Strains were grown in LB or M9NG media (Sadaf et al., 2007).

2.2. PCR amplification and production of constructs

Oligonucleotide primers used for amplification of xylanase genes were designed using NEBcutter (Vincze et al., 2003), Primer 3.0 (Rozen and Skaletsky, 2000) and OligoCalc (Kibbe, 2007). These primers, designed on the basis of domain organization given at NCBI (XynC: GenBank accession no. BAA21516) and further checked with Pfam web-server (Finn et al., 2008) are given in Table 1. Possibility of any secondary structure formation was analyzed by determining free energy values for the fragment between the ribosomal binding site and the +10 codon using Mfold web-server (Zuker, 2003).

DNA fragments encoding XynC-C, XynC-B and XynC-BC were PCR amplified by initial denaturation at 95 °C for 4 min, then 30 cycles of denaturation at 95 °C for 45 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min 45 s, and final extension was done for 25 min. PCR amplified products were run on 1% agarose gel and purified by the QIAquick gel extraction kit (QIAgen Inc., USA).

The constructs *xynC-CB* and *xynC-BCB* were produced following the scheme shown in Fig. 1. The fragment encoding the binding domain (x*ynC-B*) was amplified using F1 and R1 as forward and reverse primers, respectively. For fragments encoding XynC-C and XynC-BC the primer sets used were F2 + R2 and F3 + R2, respectively. These amplified products were purified and cloned into pTZ57R/T, producing pTZ-BC, pTZ-C and pTZ-B. The *xynC-B* obtained by restriction of pTZ-B with *Bam*HI and *Eco*RI was purified and then sub-cloned into expression vector pET22b(+) at the same restriction sites, producing pXynC-B. Similarly the inserts after digestion of pTZ-BC and pTZ-C with *Nde*I and *Bam*HI were purified and then sub-cloned into pXynC-B upstream to *xynC-B*, to produce pXynC-BCB and pXynC-CB, respectively. *E. coli* DH5α cells were transformed with the constructs thus made and further confirmed by colony PCR and restriction analysis.

2.3. Expression analysis of recombinant xylanases

10 ml LB broth containing 100 μg ml^{-1} ampicillin were inoculated with cells from a single colony of the transformed *E. coli* cells and incubated at 37 °C overnight. 3 ml of this culture was added to 100 ml LB broth containing the antibiotic and incubated at 37 °C till OD_{600} reached 0.5–0.6. Expression of xylanases was induced with 0.5 mM IPTG or 10 mM lactose when M9NG medium (Sadaf et al., 2007) was used. Culture samples were drawn at regular intervals and analyzed by SDS-PAGE.

For isolation of the expressed enzyme the cells cultivated in the presence of 10 mM lactose for 15 h were harvested and resuspended in 0.05 M phosphate buffer (pH 6.0) to an OD_{600} 50. The cells were lysed in a French Press cell disrupter (Thermo Electron Corporation) and the lysate supernatant was obtained after centrifuging at 6500 rpm for 15 min at 4 °C. For partial purification the supernatant of the lysed cells was incubated at 60 °C for 1 h and transferred to ice bath for 15 min. The precipitated proteins were then removed by centrifugation at 10,000 rpm for 15 min. Supernatant thus obtained was used for further experiments.

2.4. Xylanase activity and protein assays

Xylanase activities were determined by mixing 20.0 mg substrate in 1.0 ml enzyme sample suitably diluted in 0.05 M phosphate

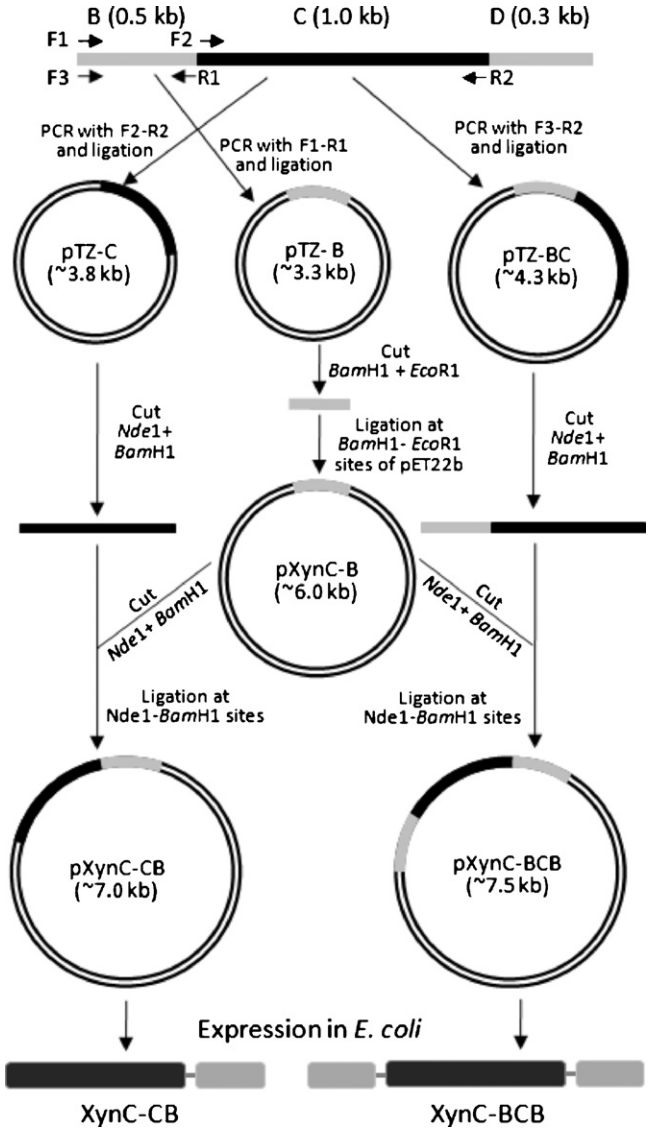

Fig. 1. Schematic diagram for producing constructs expressing XynC-CB and XynBCB of *C. thermocellum* in *E. coli*.