PROTEIN ENGINEERING OF XYLANOLYTIC ENZYMES FOR IMPROVED PROPERTIES

A THESIS SUBMITTED TO THE UNIVERSITY OF THE PUNJAB IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOLOGICAL SCIENCES

BY

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SUMMARY

*Clostridium thermocellum* is a spore-forming anaerobic thermophilic bacterium which secretes highly active cellulolytic enzymes. It produces a consortium of plant-cell wall hydrolases that form a cell-bound multi-enzyme complex called the cellulosome. The xylanase C (XynC) and xylanase Z (XynZ) are two major cellulosomal xylanases of *C. thermocellum*. A 1,857 bp *xynC* gene, encodes the xylanase of 619 amino acids with a molecular mass of 69,517. XynC contains a typical N-terminal signal peptide of 32 amino acid residues, followed by a 165-amino-acid sequence which is homologous to the thermostabilizing domain. Downstream of this domain is a family 10 catalytic domain of glycosyl hydrolase. The C terminus is separated from the catalytic domain by a short linker sequence contains a dockerin domain responsible for cellulosomal assembly. The XynZ consists of N-terminus feruloyl esterase domain, a Proline-rich linker, a family 6 carbohydrate binding module (CBM6), a dockerin and a glycoside hydrolase family 10 catalytic domain.

To study the truncated derivatives of the two xylanase components, XynC and XynZ of *C. thermocellum* were cloned and expressed with and without binding modules in *E. coli*. Two constructs of XynC, one with cellulose binding module and the catalytic domain (pXynC-BC) and the other with catalytic domain only (pXynC-C) were produced. Similarly for XynZ the constructs produced were pXynZ-BDC, which also contained dockerin domain, and pXynZ-C (only catalytic domain). Xylanase expression in *E. coli* cells when transformed with pXynC-BC and pXynZ-BDC were 30% and 25%, respectively. Transformation of *E. coli* cells with the constructs carrying only catalytic domains, the expression levels were ~45% in each case. Whereas, the specific activities for Xylanase C with and without carbohydrate binding module (CBM) were similar, in the case of XynZ the specific activity for the enzyme without CBM was
~5-fold greater than that of the enzyme with CBM. The overall activities produced, increased from 1925 U.l\(^{-1}.OD\(_{600}\)\(^{-1}\) in case of XynC-BC to 3050 U.l\(^{-1}.OD\(_{600}\)\(^{-1}\) for XynC-C. However, the overall increase in activities was ~9-fold when the enzyme was expressed as XynZ-C (32900 U.l\(^{-1}.OD\(_{600}\)\(^{-1}\)) as compared to that in the other case (3665 U.l\(^{-1}.OD\(_{600}\)\(^{-1}\)). Both the enzymes with and without CBM were found to be quite stable over a broad pH range (pH 4 - 9). XynZ-C was more thermostable as compared to XynZ-BDC as it retained 87% of xylanase activity as compared to 42% in the later case, when incubated at 70°C for 2 hrs. However, XynC-BC retained 70% activity on incubation at 70°C for 2 hrs but XynC-C lost total activity under the same conditions. \(K_m\) values for XynC-BC and XynC-C as determined on soluble xylan as substrate were 3.12 and 3.57 mg.ml\(^{-1}\), respectively, whereas these values for XynZ-BDC and XynZ-C were 33.30 and 15.38 mg.ml\(^{-1}\), respectively.

To study the influence of binding module position on activity, stability and expression of XynC the protein with the binding module at C-terminal (XynC-CB), and the one with the binding module 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 4,410 and 7,450 U \(\mu M\)\(^{-1}\) 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 module at the C-terminal of the catalytic domain enhances activity, substrate affinity as well as thermostability.

To study the influence of binding module position with respect to the catalytic domain on activity, stability and expression, of XynZ the enzyme variants with the binding module at C-terminal (XynZ-CB), N-terminal (XynZ-BC), both N- and C-terminals (XynZ-BCB), one with catalytic domain at both N- and C-terminal of the binding module (XynZ-CBC) and one with family 22 binding module at N-terminal (XynZ-B’C) were expressed in E. coli. Recombinant plasmids, pXynZ-CB, pXynZ-BC pXynZ-BCB pXynZ- CBC and pXynZ-B’C were constructed by sequential cloning of genes in pET28a(+)/pET22b(+). XynZ-CB, XynZ-BC, XynZ-BCB XynZ-CBC and XynZ-B’C were expressed at levels around 30, 30, 33 15 and 30% of the total E. coli cell proteins, respectively. The specific activities of XynZ-CB, XynZ-BC, XynZ-BCB XynZ-CBC and XynZ-B’C were 72, 68, 67 200 and 357 U mg$^{-1}$, on equimolar basis the activities were 4160, 4120, 5092 19,200 and 20,700 U µM$^{-1}$ of the enzyme and their overall yields in the culture were 3678, 3579, 3800 5400 and 19,300 U l$^{-1}$ OD$_{600}^{-1}$, respectively. Substrate binding studies showed that in case of XynZ-C 61% of the activity remained unbound to birchwood xylan, whereas in the cases of XynZ-BC, XynZ-CB, XynZ-BCB and XynZ-CBC the activities left unbound were 52, 51, 53 50 and 39%, respectively, under the assay conditions used. $K_m$ values for XynZ-CB, XynZ-BC, XynZ-BCB and XynZ-CBC against birchwood xylan were found to be 33, 32, 32 28 and 3.3 mg ml$^{-1}$, respectively.

The production of engineered and truncated derivatives of xylanase C and Z of C. thermocellum have shown that the expression level of these proteins is dependant on the size of
proteins (insipid of the stability the secondary structures over the sequence from the ribosomal binding site to the +10 codon). Larger the size of the protein smaller is the expression and vice versa. There has been no significant effect on activity against soluble substrates, whether, the enzyme posses the binding module or not. However, binding module of family 22 has shown to enhance the activity against insoluble substrates as well as thermostability. The fusion of family 22 binding module at the N-terminus of catalytic domain of XynZ (XynZ-B'C) showed enhanced activity as compared to the native form of XynZ (XynZ-BC). The molecular modeling study revealed that a new binding site which is comparatively open and more exposed to the solvent is generated by the fusion of family 22 binding module. This aggregates the catalytic domain on xylan, and rapidly releases the hydrolytic products thus enhancing the activity of XynZ. Furthermore, structure function relationship through X- ray crystallographic and NMR studies would help in elucidating this phenomenon.
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M. Imran Mahmood Khan
## Abbreviations

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<tr>
<td><em>C. thermocellum</em></td>
<td><em>clostridium thermocellum</em></td>
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<td>CBD</td>
<td>carbohydrate binding domain/module</td>
</tr>
<tr>
<td>CBM</td>
<td>carbohydrate binding module/domain</td>
</tr>
<tr>
<td>CBM6</td>
<td>carbohydrate binding module/domain family 6</td>
</tr>
<tr>
<td>CBM622</td>
<td>carbohydrate binding module/domain family 22</td>
</tr>
<tr>
<td>CD</td>
<td>catalytic domain</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
</tr>
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<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
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<td>EDTA</td>
<td>ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>GH</td>
<td>glycosyle hydrolase</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyle B-D thioglactoside</td>
</tr>
<tr>
<td>Kb</td>
<td>kilo base pair</td>
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<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>LB</td>
<td>Lauri Bertani</td>
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<tr>
<td>OD</td>
<td>optical density</td>
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<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RBS</td>
<td>ribosomal binding site</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TEME</td>
<td>N, N, N, N, tetraethylene methylene diamine</td>
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X gal 5-bromo-4-chloro-3-indolyl β-D-glactoside
XynC xylanase C
XynC-BC binding module family 6 and catalytic domain
XynC-BCB binding catalytic and binding domain
XynC-C catalytic domain only
XynC-CB catalytic domain and binding domain
XynC-CB binding catalytic and binding domain
XynZ xylanase Z
XynZ-BC binding module family 22 and catalytic domain
XynZ-BCB binding, catalytic and binding domain
XynZ-BDC with binding, dockerin and catalytic domains
XynZ-C catalytic domain only
XynZ-CB catalytic domain and binding domain
XynZ-CBC catalytic, binding and catalytic domain
1. Introduction and Review of Literature

Xylanolytic enzymes have attracted great attention in the development of eco-friendly technologies and their use has significantly enhanced the biorefinery for the production of liquid fuel from the lignocellulosic wastes. Thus the production of xylanolytic enzymes with the aim of their bioindustrial application and engineering to enhance their biochemical characteristics like substrate affinity, enhanced activity and thermostability is a subject of intense research

1.1 Xylan and its structure

The earth has plenty of renewable biomass and the second most copious is hemicellulose, which is almost 25-35% of lignocellulosic material (Saha, 2000). The term hemicellulose was first used by Schulze in 1891. Hemicelluloses are the diverse group of polymers comprising of arabinan, galactan, mannan and xylan; xylan being the dominating component. Xylan is a multifaceted polysaccharide that is composed of xylanopyranoside residues linked in β-1,4 linkages (Shallom et al., 2003). The plant cell wall contains cellulose, xylan and lignin that interact noncovalently and covalently, giving strength and integrity to the plant (Beg et al., 2001). Different plant species have different structural organization of xylan. However, generally it has homopolymeric backbone chain that is substituted to various degrees with glucopyranosyl 4-o-methyl-D-methylglucoronopyranosyl, 4-L-arabinosyl, acetyl, feruloyl and p-cumeroyl side chain (Kulkarni, et al., 1999, Li et al., 2000). The hardwood mainly is composed O-acetyl-4-O-methylglucoronoxylan while as arabino-4-O- methylglucoronoxylan in softwoods (Kulkarni et al., 1999). The grasses and annual plants are typically arabinoxylan (Kulkarni et al., 1999), while unsubstituted linear xylan is found in tobacco, esparto
grass, (Chanda et al., 1950; Eda et al., 1976) and certain marine algae (Barry et al., 1940; Nunn et al., 1973). There is also different extent of polymerization in softwood and hardwood generally comprising of 70-130 and 150-200 β-xylanopyranose residues, respectively (Kulkarni et al., 1999).

Fig 1.1 Structure of xylan and sites of hydrolysis of xylanolytic enzymes

### 1.2 Xylanolytic enzymes

Xylan degrading enzymes, xylanases, are glycoside hydrolases catalyzing the hydrolysis of 1,4-β-D-xylosidic linkage. These are involved in the production of xylose from xylan which is the primary carbon source of cell metabolism and are produced by many organisms including bacteria, algae, fungi, protozoa, gastropods and anthropodes (Prade, 1995). They were termed as pentosanase when first reported in 1955 by Whistler
then in 1961 they were recognized by International Union of Biochemistry and Molecular Biology (IUBMB) and were assigned enzyme code EC 3.2.1.8. (Puls et al., 1987; Subramaniyan et al., 2002). Various enzymes of different specificities like hydrolases and esterases are required for the complete hydrolysis of xylan. The Endo 1, 4-β-D-xylanase (EC 3.2.1.8) cleaves the backbone of xylan randomly, releasing xylose, xylo-oligosaccharides and xylobiose. The β-D-xylosidase (EC 3.2.1.37) acts on the xylo-oligosaccharides and xylobiose from non reducing ends, releasing xylose monomers. The removal of the side chain is mediated by a group of enzymes viz. α-L-arabinofuranosidase, (EC 3.2.1.55) α-D-glucoronidase (EC 3.2.1.139), ferulic acid esterase, (EC 3.1.1.73) acetylxyylan esterase, (EC 3.1.1.72) and p-cumaric acid esterase (EC 3.1.1…)(Sunna et al., 1997; Subramaniyan et al., 2002).

1.3 Classification of xylanase

Xylanases were divided in two groups according to their chemical and physical properties; one contains enzymes of low molecular mass (up to 30 kDa) and basic isoelectric point, while other group has enzymes of high molecular mass (more than 30 kDa) and acidic isoelectric point (Wong et al., 1988). On the basis of primary sequence comparison, structural fold, substrate specificity and enzyme reaction mechanism the glycoside hydrolases are divided into 125 GH families. The enzymes grouped in one family have similar sequence of catalytic domain, reaction mechanism and specificity for their substrates. The families are grouped in clans which have similar secondary structural fold of catalytic domain for the catalysis. There are 14 different clans of GH families from GH-A to GH-N and each clan has more than one family. According to this system there are six families of xylanases which have catalytic domain with truly endo-
xylanase activity, these families are 5, 7, 8, 10, 11, and 43, most of the xylanases belong to family 10 and 11. These six families belong to clan GH-A, GH-B GH-M GH-A, GH-C and GH-F, respectively (Collins et al., 2005).

1.3.1 Multidomain xylanases

Most of themophilic bacteria (Clostridia and Thermotoga specie) produce multidomain xylanases. These multidomain xylanases have a major catalytic domain and other non catalytic accessory domain like substrate binding domain, dockerin domain and in some cases the second catalytic domain of different specificity.

