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Over expression of β-1, 4-xylanase by auto-induction in *E. coli*

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Abstract: Catalytic domain of β-1,4-xylanase gene, (*xynZ.CD*) of *Clostridium thermocellum* was cloned in pET28a expression vector and over-expressed in *Escherichia coli* BL21 CodonPlus (RIL). The production of XynZ.CD in *E. coli* was optimized using different concentrations of lactose and induction of the enzyme at different stages of growth. The maximum growth of the cells and the enzyme activity were observed when the cells were induced with 10mM lactose after 8 hours of incubation. The enzyme was found to constitute >40% of the total cell proteins in the supernatant of the lysed cells transformed with recombinant pET28a/*xynZ.CD.* It was purified by heating the cell lysate at 65°C for 30 m followed by fractionation through FPLC. Molecular weight of XynZ.CD was found to be approximately 38,524 D by MALDI-TOF analysis. The enzyme variant was quite stable within broad pH range of 5.5 - 8.0 and it retained >85% of xylanase activity after 2 h incubation at 70^0C.

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INTRODUCTION

Xylan, one of the major components of plant cell wall, belongs to a complex and highly variable family of polysaccharides which are based on a β - 1, 4 linked backbone of xylopyranosyl residues substituted with 4-O-methyl-glucuronosyl, 4-O-arabinosyl, and acetate side groups. Complete degradation of xylan requires the action of several types of enzymes[1]. Endoxylanase splits internal bonds in the polysaccharide backbone and β-xylosidase hydrolyzes xylo-oligosaccharides to D-xylose.

α-glucuronidase and α-arabinofuranosidase cleave the branched sugars. Esterases which deacetylate acetyl xylan have been identified in some fungal cellulolytic systems.

In anaerobic environments and decaying plant materials, complex communities of interacting microorganisms carry out decomposition of lignocellulose. Among the lignocellulolytic bacteria, clostridia play important roles in plant biomass turnover.[2] The gram positive anaerobic thermophile *C. thermocellum* secretes several xylanases and cellulases. Eight xylanase genes have been characterized from *C. thermocellum*[3-8].

Endo-β-1,4-D-xylanases are the enzymes that cleave xylan in a random manner. XynZ is a major endoxylanase associated with the cellulosome of *C. thermocellum*. It consists of N-terminus feruloyl esterase domain (FAE_{XynZ}), a Proline- rich linker (L), a family VI CBD (CBD_{XynZ}), a dockerin and a glycoside hydrolase family 10 catalytic domain[3,4] .

In the present study only the catalytic domain, *i.e.,* XynZ.CD of *C. thermocellum* was expressed in *E. coli*, to high level in an auto induction medium. This enzyme variant was purified and characterized for biochemical and enzymological properties.

MATERIALS AND METHODS

Strains, plasmids and growth media

Chromosomal DNA of *C. thermocellum* ATCC (27405D) was used as a source of the xylanase Z gene (Accession No: M22624). pTZ57R/T vector obtained from Fermentas (Ontario, Canada) was used to clone PCR product. *E. coli* DH5α was used for vector propagation and transformation, while *E. coli* BL21 CodonPlus (RIL) and vector pET28a (used for over-expression) were obtained from Novagen (Madison, USA). InsT/Aclone PCR product cloning kit was obtained from Fermentas (Ontario, Canada). QIAgen gel extraction kit was obtained from QIAgen Inc. (USA). Strains were grown in LB and M9NG Media[9].

PCR amplification of the xynZ *gene*

For amplification of *xynZ-CD* the forward primer used was 5′-GCAG**CCATGG**ATCCGT CTGTTACTCCGACACA-3′ while the reverse primer was 5′-GC**GGATCC**TATCAATAGC CCATAAGAGCTTCC-3′, with *Nco*I and *Bam* HI sites shown in bold, respectively. PCR amplification reaction was performed in 2720 Thermal Cycler (Applied Biosystems) for 30 successive cycles with denaturation at 95°C for 5 m, annealing at 65°C for 30 s and primer extension at 72°C for 1.5 min. $MgCl_2$ was varied in the concentration range 2-3.5 mM.