The Thermotoga neapolitina xylanase (XynA) has been reported to have catalytic domain of family 10 and non catalytic domains present at N- and C- terminus of catalytic domain. These non catalytic domains are not only vital for the activity of XynA but also for the binding and thermostability. This xylanase was much stable when incubated at 90°C and retained half of its activity after incubation at 100 °C for 2 h (Zverlov et al., 1996). Rhodothermus marinus contains endoxylanase (xyn1) which has five different domains in addition to catalytic domain of family 10. The tandem repeats of cellulose binding domain belonging to family IV is located at N- terminus of the catalytic domain (Karlsson et al., 1997). The Bacillus sp. BP-23 contains multidomain xylanase (xynC) that has three domains one N-terminal thermostabilizing domain, a family 10 catalytic domain and a C- terminal family 9 carbohydrate binding domain. Deletion of N- terminal domain resulted in lower temperature optima and thermal stability (Blanco et al., 1999).

A xylanase gene (xynC) of anaerobic ruminal fungus Neocallimastix patriciarum is a multidomain enzyme, contains N-terminal family 11 catalytic domain, a C-terminal cellulose binding domain (CBD) and a dockerin domain situated between them. The
truncated derivatives revealed that presence of cellulose binding domain is necessary for its optimal activity (Liu et al., 1999). The xylanase (XynX) of Clostridium stercorarium is a multidomain enzyme comprising of a thermostabilizing domain, a family 10 catalytic domain, two family 9 carbohydrate binding domains and three S-layer homology domains (Kim et al., 2000). Caldicellulosiruptor cellulovorans xylanase (XynA) has one N-terminal xylan binding domain involved in thermostability, a catalytic domain of family 10 followed by two carbohydrate binding domain of family 3, all these domains are linked to each other through linker peptides (Sunna et al., 2000). The xylanase (Xyn10B) of Clostridium stercorarium is a multidomain enzyme comprising of two family 22 carbohydrate binding domains a family 10 catalytic domain, a family 9 carbohydrate binding domain and two S-layer homology domains (Ali et al., 2001). C. thermocellum have shown to contain eight multidomain xylanase genes: xynZ (Grepinet et al., 1988), xyn10B (Charnock et al., 2000, Fontes et al., 1995), xynC (Hayashi et al., 1997), xynX (Jung et al., 1998, Kim et al., 2000), xynA and xynB (Hayashi et al., 1999), and xynU and xynV (Fernandes et al., 1999). The N. flexuosa Xyn11A and Xyn10A xylanase consist of a catalytic module, a linker and a C-terminal true xylan binding domain, its truncated version devoid of the carbohydrate binding domain expressed in Trichoderma reesei, (Paloheimo et al., 2007). Two chimeric xylanases have been constructed by the sequential fusion of catalytic domains of xylanase and xylosidase and the other one of xylanase and arabinofuranosidase in pET29 expression vector. These chimeric xylanases showed lower pH optima than the native xylanase (Fan et al., 2009). There are several xylanases which are multidomain and contain carbohydrate binding domains belonging to different families and these domains have role in carbohydrate
binding or thermostabilizing. The \textit{Thermotoga maritima} xylanase A has family 9 carbohydrate binding domain which binds with amorphous and crystalline cellulose (Notenboom \textit{et al}., 2001). The Xyn10B of \textit{Polyplastron multivesiculatum} contains family 22 carbohydrate binding domain which not only binds xylan but also cellulose (Devillard \textit{et al}., 2003). The \textit{Paenibacillus} sp. Strain W-61 has xylanase 5 which contains one family 9 and two family 22 binding domain, due to the presence of family 9 binding domain it binds to avicel (Ito \textit{et al}., 2003). In addition to binding to carbohydrates these non catalytic domain also have considerable effect on temperature optima and thermal stability (Araki \textit{et al}., 2006).

\textbf{1.4 Applications of thermostable xylanases}

Thermostable xylanases have gained enormous attention due to their biotechnological prospective in different industrial processes, like food, feed, biorefining and paper and pulp industries (Bhat, 2000). They also have other recognized applications which include the brewing to increase filterability of wort and decreasing haze (Tikhomirov 2003), extraction of coffee and the preparation of soluble coffee (Wong \textit{et al}., 1988), in detergents (Kamal \textit{et al}., 2003), the protoplastation of plant cells (Kulkarni \textit{et al}., 1999), production of alkyl glycosides that are used as surfactants (Matsumura \textit{et al}., 1999 and Imanaka 1992) etc.

The noteworthy application of thermostable xylanases is the prebleaching of kraft pulp as they have proved as an alternative to chlorine containing toxic chemicals. (Beg \textit{et al}., 2001). Bleaching is process of removal of lignin from the chemical pulp to produce completely white finished pulp which improves the quality of paper. Bleaching of kraft pulp these days is based on large amount of chlorine containing chemicals and sodium
hydrosulfite. Chlorinated organic substances are the by products of these chemicals which are mutagenic, toxic and are source of harmful disturbance to biological system. These chemicals also cause many effluent based problems and are a great threat to the environment. Thus an alternative and cost effective biobleaching is achieved which implies the use of microorganism and enzyme for bleaching the kraft pulp (Collin et al., 2005). It is based on microorganism which can directly depolymerize the lignin or the use of enzyme which can act on hemicellulose and subsequently favouring delignification. The major enzyme required for the removal of the lignin from the kraft pulp is endo-β-xylanase however the addition of ligninase, mannase, lipase, and α-glactosidase have also shown promising outcome. (Gubtiz et al., 1997; Clarke et al., 2000). Therefore the use of endo-β-xylanase to degrade the hemicellulose of kraft pulp not only facilitates the removal of lignin but also reduces the use of chlorinated chemicals (Garg et al., 1998; Vicuna et al., 1997).

1.4.1 Need of thermostable xylanases in paper and pulp industries

In paper and pulp industry, wood that is used for production of pulp is treated at high temperature and basic pH (Jacques et al., 2000) which requires the proteins (xylanase) disrupting the plant cell wall at basic pH and high temperature that ultimately facilitates removal of lignin during prebleaching. The xylanase required for such purpose must not have cellulosytic activity, must be thermostable and small molecular weight so they can penetrated the pulp (Niehaus et al., 1999).

1.4.2 Biorefinery and use of thermostable xylanases in Biorefining

Biorefinary involves the conversion of agricultural crops or wood for the production of chemicals, commodities, and fuels by biomolecules (Fernando et al., 2006
Kamm et al., 2004). The Production of biofuel from hydrolysis of agricultural and lignocellulosic wastes is the most emerging and progressing area of biorefining. It involves the degradation of cellulosic and hemicellulosic wastes at high temperature, thus raising the desire of thermostable enzymes especially xylanases and cellulases (Bayer et al., 1998). Bioethanol is the most common renewable fuel available these days. The use of cellulases and xylanases to produce fermentable sugars raised during world oil crises in the 70s. At present its need has become more outspoken not only to become less dependant on fossil reservoirs but also to reduce green house gas emission (Turner et al., 2007). The US “Biofuel initiative” has decided to make bioethanol much cheaper upto 2012 and shift of their one third fuel requirement to biofuel by 2030, while EU has objective of having 12% renewable energy by 2010 (energy for future). The corn grain and sugarcane are the common source of bioethanol but yet cannot compete with fossil fuels. In spite of agricultural and forest biomass, urban wastes are additional sources containing 40% of cellulose in municipal waste (Tomme et al., 1995). The complex and crystalline structure of cellulose make it difficult to hydrolyze however the pretreatment at elevated temperature and use of thermostable enzymes can overcome this problem to provide fermentable sugars (Zaldivar et al., 2001).

1.5 Cellulosome and its structural organization

There are numerous anaerobic thermophilic bacteria which produce, multienzyme complex to degrade cellulosic and hemicellulosic substrates like Clostridium thermocellum, Ruminococcus albus (Lamed et al., 1983), Clostridium celllobioparum (Lamed et al., 1987), Clostridium cellulovorans (Shoseyov et al., 1992), Clostridium papyrosolvens (Pohlschröder et al., 1995), Clostridium josui (Kakiuchi et al., 1998),
Acetivibrio cellulolyticu (Ding et al., 1999), Clostridium cellulolyticum (Pages et al., 1999), Bacteroides cellulosolvens (Ding et al., 2000), Clostridium acetobutylicum (Nolling et al., 2001), Ruminococcus flavefaciens (Kirby et al., 1997: Ding et al., 2001).

The cellulosomal complex is usually found on bacterial cell wall where it binds with the insoluble substrates like cellulose and hemicellulose. The hydrolysis of insoluble substrate produces soluble products such as glucose, xylose, mannose etc. which are taken up by the bacteria and are used as primary source of metabolism.

The cellulosome is composed of three subunits an anchoring subunit, a scaffolding subunit and enzymatic subunit (Fig 1.2). The anchoring subunit contains S-layer homology domains which bind with cell wall of bacteria and a type II cohesin domain which interacts with the type II dockerin domain of scaffolding subunit. The scaffolding subunit is a collection of many type I cohesin domains which interacts with type I dockerin domain of catalytic subunit, type II dockerin domain and carbohydrate binding domain. The enzymatic subunit is an aggregate of cellulases, hemicellulases, carbohydrate binding domain and dockerin domain. The interaction of three subunits (anchoring subunit, a scaffolding subunit and enzymatic subunit) through their cohesin and dockerin domains makes cellulosomal assembly (Bayer et al., 1998).

1.6 Carbohydrate binding module and their classification

Carbohydrate binding domain/module is a domain found within the hydrolytic enzymes having activity to bind carbohydrates. Their biological role is to aggregate the enzyme on its substrate by mediating strong association between them, ultimately enhancing the catalysis of substrate. In addition to substrate binding this domain also has thermostabilizing effect and metal ions binding sites. These carbohydrate
binding domain are divided in 63 CBM families on the basis of primary sequence comparison and these families are divided in 7 fold families on the basis of the secondary structural fold involved in binding to carbohydrate.

There are different CBM families linked to xylanases like CBM2 found with Xylanase 10A and Xylanase 11A of *Cellulomonas fimi*, CBM4 with Xylanase 10A of *Rhodothermus marinus*, CBM6 with Xylanase 11A, Xylanase 10Z of *Clostridium thermocellum* and *Clostridium stercorarium*, CBM9 with Xylanase 10A of *Thermotoga maritima*, CBM10 with Xylanase 10A of *Cellvibrio japonicus*, CBM13 with Xylanase 10A of *Streptomyces olivaceoviridis*, CBM15 with Xylanase 10C of *Cellvibrio japonicus*,

---

*Fig.1.2 Cellulosomal organization of Clostridium thermocellum*
CBM22 with Xylanase 10B and Xylanase 10C of Clostridium thermocellum and CBM36 with Xylanase 43A of Paenibacillus polymyxa (Boraston et al., 2004).

1.7 Protein engineering of xylanases

Thermostable xylanases have shown great advantages as the catalysis at high temperature facilitates their better penetration and degradation of cellulosic and hemicellulosic biomass. The essential reasons of using thermostable xylanases in bioprocess are their inherent thermostability, which favors their prolonged storage at room temperature, minimum risks of contamination and less activity loss at higher temperature. The global world market for the industrial enzymes was estimated to be $ 2 billion in 2004 and predicted growth rated is 4-5%. There are two major sections of industrial enzymes based on their applications I.Techical enzymes II. Food and Feed enzymes. The Technical enzymes constitute 52% of the world enzyme market used in detergents, biorefineries and paper and pulp industries. The major technical enzymes are cellulases, xylanases, amylases and proteases (Turner et al., 2007). The exercise of thermostable xylanases for the world greatest growing bioindustries is prominent as they provide global environmental benefits. There is a wide range of protein engineering strategies from site directed mutagenesis to directed evolution to enhance the properties of xylanolytic enzymes.

1.7.1 Site-directed mutagenesis of xylanases

Site-directed mutagenesis was used to elucidate the reaction mechanism of Bacillus pumilus xylanase and three amino acids Asp-21, Glu-93 and Glu-182, were mutated. The Asp residue was mutated to either Ser or Glu, and the Glu residues to Ser or Asp. This study revealed that Glu -93 and Glu-182 are the amino acid residues present at
the active site of xylanase involved in catalysis of xylan (Ko et al., 1992). The thermostability of *Bacillus pumilus* xylanase was enhanced by random mutagenesis. The mutant in which asparagines and glycine were replaced with serine showed higher stability than wild type and kinetic analysis showed that there was increase in activation enthalpy (Arasea et al., 1993). Single mutations were made by site directed mutagenesis on xylanase (XynA) of *Streptomyces lividans* and then positive single mutations were combined to produce double mutants. The mutant proteins which were F155Y, R156E, R156K, and N173D showed 28, 10, 50, and 25% more activity with respect to wild-type protein without showing any effect on pH optima (Moreaua et al., 1994). The role of conserved histidine residues in the active site of family 10 xylanases was determined by site directed mutagenesis. Six mutants were produced by replacing histidine with alanine, glutamate, lysine, glutamine, phenylalanine and tryptophane. There was no significant effect on activity of the enzyme was observed however, there was lower pKa for the acid base catalysis and lower enzyme stability (Roberg et al., 1998). In order to improve thermophilicity and thermostability of mesophilic *streptomyces* sp. S38 Xyl1 the structural features of *Thermomonospora fusca* TfxA family 11 xylanases were introduced in Xyl1. Two tyrosine residues were introduced to increase the aromatic interaction. There was 10 °C increases in optimum temperature and half life of the enzyme at 57 °C (Georis et al., 2000). *Trichoderma reesei* endo-beta-1,4-xylanase (Xyn II) was engineered by introducing three mutations by site directed mutagenesis and all the mutants showed 5 degree increase in residual activity at pH 9 as well the brightness of the pulp (Zitting et al., 2006).The endoglucanase (Cel5A) of *Thermotoga maritima* was engineered by site directed mutagenesis and by fusion of CBM6 of *Clostridium*
stercorarium xylanase. The mutant produced showed higher pH optima and 14-18 fold higher activity against avicel (Mahadevan et al., 2008). The role of Asp60, Tyr35 and Glu141 of xylanase (Xyl1) of Scytalidium acidophilum was studied by site directed mutagenesis. The mutants D60N, Y35W and E141A were expressed in Pichia pastoris there was significant increase in the pH optimum of the mutants with enhanced specific activity (Albala et al., 2009). Two tyrosine residues were mutated with cystein at the N-terminus of xylanase II (XYN II) of Trichoderma reesei by site directed mutagenesis. The mutated xylanase showed higher temperature optima and improved thermalstability (Han et al., 2010). Substrate affinity was altered by the site directed mutagenesis of tryptophane residue of Cohnella laeviribosi HY-21. There was two fold increased activity in the presence of non ionic detergent and eight fold increased activity against xylooligosacchrid (Kim et al., 2010). The alanine at 33 position of xylanase B of Aspergillus niger strain 400264 was mutated with serine, the resultant protein showed 19% decreased activity after incubation at 85°C for 30 min. showing that alanine at this position had significant role for adoption at higher temperature (Xie et al., 2011)

1.7.2 Directed evolution and domain fusions

Directed evolution strategy was adopted to enhance the alkalophilicity of a xylanase of Neocallimastix patriciarum using error-prone PCR. Eight amino acid mutations were observed in six mutants, all the mutants showed higher activities than native enzyme at pH 8.5 and above (Chen et al., 2001). A thermostable mutant xylanase of Bacillus subtilis was identified by combining saturation mutagenesis, random DNA shuffling and point mutations (Miyazaki et al., 2006). The mutant xylanase (Xylst) showed substitutions of three amino acid residues i-e Q7H, N8F, and S179C. The Xylst
was highly thermostable as it retained its activity after incubation at 60°C for 2 hours and there was shift in optimal temperature from 55 to 65 °C. In another study error-prone PCR was used to enhance alkalophilicity and thermostability of xylanase (XynA) of *Thermomyces lanuginosus*. A library containing 960 clones was selected by Congo red method and crude filtrate of positive clones was screened at 80°C for thermostability and at pH 10 for alkaline tolerance. The most stable mutant retained 75% activity after incubation at 80 °C for 90 min while the best alkali-stable mutant retained 93% activity when assayed at pH 10 (Stephens *et al.*, 2009). Directed evolution strategy by using error-prone PCR was also used by Mchunu *et al.* (2009) for the xylanase of *Thermomyces lanuginosus*. The mutant obtained after screening, retaining 84% activity on incubation at 60 °C at pH 10 for 90 min, while the wild type enzyme retained 22% activity after 60 min. The mutant was cloned in pET22b (+) for expression in *Escherichia coli* BL21 and *Pichia pastoris*. The expression *Pichia pastoris* was 545 fold higher than expression in *E. coli*. Reitinger *et al.* (2010) employed a different strategy for the xylanase (Bcx) of *Bacillus circulans*. The xylanase gene was circularly permuted with PCR to introduce new terminal in loop regions while linking its native terminal through one or two glycine residues. Then a library of circular permutants was generated by random DNase cleavage and this library was screened for xylanase activity by Congo Red-staining. The study of 35 active circular permutants showed that many of the new termini were introduced in external loops while few loops were also observed within β-strands. In addition numerous permutations put catalytic residues at or near the new termini resulting in 4-fold increase in activity.
A xylanase gene (xynX) of *Clostridium thermocellum* has one thermostabilizing domain which is present between the catalytic domain and signal sequence. Shin *et al.* (2002) shifted the thermostabilizing domain to the C-terminus from the N terminus and the modified product showed a higher optimum temperature and was more thermostable than the one without the thermostabilizing domain. Similarly, enhanced thermostability and hydrolytic activity was achieved by domain shuffling across *Cellulomonas fimi* and *Thermomonospora alba* XylA (Wang and Xia, 2007). The fusion of a family 6 cellulose binding domain from *Clostridium stercorarium* XylA at the C-terminal of a *Bacillus halodurans* C-125 family 10 xylanase resulted in improved activity towards insoluble xylan (Mangala *et al*., 2003). The molecular engineering of *B. halodurans* xylanase with family 4 and 2b carbohydrate binding domain at C-terminus displayed higher level of catalysis as compared to parental enzyme (Farooqahmed *et al*., 2003). A chimeric xylanase (XynB-CBM2b) created by fusion of carbohydrate-binding module of family 2b from *Streptomyces thermoviolaceus* with xylanase (XynB) of *Thermotoga maritima* showed slightly higher activity against soluble xylan and there was no significant effect on activity against insoluble xylan. However, this xylanase was stable even at 90°C (Kittur *et al*., 2003). A bifunctional cellulase xylanase of *Thermotoga maritima* was prepared by sequentially fusing the genes. When xylanase gene was fused downstream of cel5C the fusion product showed both cellulase and xylanase activity but fusion of xylanase gene upstream of cel5C resulted in loss of activity (Hong *et al*., 2006). The xylanase (xynIIA) has carbohydrate binding domain and a catalytic domain. The truncated derivative containing only catalytic domain was expressed in *T. reesei*. The catalytic domain showed higher level of expression and thermostability than full length
gene (Marja et al., 2007). The carbohydrate binding domain of family 22 from *Thermotoga neapolitana* was fused with xylanase of *Bacillus halodurans* S7 and expressed in *E. coli*. The fusion of binding domain enhanced xylan binding, enzyme activity without effecting the temperature and pH optima, however, thermostability of the protein was decreased (Mamo et al., 2007). Two xylanases (xylanase A and B) of *Thermotoga maritima* were engineered by family shuffling through rational manner and twenty one different chimeric constructs were made; only sixteen of them were catalytically active. Most stable mutant was observed to have the N and C-terminal segments coming from hyperthermophilic xylanase B of *Thermotoga maritima* which showed that the interaction of N and C-terminals of protein contributed to the thermostability (Kamondi et al., 2008). Carbohydrate binding domain and polyhistidine tag was fused at the C-terminus of *Thermopolyspora flexuosa* xylanase (XynA). The fusion of CBM did not show any significant effect on the property, while polyhistidine-tag enhanced the thermostability of the protein. However, glycosylation of asparagine at position 36 lowered the stability (Anbarasan et al., 2009). Carbohydrate binding domain of family 9 from *Thermotoga maritima* was fused with the catalytic domain of family 11 xylanase of *Aspergillus niger*. The xylanase thus produced showed 4 fold and 40% increase in activity on soluble and insoluble birchwood xylan, respectively (Liu et al., 2011).

1.8 Major Xylanolytic enzymes of *Clostridium thermocellum*

*Clostridium thermocellum* is a spore-forming anaerobic thermophilic bacterium which secretes highly active cellulolytic and hemi cellulolytic enzymes. It produces a consortium of plant-cell wall hydrolases that form a cell-bound multi-enzyme complex
called the cellulosome. A 1,857 bp \textit{xynC} gene, encodes the xylanase of 619 amino acids with a molecular mass of 69,517. \textit{XynC} contains a typical N-terminal signal peptide of 32 amino acid residues, followed by a 165-amino-acid sequence which is homologous to the thermostabilizing domain. Downstream of this domain is a family 10 catalytic domain of glycosyl hydrolase. The C terminus is separated from the catalytic domain by a short linker sequence contains a dockerin domain responsible for cellulosomal assembly (Hayashi \textit{et al.}, 1997).

\textit{XynZ} is a major endo-xylanase associated with the cellulosome of \textit{C. thermocellum}. It consists of N-terminus feruloyl esterase domain (FAEXynZ), a Proline-rich linker, a family 6 CBM, a dockerin and a glycoside hydrolase family 10 catalytic domains (Grepinet \textit{et al.}, 1988; Blum \textit{et al.}, 2000).

**Objectives of the study**

The xylanolytic enzyme have attracted a great deal of attention due to their biotechnological application in various industrial processes, thus the current research study is based on following objectives.

- Production of \textit{XynZ} and \textit{XynC} of \textit{C. thermocellum} without carbohydrate binding modules
- Effect of position of binding modules on expression, activity and characteristics of \textit{XynZ} and \textit{XynC}
- Characterization of recombinant xylanolytic enzymes

The current research study was divided in three major parts; in one part of the study the truncated derivatives of xylanase C and xylanase Z, while in the second and third part the engineered derivatives were produced and expressed in \textit{E. coli}.

In the first part of the present study, the truncated derivatives of xylanase C, \textit{XynC-BC} (with binding and catalytic domains) and \textit{XynC-C} (with catalytic
domain only) while truncated derivatives of xylanase Z termed as named XynZ-BDC (with binding, dockerin and catalytic domains) and XynZ-C (with catalytic domain) from *C. thermocellum* with and without CBMs were expressed in *E. coli*.

In the second part of this study two engineered derivatives of XynC were produced. In one derivative the binding domain was transpositioned from N-terminal of the XynC catalytic domain to its C-terminal producing (XynC-CB), while in second an additional binding domain was fused to the C-terminal of XynC-BC to produce XynC-BCB (Fig.1.3)

![Diagram of XynC derivatives](image)

**Fig.1.3 Structural organization of chimras of XynC of Clostridium thermocellum produced and expressed in E. coli**

In the third part the engineered derivatives of XynZ were produced. The protein with the binding domain at C-terminal (XynZ-CB), one with the binding domain at N-terminal (XynZ-BC), one with binding domain at both N- and C-terminal (XynZ-BCB), one with catalytic domain at both N- and C-terminal (XynZ-CBC) and one with the binding module of family22 at N-terminal (XynZ- B'C) were produced and expressed in *E. coli* (Fig 1.4).
All the derivatives were produced by using genetic engineering techniques and expressed in *E. coli*. Their activity yield, temperature and pH stabilities, binding assay and kinetic parameters like $k_m$ and $V_{max}$ were studied.

![Structural organization of chimras of XynZ of *Clostridium thermocellum* produced and expressed in *E. coli*](image)

Fig. 1.4 Structural organization of chimras of XynZ of *Clostridium thermocellum* produced and expressed in *E. coli*
2.1 Bacterial strains, plasmids and Growth Media

Chromosomal DNA of *C. thermocellum* (ATCC 27405D), kindly provided by Prof. D. B. Wilson, Cornell University, Ithaca, NY, USA, was used as a source of the xylanase genes (xynC: GenBank Ac. No. D84188 and xynZ: GenBank Ac. 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 (RIPL) and vectors, pET22b(+) and pET28a(+) 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 obtained from QIAgen Inc. (USA). Strains were grown in LB or M9NG Media (Sadaf et al., 2007).

Table 2.1: Composition of supplements in bacterial culture

<table>
<thead>
<tr>
<th>S.#</th>
<th>Supplement</th>
<th>Stock</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IPTG</td>
<td>100 mM</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>2</td>
<td>X-gal</td>
<td>50 mg/mL</td>
<td>80 µg/mL</td>
</tr>
<tr>
<td>3</td>
<td>Ampicillin</td>
<td>25 mg/mL</td>
<td>50 µg/mL</td>
</tr>
<tr>
<td>4</td>
<td>Kanamycin</td>
<td>25 mg/mL</td>
<td>60 µg/mL</td>
</tr>
</tbody>
</table>

2.2 PCR amplification and production of constructs

2.2.1 PCR amplification of xylanase genes

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). Primers were designed on the basis of domain organization given at NCBI (XynC: GenBank Ac. No. BAA21516 and XynZ: GenBank Ac. No.
AAA23286) and further checked with Pfam web-server (Finn et al., 2008). 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, shown in Annexure IV (Zuker, 2003).

For xynC derivatives PCR was done with initial denaturation at 95°C for 4 min, then 30 cycles of denaturation at 95°C for 45 sec, annealing at 58°C for 30 sec and extension at 72°C for 1 min, 45 sec. The reactions for xynZ derivatives were same except for annealing, which was carried at 65°C for 30 sec. Final extension was done for 25 min in both the cases. PCR amplified products were run on 1% agarose gel, purified by the QIAquick gel extraction kit (QIAGen Inc., USA) and cloned into pTZ57R/T by InsT/Aclone PCR product cloning kit (Fermentas, USA). Competent cells of E. coli DH5α were prepared and transformed with the recombinant vectors according to the standard protocol (Sambrook and Russell, 2001).