PCR amplified product was run on a 1% agarose gel and purified by the QIAquick gel extrcation kit (QIAgen Inc., USA) and cloned into pTZ57R/T by InstaTA cloning kit (Fermentas, USA). The recombinant pTZ57R/*xynZ.CD* was purified by the alkaline lysis method[10].

Cloning and expression

The *xynZ.CD* was excised from the recombinant pTZ57R by restriction with *Nco*I and *Bam*HI. After purification using the QIAgen gel extraction kit, the insert was ligated into pET28a, previously linearized with *Nco*I and *Bam*HI. Competent cells of *E. coli*

DH5α and *E. coli* BL21 CodonPlus (RIL) were prepared by $CaCl_2$ treatment[10]. *E. coli* DH5α cells were transformed with the ligation mixture to propagate recombinant vectors and transformants were screened by the colony-pick PCR. Recombinant plasmid pET28a/*xynZ-CD* was purified and competent cells of *E. coli* BL21 CodonPlus (RIL) were transformed with this DNA. Transformed cells were then plated on LB-agar plates containing 60µg/ml kanamycin and incubated at 37°C overnight.

About 10ml of LB broth containing 60µg/ml kanamycin was inoculated with single *E. coli* colony transformed with pET28a/X*ynZ.CD* and incubated at 37°C in an orbital shaker overnight. 3ml of the overnight inoculum was then added to 100ml LB broth containing 60µg/ml kanamycin and incubated in an orbital shaker at 37°C till OD_{600nm} reached 0.5 - 0.8. Expression of XynZ.CD was induced with 0.5mM IPTG or 10mM lactose. Culture samples were drawn after 0, 2, 4, 6, 8, 10, and 12 hours of induction. The proteins in different fractions were analyzed on 12% SDS-PAGE.

Effect of induction stage

100 ml M9NG medium[10] in 1 liter Erlenmeyer flask was induced with 10 mM lactose at 0, 2, 4, 6, and 8 hours after the cell growth. 5 ml of the culture was drawn out from each flask at 2 h interval and centrifuged. The cell pellet thus obtained was washed with 5ml of 0.05 M phosphate buffer (pH 6.0). The cells after resuspension in 5ml of the same buffer were sonicated (UP 400S, ultraschallprozessor, $dr.hielscher_{GmbH}$) for 10 m giving 30 s burst at 50% amplitude with 60 s interval. Cell debris was removed by centrifuging at 6,500rpm in Eppendrof 58404R centrifuge at 4°C for 15 m. Xylanase activity of the lysate supernatant was determined by incubating with 1% (w/v) birchwood xylan pretreated with 0.1M NaOH (Sigma) at 60°C for 10 minites. The liberated reducing sugar was determined by DNS method[11]. Proteins in the samples were analysed by SDS-PAGE using 12% gel[12]. The zymogram analysis was performed after separation of proteins by native PAGE using 10% gel[13]. The gel was over-laid after electrophoresis on the xylan-agar plates containing 0.05% substrate, and incubated at 60°C for 10 minutes. The plates were stained with congo red and then destained with 1M NaCl.

For preparative scale 400ml culture was cultivetd in 2L baffled flask under the conditions given above. The cells were harvested by centrifugation after 12 hours induction. After suspension in 0.05M phosphate buffer (pH 6.0), the cells were lysed in a French Press Cell Disrupter (Thermo Electron Corporation) and centrifuged to obtain cell lysate supernatant for further experiments.

Purification of XynZ.CD

The cell lysate heated at 65°C for 30 minutes at which most of the proteins were precipitated while retaining most of the enzyme activity. After centrifugation the clear supernatant was used for purification by FPLC (Amersham Biosciences). The sample was concentrated by lyophilization before loading on the Superdex 200 10/300 GL column with 1.5Mpa and flow rate 0.3ml/min, previously equilibrated with 0.05 M phosphate buffer (pH 6.0). Active fractions were pooled and analyzed by 12% SDS-PAGE.

Xylanase assay

Xylanase activity was determined by incubating the enzyme at 60°C for 10 minutes with 1% (w/v) birchwood xylan pretreated with 0.1M NaOH, as the substrate in 0.05 M phosphate buffer (pH 6.0). Reducing sugar thus released was measured by DNS method[11]. One unit of xylanase activity is defined as the amount of enzyme that released 1µmol of xylose equivalents. For assay on insoluble substrate enzyme was incubated with 10mg/ml Birchwood xylan for different time periods up to 2 hours and the liberated xylose was estimated.