Table 2.2: PCR Conditions for the amplification of xylanase genes

<table>
<thead>
<tr>
<th>S.#</th>
<th>Component</th>
<th>Stock Conc.</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Forward Primer</td>
<td>10µM</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Reverse Primer</td>
<td>10µM</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>DNA (template)</td>
<td>varies</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>dNTPs</td>
<td>10 mM</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>MgCL₂</td>
<td>25 mM</td>
<td>variable</td>
</tr>
<tr>
<td>6</td>
<td>Taq DNA polymerase</td>
<td>1 U/µL</td>
<td>0.4</td>
</tr>
<tr>
<td>7</td>
<td>Deionized water</td>
<td>-</td>
<td>35.6</td>
</tr>
</tbody>
</table>
2.2.2 Production of truncated derivatives of xylanases C and xylanase Z of *C. thermocellum*

The truncated derivatives of xylanase C termed as XynC-BC (with binding and catalytic domains) and XynC-C (with catalytic domain only) while truncated derivatives of xylanase Z termed as XynZ-BDC (with binding, dockerin and catalytic domains) and XynZ-C (with catalytic domain). The genes were amplified with the primers as shown in table 2.4. The amplified product were purified and cloned into pTZ57R/T, producing pTZ57R/xynC-BC, pTZ57R/xynC-C, pTZ57R/xynZ-BDC and pTZ57R/xynZ-C, respectively. The XynC-BC, XynC-C, XynZ-BDC and XynZ-C obtained by restriction of pTZ57R with *NdeI* and *EcoRI* for xylanase C while with *NcoI* and *BamHI* for xylanase Z were purified and then sub-cloned into expression vector pET22b(+) and pET28a(+) at the same restriction sites, producing pXynC-BC, pXynC-C, pXynZ-BDC, pXynZ-C, respectively.

Recombinant plasmids were purified and used to transform competent cells of *E. coli* BL21 CodonPlus (RIPL).
Table 2.4: Primers used for amplification of xynC and xynZ of *C. thermocellum*

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer</th>
<th>Sequence</th>
<th>Restriction site&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>xynC-C-F</td>
<td>5′‑CACATATGGCTTTGAAAGATGTTTTGC‑3’</td>
<td>NdeI</td>
</tr>
<tr>
<td>2</td>
<td>xynC-BC-F</td>
<td>5′‑CACATATGGCAGCTGATTACGATGA‑3’</td>
<td>NdeI</td>
</tr>
<tr>
<td>3</td>
<td>xynC-BC-R</td>
<td>5′‑GGAAAATCTCAGTTAACTATAGCATAAAATGC‑3’</td>
<td>EcoRI</td>
</tr>
<tr>
<td>4</td>
<td>xynZ-C-F</td>
<td>5′‑GCAGCCATGGATCGTCTGTACTCGACACA‑3’</td>
<td>NcoI</td>
</tr>
<tr>
<td>5</td>
<td>xynZ-BDC-F</td>
<td>5′‑CTCCATGGCAAAGCCGACTACACAC‑3’</td>
<td>NcoI</td>
</tr>
<tr>
<td>6</td>
<td>xynZ-BDC-R</td>
<td>5′‑GGGGATCCTATCAATAGCCGATGCTTCC‑3’</td>
<td>BamHI</td>
</tr>
</tbody>
</table>

<sup>a</sup> Restriction sites are underlined in the sequences.

2.2.3 Production of engineered derivatives of xylanases C of *C. thermocellum*

The engineered derivatives of xylanase C termed as XynC-CB (Catalytic domain at N-terminal and binding module at C-terminal) and XynC-BCB (binding modules on both N and C-terminal of catalytic domain). The constructs xynC-CB and xynC-BCB were produced following the scheme shown in Fig. 2.1. The fragment encoding the binding module (xynC-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 (Table 2.5).
Table 2.5: Nucleotide sequence of PCR primers used for amplification of different fragments used for the production of engineered derivatives of xylanases C of *C. thermocellum*

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer</th>
<th>Sequence</th>
<th>Restriction site</th>
<th>ΔG (kcal mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>xynC-B-F1</td>
<td>5' -CTGGATCCGCAGCTCTGATTTACGATG-3'</td>
<td>BamHI</td>
<td>-1.5</td>
</tr>
<tr>
<td>2</td>
<td>xynC-B-R1</td>
<td>5' -GAGAATTCATGCAGGTGGCTGTGAATC-3'</td>
<td>EcoRI</td>
<td>−</td>
</tr>
<tr>
<td>3</td>
<td>xynC-C-F2</td>
<td>5' -CACATATGAGCTTTGAAGACGTCCTTTGC-3'</td>
<td>NdeI</td>
<td>-1.2</td>
</tr>
<tr>
<td>4</td>
<td>xynC-BC-F3</td>
<td>5' -CACATATGGCAGCTCTGATTACGATGA-3'</td>
<td>NdeI</td>
<td>-1.5</td>
</tr>
<tr>
<td>5</td>
<td>xynC-C-R2</td>
<td>5' -CTGGATCCGCAGCTTTGTCG-3'</td>
<td>BamHI</td>
<td>−</td>
</tr>
</tbody>
</table>

*Restriction sites are underlined in the sequences.

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 *BamHI* and *EcoRI* 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 *NdeI* and *BamHI* 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.
Fig 2.1: Schematic diagram for producing constructs expressing XynC-CB and Xyn-BCB of *C. thermocellum* in *E. coli*.
2.2.4 Production of engineered derivatives of xylanases Z of *C. thermocellum*

The engineered derivatives of xylanase Z termed as XynZ-CB (catalytic domain at N-terminal and binding module at C-terminal), XynZ-BC (binding module at N-terminal and catalytic domain at C-terminal), XynZ-BCB (binding modules at both N- and C-terminal of catalytic domain), XynZ-CBC (Catalytic domains at both N- and C-terminal of binding module), XynZ-BCB (binding modules on both N and C-terminal of catalytic domain) and XynZ-BC (binding module of family 22 at N-terminal and catalytic domain at C-terminal). The constructs XynZ-BC, XynZ-CB, XynZ-BCB and XynZ-CBC were produced following the scheme shown in Fig. 2.2 and primers shown in table 2.6.

For the construction of XynZ-BC the fragment encoding the binding module (XynZ-B) was amplified using B-Fn and B-Rn, respectively. The amplified product was purified and cloned into pTZ57R/T, producing pTZ57R/Bn, the XynZ-B obtained by restriction of pTZ57R/Bn with *NcoI* and *BamHI* was purified and then sub-cloned into expression vector pET28a(+) at the same restriction sites, producing pCBMn. While the catalytic domain (XynZ-C) was amplified using C-Fc and C-Rc′ as forward and reverse primers, respectively. The amplified product was purified and cloned into pTZ57R/T, producing pTZ57R/xynZCc. The XynZ-Cc obtained by restriction of pTZ57R/xynZCc with *BamHI* and *EcoRI* was purified and then sub-cloned to the downstream of pCBMn at the same restriction sites, producing pXynZ-BC.

For the construction of XynZ-CB the fragment encoding the binding module (XynZ-B) was amplified using B-Fc and B-Rc, as forward and reverse primers, respectively. The amplified product was purified and cloned into pTZ57R/T, producing
pTZ57R/Bc, the XynZ-B obtained by restriction of pTZ57R/Bc with BamHI and SacI was purified and then sub-cloned into expression vector pET28a(+) at the same restriction sites, producing pCBMc. While the catalytic domain (XynZ-C) was amplified using C-Fn and C-Rn as forward and reverse primers, respectively. The amplified product was purified and cloned into pTZ57R/T, producing pTZ57R/xynZ-Cn. The XynZ-Cc obtained by restriction of pTZ57R/xynZ-Cc with Nco1 and BamHI was purified and then sub-cloned to the upstream of pCBMc at the same restriction sites, producing pXynZ-CB.

For the construction of XynZ-BCB the fragment encoding the binding and catalytic domain (XynZ-BC) was amplified using B-Fn and C-Rc", as forward and reverse primers, respectively. The XynZ-BC obtained after restriction digestion of pTZ57R/xynZ-BC with Nco1 and EcoRI was cloned into expression vector pET28a(+) at the same restriction sites, producing pXynZ-BC2. While the binding module (XynZ-B) was amplified using B-Fe' and B-Re', as forward and reverse primers, respectively. The XynZ-B obtained after restriction with EcoRI and HindIII was sub-cloned to the downstream of pXynZ-BC2 at the same restriction site producing pXynZ-BCB.

For the construction of XynZ-CBC the fragment encoding the catalytic domain (XynZ-Cc2) was amplified using C-Fc' and C- Rc"", as forward and reverse primers, respectively. The XynZ-Cc2 obtained after restriction with SacI and XhoI was cloned into expression vector pET28a(+) at the same restriction sites, producing pXynZ-C2. While the catalytic and binding module (XynZ-CB) was amplified using C-Fn and B-Re", as forward and reverse primers, respectively. The XynZ-CB obtained after restriction with NcoI and SacI was sub-cloned to the upstream of pXynZ-C2 at the same restriction site producing pXynZ-CBC.
For the production of XynZ-BC the binding domain of family 22 was amplified using XynC-BC-F and XynC-BC-F, as forward and reverse primers, respectively. The fragment obtained after amplification was cloned upstream of the catalytic domain of XynZ in pET22b to produce pXynZ-BC. *E. coli* DH5α cells were transformed with the constructs thus made and further confirmed by colony PCR and restriction analysis.

Table 2.6: Nucleotide sequence of PCR primers used for amplifications of different fragments used for the production of engineered derivatives of xylanases Z of *C. thermocellum*

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer</th>
<th>Sequence</th>
<th>Restriction site(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>xynZ- B-Fn</td>
<td>5'–CTCCATGGCAAGCCGCTAACACA–3'</td>
<td>NcoI</td>
</tr>
<tr>
<td>2</td>
<td>xynZ-B-Rn</td>
<td>5'–GAGGATCCGCTTTCAACGCCAAAAGTG–3'</td>
<td>BamHI</td>
</tr>
<tr>
<td>3</td>
<td>xynZ-C-Fc</td>
<td>5’–CAGGATCCATCCGTCTTGTTACTCGA–3’</td>
<td>BamHI</td>
</tr>
<tr>
<td>4</td>
<td>xynZ-C-Rc</td>
<td>5’–CTGAAATTCTCATTAGCCCATAGAGCTTC–3’</td>
<td>EcoRI</td>
</tr>
<tr>
<td>5</td>
<td>xynZ-C-Fn</td>
<td>5’–CTCCATGGCAATCCGTCTTGTTACTCGA–3’</td>
<td>NcoI</td>
</tr>
<tr>
<td>6</td>
<td>xynZ-C-Rn</td>
<td>5’–GAGGATCCGCTTTCAACGCCAAAAGTG–3'</td>
<td>BamHI</td>
</tr>
<tr>
<td>7</td>
<td>xynZ-B-Fc</td>
<td>5’–CTGGAATCCAGCGGCTAACAACAG–3’</td>
<td>BamHI</td>
</tr>
<tr>
<td>8</td>
<td>xynZ-B-Rc</td>
<td>5’–CTGGAATCCAGCGGCTAACAACAG–3’</td>
<td>SacI</td>
</tr>
<tr>
<td>9</td>
<td>xynZ-C-Rc’</td>
<td>5’–CTGAAATTCCATGCCCATAGAGCTTC–3’</td>
<td>EcoRI</td>
</tr>
<tr>
<td>10</td>
<td>xynZ-B-Fe</td>
<td>5’–CTGGAATCCAGCGGCTAACAACAG–3’</td>
<td>EcoRI</td>
</tr>
<tr>
<td>11</td>
<td>xynZ-B-Re’</td>
<td>5’–AAGGCTTGGCTTTCAACGCCAAAAGTG–3’</td>
<td>HindIII</td>
</tr>
<tr>
<td>12</td>
<td>xynZ-C-Fc’</td>
<td>5’–CGGAGCTCAATCCGTCTTGTTACTCGA–3’</td>
<td>SacI</td>
</tr>
<tr>
<td>13</td>
<td>xynZ-C-Rc’’</td>
<td>5’–CTCAGGTCAATAGCCCCATAAGAGCTTC–3’</td>
<td>XhoI</td>
</tr>
<tr>
<td>14</td>
<td>xynZ-B-Re’’</td>
<td>5’–CTCAGGTCAATAGCCCCATAAGAGCTTC–3’</td>
<td>SacI</td>
</tr>
</tbody>
</table>

*Restriction sites are underlined in the sequences.*
Fig. 2.2: Schematic diagram for producing constructs expressing XynZ-BDC, XynZ-C, XynZ-BC, XynZ-CB, XynZ-BCB and XynZ-CBC of *C. thermocellum* in *E. coli*.
2.3 Cloning of PCR products to Cloning Vector (pTZ57R/T)

After amplification and purification of xylanase genes, they were cloned in pTZ57R/T (Annex I). TA cloning was carried out using Fermentas InstaT/AcloneTM PCR Product Cloning kit (K1214) according to the manufacturer’s instructions. Preparation of competent cells of E. coli DH5α and transformation of these cells was done using Fermentas TransformAidTM Bacterial Transformation kit (K2711) according to the manufacturer’s instructions. Transformed cells were then plated on LB-agar plates (1.5% agar) containing ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) with final concentrations of 50µg/ml, 0.5 mM and 20µg/ml respectively, and then incubated at 37°C overnight. Positive clones containing recombinant plasmid (pTZ57R/xyn) were screened by “Blue-White screening”. Ten well-separated white colonies (W1-W10) were further spotted on fresh LB-agar plates (1.5% agar) containing ampicillin, IPTG and X-gal with final concentrations of 50µg/ml, 0.5 mM and 20µg/ml respectively, and then incubated at 37°C overnight.

2.3.1 Selection of positive clones by colony PCR

In order to confirm that xylanase genes have been successfully inserted in the cloning vector or expression vector, Colony PCR was performed. The composition and programming of PCR reaction was same as given in the previous Table 2.2, with exception that a single colony from the plates was picked using autoclaved tip and dipped in PCR tube containing the reaction ingredients. This colony was used as a template during the colony PCR. The results of the colony PCR were observed on agarose gel
electrophoresis. The colonies showing positive result after colony PCR were employed for the further culturing and isolation of plasmid DNA.

Table 2.7: Colony PCR Programming for amplification of xylanase genes

<table>
<thead>
<tr>
<th>S.#</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>94</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>50-56*</td>
<td>1-2.3*</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>cycle repeat (2-4), 30 times</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>72</td>
<td>5</td>
</tr>
</tbody>
</table>

2.3.2 Isolation and purification of pTZ57R/xyn

10 ml of LB broth containing 100 μg/ml ampicillin were inoculated with the cells of positive colony and incubated at 37°C overnight (15-18 hours). 1.5ml of the overnight culture was centrifuged at 12,000 rpm for 30 seconds at 4°C. Supernatant was discarded and dry pellet was resuspended in 100μl of ice-cold alkaline lysis solution I; ALS I (Annex VI) by vigorous vortex. 200μl of freshly prepared alkaline lysis solution II; ALS II (Annex VI) was then added. It was gently mixed by inverting the tubes 10-20 times and then left for 5 minutes at room temperature. 150 μl of ice-cold alkaline lysis solution III; ALS III (Annex VI) was added and gently mixed by inverting and left for 5 minutes on ice. The mixture was centrifuged at 12,000 rpm for 5 minutes at 4°C. Supernatant containing plasmid was transferred to fresh microfuge tube; equal volume of
phenol/chloroform was added and gently mixed by inverting the tubes 5-10 times. Then centrifuged at 12,000 rpm for 2 minutes. Upper aqueous phase was carefully transferred to fresh microfuge tube and 2 volumes of distilled ethanol were added and allowed to stand for 2 min to precipitate the plasmid. It was then centrifuged at 12,000 rpm for 5 min at 4°C. Supernatant was gently removed and the tube was inverted on blotting paper to dry the DNA pellet. 1ml of 70% ethanol was added to wash the pellet and the closed tube was inverted several times. DNA was recovered by centrifugation at 12,000 rpm for 2 min at 4°C. Supernatant was again removed and the pellet was left to dry (Atleast 15 minutes). The pellet was then dissolved in 50µl of TE buffer (pH = 8.0) containing 20 µg/ml DNase free RNase A and incubated at 37°C for 2-3 hours to degrade any RNA present. Finally, plasmid isolation was checked on 1% agarose gel in 1X TAE buffer (pH = 8.5) and plasmid was stored at -20°C (Annex VI).

2.3.3 Double restriction digestion of pTZ57R/xyn

Purified plasmid (pTZ57R/xyn) was first linearized with EcoRI/Ndel/NcoI to check its length (bp). Reaction mixture (total volume 10µl) contained 5µl plasmid (pTZ57R/xyn), 1µl 10X EcoRI buffer, 0.5µl EcoRI/Ndel/NcoI (10U/µl) and 3.5µl sterile ddH₂O. The reaction mixture was then incubated at 37°C for 4 hours.

For double digestion of pTZ57R/xyn, the reaction mixture (total volume 50 µl) consisted of 35µl pTZ57R/xyn, appropriate buffer (10X), 2.5 µl Enzyme I (10U/µl), 2.5 µl Enzyme II (10U/µl) and 10µl sterile ddH₂O. The reaction mixture was then incubated at 37°C for 6 hours or overnight as shown in table 2.8.
Table 2.8: Double restriction digestion of various recombinant cloning vectors of XynC and XynZ of *C. thermocellum*, their respective restriction enzymes, buffer and incubation time

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Recombinant cloning vector</th>
<th>Restriction Enzyme I</th>
<th>Restriction Enzyme II</th>
<th>Buffer type/concen.</th>
<th>Incubation time (37°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pTZ57R/xynC-BC</td>
<td>NdeI</td>
<td>EcoRI</td>
<td>2X Tango</td>
<td>overnight</td>
</tr>
<tr>
<td>2</td>
<td>pTZ57R/xynC-C</td>
<td>NdeI</td>
<td>EcoRI</td>
<td>2X Tango</td>
<td>overnight</td>
</tr>
<tr>
<td>3</td>
<td>pTZ57R/xynZ-BDC</td>
<td>NcoI</td>
<td>BamHI</td>
<td>1X Tango</td>
<td>6 hours</td>
</tr>
<tr>
<td>4</td>
<td>pTZ57R/xynZ-C</td>
<td>NcoI</td>
<td>BamHI</td>
<td>1X Tango</td>
<td>6 hours</td>
</tr>
<tr>
<td>5</td>
<td>pTZ-BC</td>
<td>NdeI</td>
<td>BamHI</td>
<td>2X Tango</td>
<td>overnight</td>
</tr>
<tr>
<td>6</td>
<td>pTZ-C</td>
<td>NdeI</td>
<td>BamHI</td>
<td>2X Tango</td>
<td>overnight</td>
</tr>
<tr>
<td>7</td>
<td>pTZ-B</td>
<td>BamHI</td>
<td>EcoRI</td>
<td>Buffer R</td>
<td>4 hour</td>
</tr>
<tr>
<td>8</td>
<td>pTZ57R/Bn</td>
<td>NcoI</td>
<td>BamHI</td>
<td>1X Tango</td>
<td>6 hours</td>
</tr>
<tr>
<td>9</td>
<td>pTZ57R/xynZ-Cc</td>
<td>BamHI</td>
<td>EcoRI</td>
<td>Buffer R</td>
<td>4 hour</td>
</tr>
<tr>
<td>10</td>
<td>pTZ57R/Bc</td>
<td>BamHI</td>
<td>SacI</td>
<td>Buffer BamHI</td>
<td>4 hour</td>
</tr>
<tr>
<td>11</td>
<td>pTZ57R/xynZ-Cn</td>
<td>NcoI</td>
<td>BamHI</td>
<td>1X Tango</td>
<td>6 hours</td>
</tr>
<tr>
<td>12</td>
<td>pTZ57R/xynZ-BC</td>
<td>NcoI</td>
<td>EcoRI</td>
<td>1X Tango</td>
<td>6 hours</td>
</tr>
<tr>
<td>13</td>
<td>pTZ57R/xynZ-Bc</td>
<td>EcoRI</td>
<td>HindIII</td>
<td>Buffer R</td>
<td>6 hours</td>
</tr>
<tr>
<td>14</td>
<td>pTZ57R/xynZ-Bn</td>
<td>NcoI</td>
<td>BamHI</td>
<td>1X Tango</td>
<td>6 hours</td>
</tr>
<tr>
<td>15</td>
<td>pTZ57R/xynZ-Cc2</td>
<td>SacI</td>
<td>XhoI</td>
<td>1X raised to 2X</td>
<td>6 hours</td>
</tr>
</tbody>
</table>

2.4 Cloning of xylanase genes into expression vector

The xylanase genes were excised from the pTZ57R/T plasmid by double digestion with their respective enzymes and cloned in pET-28a(+) (Annex II) or pET-22b(+) (Annex II). Expression vectors were also digested with same restriction enzymes as shown in table 2.5. The xylanase gene was purified from agarose gel with the help of Qiagen gel extraction kit prior to ligation in pET-28a(+) or pET-22b(+).

2.4.1 Ligation of xylanase gene into expression vector

The ratio of 3:1 (molar) insert to vector was used for the ligation with following conditions.
Table 2.9: Composition of ligation mixture

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vector ends</td>
<td>60 (fmol)</td>
</tr>
<tr>
<td>2</td>
<td>Insert ends</td>
<td>180 (fmol)</td>
</tr>
<tr>
<td>3</td>
<td>DNA Ligase</td>
<td>1 (unit)</td>
</tr>
</tbody>
</table>

The ligated mixture was incubated at 22°C for 16 hours.

2.4.2 Transformation in *E. coli* (DH5α)

Transformation was performed as described in the section 2.3, except ampicilline or kanamycin was used as antibiotic. The colonies of expression vector containing xylanase gene were screened by colony PCR and double restriction digestion.

2.4.3 Plasmid DNA isolation

The plasmid DNA was isolated from the positive (white) colonies after overnight culturing following the conditions as described in section 2.3.2. The plasmid DNA was confirmed by restriction digestion with appropriate enzyme followed by agarose gel electrophoresis.

2.4.4 Restriction analysis of expression vector

Double digestion of expression vector was performed to confirm the presence of gene insert. For this purpose, restriction enzymes corresponding to the constructs were used to cut the plasmid DNA as shown in table 2.10. After double digestion of the plasmid DNA, vector and insert were analyzed on 0.8% agarose gel by electrophoresis.

2.4.5 Transformation of *E. coli* BL21 (DE3) with expression vector (pET-28a(+)/pET-22b(+))
Preparation of competent cells of *E. coli* BL21 (DE3) and transformation of these cells was done using Fermentas TransformAid™ Bacterial Transformation kit (K2711) according to the manufacturer’s instructions. Transformed cells were then plated on LB-agar plates (1.5% agar) containing ampicilline or kanamyicine with final concentrations of 50 or 60 µg/ml, respectively, and then incubated at 37°C overnight. Ten of the colonies were further spotted on LB-agar plates containing 50 or 60 µg/ml ampicilline or kanamyicine respectively, and incubated at 37°C overnight.

Table 2.10: Double restriction digestion of various recombinant expression vectors of XynC and XynZ of *C. thermocellum*, their respective restriction enzymes, buffer and incubation time

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Recombinant expression vector</th>
<th>Restriction Enzyme I</th>
<th>Restriction Enzyme II</th>
<th>Buffer type/concen.</th>
<th>Incubation time (37°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pXynC-BCD</td>
<td>NdeI</td>
<td>EcoRI</td>
<td>2X Tango</td>
<td>6 hours</td>
</tr>
<tr>
<td>2</td>
<td>pXynC-BC</td>
<td>NdeI</td>
<td>EcoRI</td>
<td>2X Tango</td>
<td>6 hours</td>
</tr>
<tr>
<td>3</td>
<td>pXynC-C</td>
<td>NdeI</td>
<td>EcoRI</td>
<td>2X Tango</td>
<td>6 hours</td>
</tr>
<tr>
<td>4</td>
<td>pXynC-BCB</td>
<td>NdeI</td>
<td>EcoRI</td>
<td>2X Tango</td>
<td>6 hours</td>
</tr>
<tr>
<td>5</td>
<td>pXynC-CB</td>
<td>NdeI</td>
<td>EcoRI</td>
<td>2X Tango</td>
<td>6 hours</td>
</tr>
<tr>
<td>6</td>
<td>pXynZ-BDC</td>
<td>NcoI</td>
<td>BamHI</td>
<td>1X Tango</td>
<td>4 hours</td>
</tr>
<tr>
<td>7</td>
<td>pXynZ-C</td>
<td>NcoI</td>
<td>BamHI</td>
<td>1X Tango</td>
<td>4 hours</td>
</tr>
<tr>
<td>8</td>
<td>pXynZ-BC</td>
<td>NdeI</td>
<td>EcoRI</td>
<td>2X Tango</td>
<td>6 hours</td>
</tr>
<tr>
<td>9</td>
<td>pXynZ-BC</td>
<td>BamHI</td>
<td>EcoRI</td>
<td>2X Tango</td>
<td>4 hours</td>
</tr>
<tr>
<td>10</td>
<td>pXynZ-CB</td>
<td>NcoI</td>
<td>SacI</td>
<td>1X Tango</td>
<td>6 hours</td>
</tr>
<tr>
<td>11</td>
<td>pXynZ-CB</td>
<td>NcoI</td>
<td>BamHI</td>
<td>1X Tango</td>
<td>4 hours</td>
</tr>
<tr>
<td>12</td>
<td>pXynZ-BCB</td>
<td>BamHI</td>
<td>EcoRI</td>
<td>2X Tango</td>
<td>4 hours</td>
</tr>
<tr>
<td>13</td>
<td>pXynZ-BCB</td>
<td>NcoI</td>
<td>EcoRI</td>
<td>1X Tango</td>
<td>4 hours</td>
</tr>
<tr>
<td>14</td>
<td>pXynZ-BCB</td>
<td>NcoI</td>
<td>HindIII</td>
<td>1X Tango</td>
<td>4 hours</td>
</tr>
<tr>
<td>15</td>
<td>pXynZ-CBC</td>
<td>NcoI</td>
<td>XhoI</td>
<td>2X Tango</td>
<td>4 hours</td>
</tr>
<tr>
<td>16</td>
<td>pXynZ-CBC</td>
<td>NcoI</td>
<td>SacI</td>
<td>1X Tango</td>
<td>6 hours</td>
</tr>
<tr>
<td>17</td>
<td>pXynZ-CBC</td>
<td>NcoI</td>
<td>BamHI</td>
<td>1X Tango</td>
<td>4 hours</td>
</tr>
</tbody>
</table>
2.5 Expression of xylanase genes in *E. coli* (BL21) strain

*E. coli* (BL21) strain was selected for the expression of xylanase gene. For this purpose, LB medium (20 mL), containing 100µg/mL ampicilline or 60µg/mL kanamycin, was inoculated by a single colony and incubated at 37 °C overnight.