For pH optimization the enzyme was diluted with phosphate buffer (pH 5.5-7.5) and Tris buffer (pH 8.0-9.0) and then assayed against pretreated birchwood xylan.

Enzyme stability was determined by incubating aliquotes of the sample at pH 6.0 but different temperatures for 2 hours and determining the residual activity at 60°C and pH 6.0. Effect of pH on stability was studied by incubating the sample aliquotes at 60°C but different pH for 2 hours and determining the residual activity at 60°C and pH 6.0.

Protein concentration was determined by Bradford Assay with BSA as standard[14].

RESULTS AND DISCUSSION

Cloning and expression of* xynZ *gene

For the in frame insertion of *xynZ-CD* in the expression vector, primers were designed with *Nco*I and *Bam*HI sites, and used to amplify the gene. The PCR products were analyzed on 1% agarose gel and were found to be of appropriate size i.e. 1 kb. After its gel purification, cloning in pTZ57R/T, restriction of the recombinant plasmid pTZ57R/*xynZ.CD* with *Nco*I and *Bam*HI , isolation of the gene insert and its ligation into pET28a produced the recombinant plasmid pET28a/X*ynZ.CD* (Figure1).

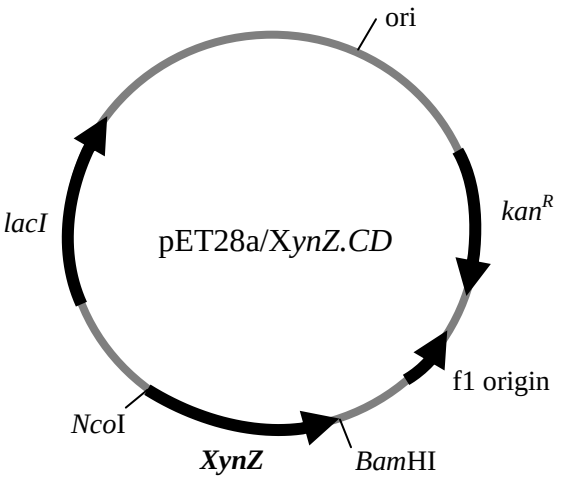


Figure 1: Recombinant plasmid produced by inserting the gene encoding XynZ.CD in pET28a between the *Nco*I and *Bam*HI sites.

E. coli BL21 CodonPlus (RIL) competent cells were then transformed with this plasmid. Transformants were found to express a 38kDa protein when induced with 10mM lactose. 12% SDS-PAGE showed that expression level was approximately 45% of the total cell protein (Figure 2).

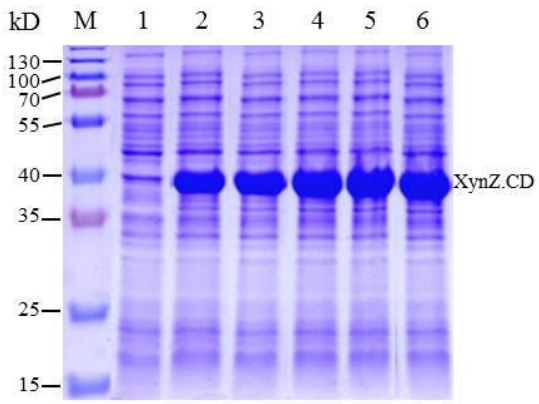


Figure 2: Expression of XynZ.CD in *E. coli* after induction with 10 mM lactose; M: protein markers; Lane 1: uninduced sample; Lanes 2-6: cell proteins at 2, 4, 6, 8 and 10 hours after induction.

Effect of inducer concentration

E. coli BL21 CodonPlus (RIL) cells harboring recombinant plasmid were induced with 5, 10, 15 and 20mM lactose concentration. The protein was expressed at same level in all cases. The culture $O.D_{600}$ was found to be 1.06, 1.12, 1.09 and 1.06, while the enzyme activity was 38, 43, 41 and 42 Uml^{-1} OD_{600}^{-1} for the cells induced with 5, 10, 15 and 20mM lactose, respectively (Table 1). Although, there was no significant effect on the growth of *E. coli* by the different concentration of lactose, 10mM lactose was found to be optimum for growth and activity yield.

Induction at different growth stages

The cells which were induced at the time of inoculation (0 hour) or after 2 hours growth showed a slow initial rate of growth, but the growth continued till later stages up to 18 h. The cells induced after 4 h growth continued to grow up to 16 h showing a maximum OD_{600} of 10.0 at that stage. The cells induced aftert 6 h growth showed a maximum OD_{600} of 14.0. The maximum growth, *i.e.* OD_{600} 17.0, was observed when induction was done after 8 h of growth (Figure 3).

As the enzyme activity is concerned, the cells which were induced at 0, 2 and 4 hours showed consistent activity (~ 40%) up to 18 hours. However, the cells which were induced after 6 and 8 hours showed comparable activity (~85%). The highest activity was observed in case of cells which were induced after 8 hours of inoculation.

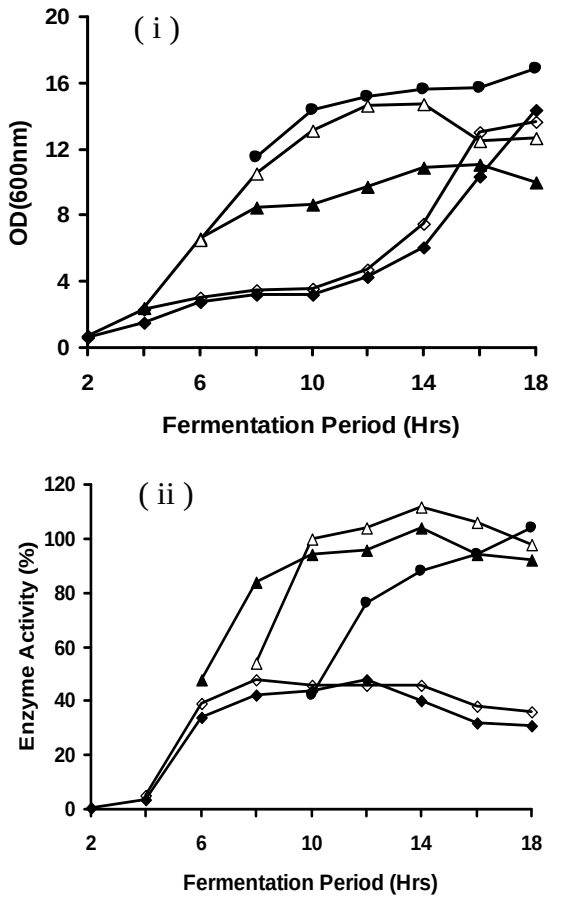


Figure 3: Effect of induction at different intervals on growth of *E. coli* and expression of XynZ.CD. (i) $O.D_{600nm}$ of the *E. coli* culture and (ii) XynZ activity in cell lysates (U/ml^{-1} $O.D_{600nm}^{-1}$) when induced with 10mM lactose at 0 (■), 2 (□), 4 (▲), 6 (Δ), and 8h (●) culture growth.

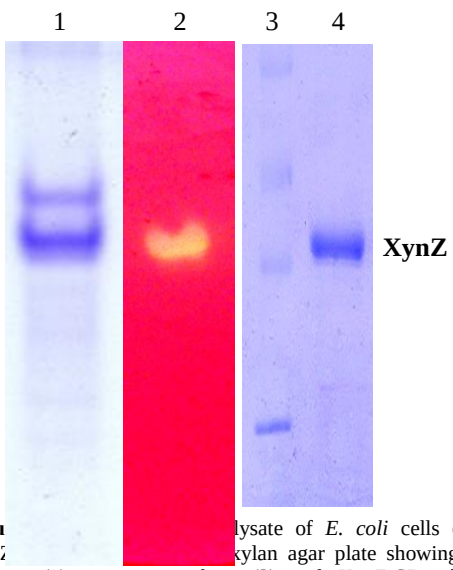
The maximum activity was observed after 18 hours of fermentation, for the cells which were induced after 6 and 8 hours of incubation. Thus induction after 8 hours of inoculation showed maximum growth of *E. coli* and activity yield of the enzyme. Olaefe *et al.*[15], reported that induction of *E. coli* after 8 h of fermentation lead to a higher culture density and the enzyme production. Khan *et al.*[16], showed the optimal production of xylalanase C of *C. thermocellum* with 10mM IPTG concentration[16].