2.6 Fermentation conditions

To 1 ml of overnight culture, 50 mL of freshly prepared LB medium with kanamycin (100 µg/ml ) was added. The culture was incubated at 37 °C and 220 rpm in an orbital shaker till OD$_{600nm}$ of the culture reached 0.6. The culture was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) with final concentration of 0.5 mM. After addition of IPTG, fractions of the medium were collected at intervals of 2, 4, 6, 8 and 10 hours for measuring the absorbance and SDS-PAGE analysis. The culture was allowed to incubate up to 16 hours at 37 °C. One milliliter culture from each flask was collected and centrifuged for 3 min at 3000 rpm. The pellets were resuspended and subjected to expression analysis by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE.).

2.6.1 Overexpression of the xylanases

The enzymes were also induced with lactose, an economically feasible inducer, in M9NG medium (Sadaf *et al.*, 2007). In order to study the effect of inducer on the expression of Xylanase at different time of induction, 500 ml M9NG medium (100 ml each in 1 liter Erlenmeyer flask) was induced with 10 mM lactose at 0, 2, 4, 6, and 8 hrs after inoculation. The OD$_{600}$ was calculated from the time of induction to maximum 18 hrs. 5 ml of the culture was drawn out from each flask with 2 hour interval. To determine the enzyme activity in the supernatant, cell pellet obtained after centrifugation was
washed with 5 ml of 0.05 M phosphate buffer (pH 6.0). The cell pellet after suspension in 5 ml buffer was sonicated (UP 400 S, ultraschallprozessor, dr.hielscher GmbH) for 10 minutes by giving 30 sec burst at 50% amplitude with 60 sec interval. Cell debris was removed by centrifuging at 6,500 rpm (5432 rcf) in Eppendorf 58404R centrifuge at 4°C for 15 min.

For preparative scale experiment 400 ml culture in 2 liter baffled flask was induced with 10 mM lactose and kanamycin (60 µg/ml). The cells were harvested after 12 hrs induction. The cell pellet was obtained by centrifuging at 6,500 rpm (5432 rcf) in Eppendorf 58404R centrifuge for 15 min at 4°C. To separate the soluble and insoluble fractions cell pellet was suspended in 0.05 M phosphate buffer (pH 6.0). The cells were lysed under high pressure of 25,000 psi in FrenchPress cell disrupter (Thermo Electron Corporation). Supernatant was obtained after centrifuging at 6500 rpm (5432 rcf) for 15 min at 4°C.

2.6.2 Total Cell Protein Analysis by SDS-PAGE

1 ml of each of the post-induction samples was taken in microfuge tubes. These were then centrifuged at 12,000 rpm for 1 minute. Supernatant was decanted off and cell pellets were resuspended in 75 µl of 1X phosphate buffered saline (PBS, Annex VI). To each sample an equal volume of 2X SDS sample buffer (Annex VI) was added. Each sample was then passed through a 27-gauge needle 10 -20 times and then heated at 85°C for 5- 10 minutes in water bath.

For sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) first gel assembly was prepared by placing spacers in-between the sides of the two glass plates and holding them with the help of clamps. 12% resolving gel (total volume 10ml)
was prepared by mixing 4ml of 30% acrylamide solution (Annex VI), 2.5ml 1.5 M Tris-Cl pH 8.8 (Annex VI), 3.3ml distilled H₂O, 100µl 10% SDS solution, 150µl 10% ammonium persulphate (APS) and 6µl TEMED. Then the gel solution was immediately poured into the gel assembly by leaving space of about 1.5 cm for stacking gel. 100 - 200µl of distilled H₂O was overlaid at the top of the gel to give smooth surface. The resolving gel was left undisturbed for 30-40 minutes to polymerize.

In the mean time 5% stacking gel (total volume 3ml) was prepared by mixing 0.5ml 30% acrylamide solution, 0.38ml 1M Tris-Cl pH 6.8 (Annex VI), 2.1ml distilled H₂O, 30µl 10% SDS solution, 30µl bromophenol blue (0.4%), 45µl 10% ammonium persulphate (APS) and 4µl TEMED. After polymerization of resolving gel the overlaid dH₂O was removed and stacking gel was poured. A comb of proper size was inserted in the stacking gel and allowed to polymerize. After polymerization comb was carefully removed and the wells were washed with 1X Tris-glycine buffer (Annex VI) three times. Gel assembly was placed in the electrophoresis tank. 1X Tris-glycine buffer was poured in both upper and lower chambers. 10µl of each protein sample and protein marker were loaded. The gel was electrophoresed at constant voltage of 100V till the dye reached at the bottom of the gel.

The gel was stained with coomassie brilliant blue R250 solution for 15 min and then destained with destaining solution (Annex VI) till the background of the gel became transparent.

2.6.3 Sub-Fractionation of Total Cell Proteins

The fractions of 10 ml taken after 0, 1, 2, 4, 6, 8 and 10 hours of induction were centrifuged at 6,500 rpm for 10 minutes. Supernatant (extracellular enzyme fraction) of
each of the fractions was shifted to fresh falcon tube and concentrated 10 fold with 100% trichloroacetic acid (TCA). Each pellet was resuspended in 5ml of lysis buffer, pH 6.0 (Annex VI). Cells were then sonicated at 50 amplitude for 0.5 second cycle for 4 minutes. Sonication was checked by taking a drop of cell suspension on a glass slide and observing under microscope. Cell suspensions were again centrifuged at 6,500 rpm for 10 minutes to separate soluble enzyme fractions (supernatant) from insoluble enzyme fractions (pellet). Soluble enzyme fractions were transferred to fresh falcon tubes and each insoluble enzyme fraction was resuspended in 5ml lysis buffer, pH 6.0, (without lysozyme). Enzyme activity in each fraction was then calculated.

A culture of *E. coli* BL21 (DE3) cells transformed with pET22b(+) vector was used as a negative control. The proteins in different fractions of 6 hours induced sample were analyzed on 12% SDS-PAGE.

### 2.7 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 buffer (pH 6.0), and incubating the mixture in a shaking water bath at 60°C for 30 minutes. The reducing sugars thus liberated were determined by DNS method (Ghose, 1987). The substrates used were birchwood and oat spelt xylan (Sigma, Germany) or pretreated bagasse, prepared by autoclaving ground bagasse powder (100 mesh) with 0.5% H₂SO₄ at 15 lb in² for three hours, followed by washing with water to neutrality and drying.

One unit of xylanase activity is defined as the amount of enzyme that released 1 µmol of xylose equivalent per minute under the assay conditions. Protein concentration
was determined by dye binding method using bovine serum albumin as a standard 
(Bradford, 1976). All the assays were done in triplicate.

2.8 Characterization of xylanases

2.8.1 Determination of optimum temperature.

The effects of pH on the enzymatic activity of the partially purified xylanase the 
preparation were investigated within a temperature range between 50°C to 80°C and the 
residual activity was assayed at regular intervals.

2.8.2 Determination of Optimum pH.

The effects of pH on the enzymatic activity of the partially purified xylanase the 
preparation were investigated within a pH range between pH 4.6 and 8.0 using 0.1 M 
sodium acetate buffer (pH 4.6 and 5.4), 0.1 M sodium citrate buffer (pH 6.0 and 6.8), 0.1 
M Na-phosphate buffer (pH 7.2 and 7.4) and 0.1 M Tris-HCl buffer (pH 8.0 and 10).

2.8.3 Determination of Thermal Stability

Thermal stability of the xylanases was determined by incubating an aliquot of the 
sample at 50, 55, 60, 65, 70, and 75°C for different periods up to 120 min and 
determining the residual activity at 60°C.

2.8.4 Determination of pH Stability

For determining pH stability, the enzyme samples were incubated at room 
temperature (30°C) at pH 3 to 10, for 120 min and the residual activity was assayed at 
regular intervals.

2.8.5 Determination of $K_m$ and $V_{max}$ of recombinant xylanases

To determine the $K_m$ and $V_{max}$ of recombinant xylanases, different substrate 
concentrations ranging from 1 - 50 mg.ml$^{-1}$ in 0.05 M phosphate buffer (pH 6.0) were
used and the activities were determined. Lineweaver-Burk plot was used to find the $K_m$ and $V_{\text{max}}$.

### 2.8.6 Binding assays

The binding of the xylanase variants to insoluble birchwood and oat spelt xylans were determined by mixing 20.0 mg of the insoluble substrate with 1.0 ml of the enzyme sample, suitably diluted in 0.05 M phosphate buffer (pH 6.0), and incubating the mixture in a shaking water bath at 4°C for 30 min (Irwin et al. 1994). The mixture was then centrifuged at 12,000 rpm for 10 min and the unbound xylanase activity in the supernatant was determined against the corresponding insoluble substrate.

### 2.8.8 Molecular modeling

Crystal structures of two Xylan binding modules, CBM6 (PDB Id: 1GMM) (Czjzek et al., 2001) and CBM22 (PDB Id: 1DYO) (Charnock et al., 2000) were used as templates to model the proteins CBM6 and CBM22 respectively using Modeller (Eswar et al., 2007, Fiser, et al., 2000). For protein-protein interaction studies, the crystal structure of catalytic core of xylanase XynZ (PDB Id: 1XYZ) (Dominguez et al., 1999) was docked with the models of CBM6 and CBM22. This was done by first generating protein interaction sites using CPORT (De Vries et al., 2011) for each protein and then the HADDOCK (De Vries et al., 2010) webserver was used to perform the dockings and generate the complexes XynZ-BC and XynZ-BC. The linkers were modeled between these complexes, using Modeller, and fifty models were generated (Eswar et al., 2007, Fiser, et al., 2000). These models were clustered into groups using NMRCLUST (Kelley et al., 1996) based on the root mean square distance (RMSD) between the corresponding residues in their structures. Representative models from each cluster and outliers,
ERRAT score (Colovos et al., 1993) and MODELLER calculated energy score of the models were used as criteria to select one “best” model for each complex i.e., XynZ-BC and XynZ-BC (denoted B6-l-XYNZ and B22-l-XYNZ).

The Xylan molecule was built in ChemBioOffice ultra 2010 (CambridgeSoft, USA) (Kerwin et al., 2010) and optimized with MMFF94 forcefield (Halgren et al., 1996). GOLD software (Cambridge Crystallographic Data Centre, UK) (Verdonk et al., 2003) was used with default parameters for docking the Xylan molecule with the B6-l-XYNZ and B22-l-XYNZ models. The binding site was selected as a space of 20Å around His$^{596}$ (residue numbered as in PDB ID: 1XYZ). Chemscore (Eldridge et al., 1997, Baxter et al., 1998) was used as scoring whereas Goldscore (Jones et al., 1997, Jones et al., 1995) was used as rescoring functions.
3.1 PCR amplification of xylanase genes

The xylanase genes ((XynC: GenBank Ac. No. **BAA21516** and XynZ: GenBank Ac. No. **AAA23286**) were amplified from the genomic DNA of the *C. thermocellum* (ATCC **27405D**) by using PCR conditions as in section 2.2.1 of material and methods. The extension time for *xynC*-BCD and *xynC*-C amplification was 2 and 1 min, while for *xynC*-B it was 30 seconds. The extension time for *xynZ*-BDC and *xynZ*-C, amplification was 1.5 and 1 min, respectively, while for *xynZ*-B it was 30 seconds. These entire genes were amplified by using different sets of primers having different restriction sites. The amplified PCR products were resolved by 0.8% agarose gel electrophoresis. It showed that *xynC*-BCD, *xynC*-BC, *xynC*-C and *xynC*-B were 1.8 (Fig. 3.1), 1.5, 1, and 0.5 kb (Fig. 3.2), respectively. In case of *xynZ* it showed that *xynZ*-BDC, *xynZ*-C and *xynZ*-B was 1.6 (Fig. 3.4), 1 (Fig. 3.5) and 0.5 kb (Fig. 3.6), respectively.