Table 1: Activity yield of XynZ.CD of *C. thermocellum* after induction with different concentration of lactose.

Lactose mM	Enzyme activity $Uml^{-1}.OD600^{-1}$	Specific activity $Umg^{-1}.OD600^{-1}$
5	38	180
10	43	215
15	41	190
20	42	154

Enzyme properties

Purification of XynZ.CD through FPLC showed a single band as shown in Figure 4 (lane 4). The purified fraction when analysed corresponded to the active enzyme band as analysed zymographically (Figure 4).



Figu lysate of *E. coli* cells expressing Xyn xylan agar plate showing xylanase activity (2), size markers (3) and XynZ.CD after FPLC purification (4).

XynZ.CD showed maximum activity at 60°C and pH 6.0 under the assay conditions used. It retained 90, 80, 75 and 65 % of its activity when heated for 30 m at 65, 70, 75 and 80°C, respectively (Fig. 5A). The effect of pH showed that the enzyme was stable over a rather broad pH range, losing <15% activity when incubated at pH 5.5-8.0 at 70°C for 2 h (Fig. 5B).

Analysis by MALDI-TOF spectrometry showed a major fraction in the sample with a mass of 38,524 D (Figure 6). Fractions of 38,309 and 37,674 D also appeared. Fraction of 38,309 D seem to be of the molecule with a methionine residue cleaved at the N-terminal, which occurs commonly. The fraction appearing as 37,674 D could be due to further truncation of the molecule or it could just be an impurity.

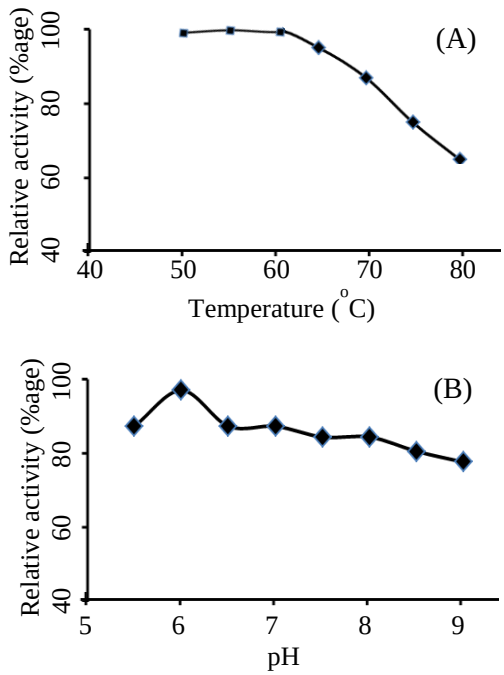


Figure 5: Effect of temperature (A) and pH (B) on the activity of XynZ.CD.

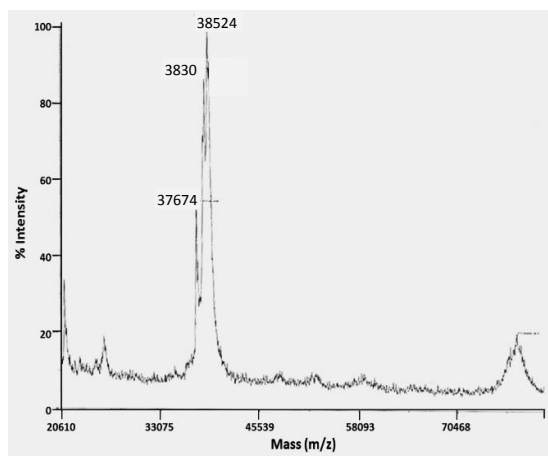


Figure 6: MALDI-TOF-MS analysis of XynZ.CD.

CONCLUSION

Induction of the *E. coli* cells transformed with pET28a/*XynZ.CD*, when allowed to grow for 8 hours

and then auto-induced with 10 mM lactose produced highest amount of XynZ.CD activity OD_{600}^{-1} l^{-1} of the culture. This seemed to be due to achieving a higher cell mass which was induced efficiently under these conditions.

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