![Figure 3.1: Analysis of PCR amplified product of *xynC* of *C. thermocellum* resolved on 0.8% agarose gel. Lane M: 1 kb DNA ladder; Lane 1: *xynC*-BCD](image-url)
Fig. 3.2: Analysis of PCR amplified truncated derivatives of \textit{xynC} of \textit{C. thermocellum} resolved on 0.8% agarose gel. Lane M: 1 kb DNA ladder; Lane 1: XynC-B; Lane 2: XynC-BC; Lane 3, 4: XynC-C

Fig. 3.3: Analysis of PCR amplified XynC-BC, XynC-C, XynC-B of \textit{C. thermocellum} resolved on 0.8% agarose gel. Lane M: lamda DNA ladder; Lane 1: XynC-BC; Lane 2: XynC-C; Lane 3: XynC-B
3.2 Cloning into pTZ57R/T vector

The PCR amplified products were purified after resolving on agarose gel and ligated into the pTZ57R/T cloning vector (Annexure). DH5α strain of E. coli was transformed with cloning vector pTZ57R/T with the xlanase genes. The transformed cultures were spread on IPTG-Xgal-Agar-Ampicillin plates as described in section 2.3. The colonies were screened by Blue/White
Screening method for positive transformants. A large number of white colonies were observed on the LB agar plates showing high efficiency of ligation. In order to confirm the insertion of gene in cloning vector, the positive clones were recultured on plates having Ampicillin concentration of 100µg/mL for isolation of recombinant plasmid.

### 3.2.1 Confirmation by colony PCR

The cloning of xylanase gene into pTZ57R/T vector was also confirmed by colony PCR. The reaction conditions of colony PCR were same as described earlier in the section 2.3.1., with additional step of denaturation of colonies at 94°C for 5 min to release the DNA to be used as template in PCR reaction. The amplified size of different variants of xylanase genes fragments by colony PCR was found to be the same as expected. The results of colony PCR are shown in Fig. 3.7, 3.8, 3.9, 3.10.

![Fig. 3.7: Analysis of colony PCR of positive transformants of XynC-BCD of *C. thermocellum* resolved on 0.8% agarose gel. Lane M: DNA ladder Mix; Lane 1-8: colony PCR products.](image)

![Fig. 3.7](image)
3.2.2 Restriction analysis of recombinant vector (pTZ57R/T)

For screening of the recombinant vector having insert of expected gene. The white colonies were selected and cultured in LB medium containing ampicillin (100 µg/mL) overnight. After overnight incubation at 37°C, the plasmid DNA was isolated and double
digestion with corresponding restriction enzymes were carried out. When pTZ57R/XynC-BCD, pTZ57R/XynC-BC and pTZ57R/XynC-C were cut with NdeI and EcoRI, the recombinant vector released the insert and showed two bands, one 2.8 kb fragment representing pTZ57R and other 1.8 (Fig. 3.11), 1 (Fig. 3.12), 1.5 (Fig. 3.13), kb fragments of XynC-BCD, xynC-C and XynC-BC, respectively. The restriction digestion of pTZ57R/XynZ-Cn, pTZ57R/XynZ-Cc, pTZ57R/XynZ-Cc2, pTZ57R/XynZ-Bc and pTZ57R/XynC-Bn was done with NcoI+BamHI, BamHI+EcoRI, SacI+XhoI, BamHI+SacI and NcoI+BamHI. The recombinant vectors released inserts and showed band of 2.8 kb fragment representing pTZ57R and 1 and 0.5 kb fragments of XynZ-C and XynZ-B, respectively (Fig. 3.14). Thus these results show that different variants of XynC and XynZ were successfully cloned into pTZ57R/T.

Fig. 3.11: Analysis of double restriction digestion of XynC-BCD of C. thermocellum resolved on 0.8% agarose gel. Lane M: lamda DNA ladder; Lane 1, 2: double restriction digestion of pTZ57R/xynC-BCD
Fig. 3.12: Analysis of double restriction digestion of xynC-C of *C. thermocellum* resolved on 0.8% agarose gel. Lane M: 1 kb DNA ladder; Lane 1: double restriction digestion of pTZ57R/xynC-C with *Nde*I and *EcoR*I.

Fig. 3.13: Analysis of double restriction digestion of xynC-BC of *C. thermocellum* resolved on 0.8% agarose gel. Lane M: 1 kb DNA ladder; Lane 1: double restriction digestion of pTZ57R/xynC-CB with *Nde*I and *EcoR*I.
Fig. 3.14: Analysis of double restriction digestion of xynZ-C and xynZ-B of *C. thermocellum* resolved on 0.8% agarose gel. Lane M: DNA ladder Mix (# SM1173); Lane 1, 2, 3: double restriction digestion of pTZ57R/xynZ-Cn, pTZ57R/xynZ-Cc and pTZ57R/xynZ-Cc2 with *NcoI* + *BamHI*, *BamHI* + *EcoRI* and *SacI* + *XhoI*, respectively; Lane 4, 5 double restriction digestion of pTZ57R/Bc and pTZ57R/Bn with *BamHI* + *SacI* and *NcoI* + *BamHI*, respectively.

### 3.3 Cloning of xylanase genes in expression vector (pET-28a(+)/ pET-22b(+))

The cloned xylanase genes were expressed in *E. coli* by using expression pET-28a(+) or pET-22b(+) vector according the instructions as given in the manual of manufacturer (Invitrogen). The presence of restriction sites and multiple cloning sites is shown in the restriction map of pET-28a(+) expression vector (Annexure II). Before ligation into the expression vector, both the gene and expression vector were digested with corresponding restriction enzymes. Digestion was performed overnight for *NdeI* and *NcoI* as both restriction enzymes are slow cutter and show better digestion after overnight incubation at 37°C. The DH5α strain of *E. coli* was transformed with these ligation mixtures.

The recombinant expression vectors with cloned gene were plated on kanamycine or ampicillin LB plates and incubated at 37°C for overnight. The successfully transformed white colonies were selected for further analysis. The plasmid DNA was isolated and digested with corresponding restriction enzymes as section 2.4.4. A fraction of the digested plasmid DNA was run on 0.8% agarose gel and cloning of xylanase genes in pET-28a(+) or pET-22b(+) expression vector was confirmed.

The pXynC-BCD, pXynC-C, pXynC-BC, pXynC-CB and pXynC-BCB were cut with *NdeI* and *EcoRI*. The restriction with these enzymes resulted in the release of 1.8 (Fig. 3.15), 1 (Fig. 3.16) 1.5 (Fig. 3.17) 1.5 (Fig. 3.19) and 2 kb (Fig. 3.23) fragments, respectively, from pET-22b(+). The colony PCR from the recombinant expression vectors also showed the insert of same size as shown in Fig. 3.18, 3.20, 3.21, and 3.22.
Fig. 3.15: Analysis of double restriction digestion of XynC-BCD of *C. thermocellum* resolved on 0.8% agarose gel. Lane M: DNA ladder Mix; Lane 1, 2: double restriction digestion of pXynC-BCD.

Fig. 3.16: Analysis of double restriction digestion of XynC-C of *C. thermocellum* resolved on 0.8% agarose gel. Lane M: 1 kb DNA ladder; Lane 1: double restriction digestion of pxynt-C with with NdeI and EcoRI.
Fig. 3.17: Analysis of double restriction digestion of xynC-C of *C. thermocellum* resolved on 0.8% agarose gel. Lane M: 1 kb DNA ladder; Lane 1: double restriction digestion of pxynC-BC with NdeI and EcoRI.

Fig. 3.18: Analysis of colony PCR of positive transformants of xynC-C of *C. thermocellum* after transformation of *E. coli* cells with recombinant vector pXynC-C, resolved on 0.8% agarose gel. Lane M: 1 kb DNA ladder; Lane 1-9: colony PCR products.
Fig. 3.19: Analysis of colony PCR of positive transformants of XynC-BC of *C. thermocellum* after transformation of *E. coli* cells with recombinant vector pXynC-BC, resolved on 0.8% agarose gel. Lane M: 1 kb DNA ladder; Lane 1-3: colony PCR products

Fig. 3.20: Analysis of colony PCR of various *E. coli* cells, transformed with recombinant vector pxynC-BC of *C. thermocellum*, resolved on 0.8% agarose gel. Lane +: a positive control; Lane 1-9: colony PCR products

Fig. 3.21: Analysis of colony PCR of various *E. coli* cells, transformed with recombinant vector pxynC-C of *C. thermocellum*, resolved on 0.8% agarose gel. Lane +: a positive control; Lane 1-10: colony PCR products

Fig. 3.22: Analysis of colony PCR of various *E. coli* cells, transformed with recombinant vector pxynC-CB and pxynC-BCB of *C. thermocellum*, resolved on 0.8% agarose gel. Lane +: a positive control; Lane 1-9: colony PCR products amplifying only xynC-B
The double restriction analysis of pXynZ-BC, pXynZ-BC, pXynZ-BCB and pXynZ-CBC was also carried to confirm the precision of the insert. When pXynZ-BC was cut with BamHI + EcoRI it produced 1 kb fragment while restriction with NcoI + EcoRI produced 1.5 kb fragment (Fig. 3.26). The restriction digestion of pXynZ-CB with NcoI + SacI produced 1.5 kb fragment. The restriction analysis of pXynZ-BCB with BamHI + EcoRI produced 1 kb fragment and with NcoI + EcoRI produced 1.5 kb fragments. When cut with NcoI + HindIII produced 2kb fragment (Fig. 3.27). The digestion of pXynZ-CBC with only NcoI produced 8 kb fragment, when cut with NcoI + XhoI released 2.5 kb fragment, while restriction with NcoI + SacI and NcoI + BamHI released 1.5 kb and 1 kb fragments, respectively, (Fig. 3.29). The colony PCR from pXynZ-C and pXynZ-B showed the fragment size of 1 and 0.5 kb, respectively (Fig. 3.24, 3.25).
Fig. 3.24: Analysis of colony PCR of various *E. coli* cells, transformed with recombinant vector pET28a/ XynZ-C of *C. thermocellum*, resolved on 0.8% agarose gel. Lane +: a positive Control; Lane 1-9: colony PCR products amplifying only XynZ-C

Fig. 3.25: Analysis of colony PCR of various *E. coli* cells, transformed with recombinant vector pET28a/ xynZ-B of *C. thermocellum*, resolved on 0.8% agarose gel. Lane +: a positive Control; Lane 1-9: colony PCR products amplifying only xynZ-B

Fig. 3.26: Analysis of double restriction digestion of recombinant vector of engineered derivative (xynZ-BC) of xynZ of *C. thermocellum* resolved on 0.8% agarose gel. Lane M: 1 kb DNA ladder (#SM1163); Lane 1: double restriction digestion of pXynZ-BC with *Ncol* and *EcoRI*; Lane 2: double restriction digestion of pXynZ-BC with *BamHI* and *EcoRI*. 
Fig. 3.27: Analysis of double restriction digestion of recombinant vector of engineered derivative (xynZ-CB) of xynZ of *C. thermocellum* resolved on 0.8% agarose gel. Lane M: 1 kb DNA ladder (#SM1163); Lane 1: double restriction digestion of pXynZ-CB with *NcoI* and *SacI*; Lane 2: double restriction digestion of pXynZ-CB with *NcoI* and *BamHI*.

Fig. 3.28: Analysis of double restriction digestion of recombinant vector of engineered derivative (xynZ-BCB) of xynZ of *C. thermocellum* resolved on 0.8% agarose gel. Lane M: 1 kb DNA ladder (#SM1163); Lane 1: double restriction digestion of pXynZ-BCB with *BamHI* + *EcoRI*; Lane 2: double restriction digestion of pXynZ-BCB with *NcoI* + *EcoRI*; Lane 3: double restriction digestion of pXynZ-BCB with *NcoI* + *HindIII*.
3.4 Expression analysis of recombinant xylanases

10 ml of LB broth, each containing either 100 µg.ml⁻¹ ampicillin for XynC constructs or 60 µg.ml⁻¹ kanamycin for XynZ constructs were inoculated with cells from a single colony of the transformed E. coli and incubated at 37°C overnight. 3 ml of the this culture was added to 100 ml LB broth containing the antibiotic and incubated at 37°C till OD₆₀₀ 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 after 2, 4, 6, 8, 10 and 12 hours of induction and analyzed by SDS-PAGE. The percentage of the expressed enzyme in the total cell proteins were determined by photodensitometric analysis using gel Documentation System (Syngene).

SDS-PAGE analysis of the proteins in the IPTG as well as lactose induced cells showed successful expression of all the recombinant xylanases. The XynC-BCD (67 kDa), XynC-C (38 kDa), XynC-BC (58 kDa), XynC-CB (58 kDa) and XynC-BCB (76 kDa) showed expression of about 15, 45, 30, 33 and 30% of the total cellular protein of E. coli, respectively, as shown in Fig. 3.29, 3.30, 3.31, 3.32, 3.33, 3.34, 3.35 and 3.36.
Fig. 3.30: SDS-PAGE analysis of total proteins of *E. coli* cells after induction with 0.5 mM IPTG, expressing XynC-BCD. M: protein markers; lane 1: uninduced sample; lanes 2-6: total cell protein after 2-10 hours of induction.

Fig. 3.31: SDS-PAGE analysis of total proteins of *E. coli* cells after induction with 10 mM lactose in M9NG media, expressing XynC-C. M: protein markers; lanes 1-6: total cell protein after 2-12 hours of induction;

Fig. 3.32: SDS-PAGE analysis of total proteins of *E. coli* cells after induction with 10 mM lactose in M9NG media, expressing XynC-BC. M: protein markers; lane 1: uninduced sample; lanes 2-6: total cell protein after 2-10 hours of induction;
Fig. 3.33: SDS-PAGE analysis of total proteins of *E. coli* cells after induction with 0.5 mM IPTG, expressing XynC-C. M: protein markers; lane 1: uninduced sample; lanes 2-6: total cell protein after 2-10 hours of induction;

Fig. 3.34: SDS-PAGE analysis of total proteins of *E. coli* cells after induction with 0.5 mM IPTG, expressing XynC-BC. M: protein markers; lanes 1-6: total cell protein after 2-12 hours of induction;
Fig. 3.35: SDS-PAGE analysis of proteins of *E. coli* cells expressing XynC-CB after induction with 10 mM lactose. Lanes 1-6: cell proteins at 2, 4, 6, 8, 10 and 12 hours post-induction.

In case of XynZ, induced *E. coli* cells were found to express XynZ-C (38 kDa), XynZ-BDC (60 kDa) XynZ-CB (58 kDa), XynZ-BC (58 kDa), XynZ-BCB (76 kDa) and XynZ-CBC (96 kDa) and XynZ-BC proteins at 45, 25, 30, 30, 33, 15 and 30% of the total cell proteins, respectively (Fig. 3.37, 3.38, 3.39).

Fig. 3.36: SDS-PAGE analysis of proteins of *E. coli* cells expressing XynC-BCB after induction with 10 mM lactose. M: protein markers; Lanes 2-6: cell proteins at 2, 4, 6, 8, 10 and 12 hours post-induction.

Fig. 3.37: Expression of XynZ-C in *E. coli* BL21 CodonPlus (RIL) after induction with 0.5 mM IPTG; M: Protein Marker; Lane 1: Uninduced sample; Lanes 2-6: cell proteins at 2, 4, 6, 8 and 10 hours post-induction.
Fig. 3.38: SDS-PAGE analysis of total proteins of *E. coli* cells after induction with 10 mM lactose in M9NG media, expressing XynZ-BC, XynZ-CB, XynZ-BCB, XynZ-CBC. M: protein markers; Lanes 1: uninduced; Lanes 2, 3: XynZ-BC; Lanes 4: XynZ-CB; Lanes 5: XynZ-BCB; Lanes 6: XynZ-BCB

Table: SDS-PAGE Protein Bands

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Fig. 3.39: SDS-PAGE analysis of proteins of *E. coli* cells expressing XynZ-B’C after induction with 0.5 mM IPTG. M: protein markers; Lanes 2-6: cell proteins at 2, 4, 6, 8 and 10 hours post-induction

For preparative scale experiment, 400 ml M9NG medium in 2 L baffled flasks was inoculated with overnight culture of transformed *E. coli* and induced with 10 mM lactose in M9NG medium. The cells were harvested after induction for 15 hrs by centrifugation at 6,500 rpm for 15 min at 4°C. The cell pellet was resuspended in 0.05 M phosphate buffer (pH 6.0) to an OD600 50, and lysed in French Press cell disrupter (Thermo Electron Corporation, Ohio, USA). Cell lysate supernatant was obtained after centrifuging at 6,500 rpm for 15 min at 4°C and further incubated at 65°C for 30 min. Supernatant after partial purification by heat treatment was then used for further experiments. All variant of XynC and XynZ were found in the soluble fractions as shown in Fig. 3.40, 3.41, 3.43, 3.44, 3.45, 3.46, 3.47 and 3.48. There was no band corresponding to XynC and XynZ in the insoluble fractions.
Fig. 3.40: SDS-PAGE analysis of soluble fractions obtained after sonication of the total proteins of *E. coli* cells after induction with 0.5 mM IPTG, expressing XynC-BCD. M: protein markers; lane 1: uninduced sample; lanes 2-8: total cell protein after 0-10 hours of induction;

![SDS-PAGE analysis](image)

Fig. 3.41: SDS-PAGE analysis of insoluble fractions obtained after sonication of the total proteins of *E. coli* cells after induction with 0.5 mM IPTG. M: protein markers; lane 1: uninduced sample; lanes 2-7: total cell protein after 0-10 hours of induction;

![SDS-PAGE analysis](image)

Fig. 3.42: SDS-PAGE analysis of soluble fractions obtained after sonication of the total proteins of *E. coli* cells after induction with 10 mM lactose, expressing XynC-BCD. M: protein markers; lane 1: uninduced sample; lanes 2-8: total cell protein after 0-12 hours of induction;

![SDS-PAGE analysis](image)
Fig. 3.43: SDS-PAGE analysis of total proteins of *E. coli* cells after induction with 10 mM lactose, expressing XynC-C and XynC-BC lane M: protein markers; lane 1: uninduced sample; lanes 2, 6: total cell protein; lanes 3, 7: cell lysate supernatant; lanes 4, 8: insoluble pellet; lanes 5, 9: cell lysate supernatant after heat treatment at 65°C for 30 min.

![SDS-PAGE analysis of total proteins of *E. coli* cells](image)

Fig. 3.44: SDS-PAGE analysis of proteins of *E. coli* cells expressing XynC-CB after induction with 10 mM lactose. M: protein markers; 1: uninduced sample; 2: total cell proteins; 3: cell lysate supernatant; 4: insoluble pellet; 5: cell lysate supernatant after heat treatment at 65°C for 30 min.

![SDS-PAGE analysis of proteins of *E. coli* cells expressing XynC-CB](image)

Fig. 3.45: SDS-PAGE analysis of proteins of *E. coli* cells expressing XynC-BCB after induction with 10 mM lactose. M: protein markers; 1: uninduced sample; 2: total cell proteins; 3: cell lysate supernatant; 4: insoluble pellet; 5: cell lysate supernatant after heat treatment at 65°C for 30 min.

![SDS-PAGE analysis of proteins of *E. coli* cells expressing XynC-BCB](image)
Fig. 3.46: SDS-PAGE analysis of total proteins of *E. coli* cells after induction with 10 mM lactose, expressing XynZ-C and XynZ-BDC; lane M: protein markers; lane 1: uninduced sample; lanes 2, 6: total cell protein; lanes 3, 7: cell lysate supernatant; lanes 4, 8: insoluble pellet; lanes 5, 9: cell lysate supernatant after heat treatment at 65°C for 30 min.

Fig. 3.47: SDS-PAGE analysis of proteins of *E. coli* cells expressing variants of XynZ after induction with 10 mM lactose. M: protein markers; 1, 2, 3, 4: cell lysate supernatant after heat treatment at 65°C for 30 min. of XynZ-BC, XynZ-CB, XynZ-BCB and XynZ-CBC, respectively.

Fig. 3.48: SDS-PAGE analysis of proteins of *E. coli* cells expressing XynZ-B’C after induction with 10 mM lactose. M: protein markers; 1: uninduced sample; 2: total cell proteins; 3: cell lysate supernatant; 4: insoluble pellet.
3.5 Effect of Lactose Induction at different growth levels of XynZ of *C. thermocellum*

To study the effect of inducer at different time of induction on the expression, and activity of XynZ-C and growth of *E. coli*. Five, one liter flasks containing 100ml M9NG medium were induced with 10 mM Lactose at 0, 2, 4, 6, and 8 hrs after inoculation with starter culture. The OD\textsubscript{600nm}, activity and specific activity were calculated from the time of induction to maximum 18 hours.

The cells which were induced at the time of inoculation (0 hours) showed gradual increase in the growth up to 18 hours of fermentation. The cells which were induced after 2 hours also showed similar pattern. The cells which were induced after 4 hours showed gradual increase in the growth up to 16 hours the maximum O.D\textsubscript{600} observed was 10. The cells which were induced after 6 hours showed gradual increase in the growth up to 14 hours after that the growth dropped down. The maximum 14 O.D\textsubscript{600} was observed in these cells. The maximum growth (O.D\textsubscript{600}17) was observed in the cells which were induced after 8 hours as shown in Fig. 3.49.

![Fig. 3.49: O.D\textsubscript{600}nm of XynZ batch, induced with 10mM Lactose, in 5 different flasks induced with 10mM Lactose at 0(●), 2 (∗), 4 (▲), 6 (∆), and 8 (○) hrs of inoculation.](image_url)
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 (Fig. 3.50).

![Graph showing enzyme activity over fermentation period](image)

**Fig. 3.50:** XynZ-C percentage Activity in 5 different flasks induced with 10mM Lactose at 0 ( ), 2 ( ), 4 ( ), 6 ( ), and 8 ( ) hrs of inoculation.

The specific activity profile was quiet different as compared to the growth and activity profile. The maximum specific activity was observed in the cells which were induced after 2 and 4 hours. However, the maximum specific activity was observed after 8 and 10 hours of fermentation (Fig. 3.51). The maximum activity was observed after 18 hours of fermentation, for the cells which were induced after 6 and 8 hours, and it was only 70 and 50% as compared to the cells induced after 2 and 4 hours, respectively (Fig. 3.51).
3.6 Activity yields of the different variants of xylanase C and xylanase Z of *C. thermocellum*

The overall activities of XynC-BC, XynC-C, XynC-CB and XynC-BCB produced in the culture were 2150, 850, 2370, 6640 U l\(^{-1}\) OD\(_{600}\)\(^{-1}\) on pretreated bagasse while 3490, 1490, 3860, 5730 U l\(^{-1}\) OD\(_{600}\)\(^{-1}\) on oat spelt xylan likewise 3400, 1530, 3660, 5430 U l\(^{-1}\) OD\(_{600}\)\(^{-1}\) on birchwood xylan, respectively. The activities of XynC-BC, XynC-C, XynC-CB and XynC-BCB against birchwood xylan on equimolar basis are 3,890, 720, 4,410 and 7,450 U µM\(^{-1}\), respectively. Activities against oat spelt xylan are 4,000, 720, 4,640 and 7,830, while against pretreated bagasse these are 2,440, 420, 2,840 and 9,120 U µM\(^{-1}\), respectively. The specific activity was 67, 19, 76 and 98 on birchwood and 69, 19, 80 and 103 on oat spelt xylan, while against pretreated bagasse these were 42, 11, 49 and 120 U mg\(^{-1}\), respectively (Table3.1).
Table 3.1: Activity yields of the different variants of xylanase C of *C. thermocellum* expressed in *E. coli*, as determined against insoluble substrates

<table>
<thead>
<tr>
<th>Xylanase variants (kDa)</th>
<th>Cell protein (mg l(^{-1}) OD(_{600}^{-1}))</th>
<th>Xylanase content (mg l(^{-1}) OD(_{600}^{-1}))</th>
<th>Xylanase activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Birchwood xylan</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>U mg(^{-1}) enzyme</td>
</tr>
<tr>
<td>XynC-BC (58)</td>
<td>170</td>
<td>51</td>
<td>67</td>
</tr>
<tr>
<td>XynC-C (38)</td>
<td>175</td>
<td>79</td>
<td>19</td>
</tr>
<tr>
<td>XynC-CB (58)</td>
<td>160</td>
<td>48</td>
<td>76</td>
</tr>
<tr>
<td>XynC-BCB (76)</td>
<td>168</td>
<td>55</td>
<td>98</td>
</tr>
</tbody>
</table>

The overall activities of XynC-BC, XynC-C, XynC-CB and XynC-BCB produced in the culture against solubilised oat spelt and birchwood xylan were 4,550, 6,700, 4,580, 4,520, U l\(^{-1}\) OD\(_{600}^{-1}\) and 4,447, 6,670, 4,500, 4,450 U l\(^{-1}\) OD\(_{600}^{-1}\), respectively. The activities of XynC-BC, XynC-C, XynC-CB and XynC-BCB against birchwood xylan on equimolar basis are 5046, 3192, 5220 and 6150 U µM\(^{-1}\), respectively. Activities against oat spelt xylan are 5162, 3230, 5336 and 6232 U µM\(^{-1}\), respectively. The specific activity was 87, 84, 90 and 81 against birchwood and 89, 85, 92 and 82 U mg\(^{-1}\), against oat spelt xylan, respectively (Table 3.2).

The overall activities of XynZ-BDC, XynZ-C, XynZ-BC, XynZ-CB, XynZ-BCB and XynZ-CBC produced in the culture were 3570, 33000, 3760, 3600, 3700 and 5200 U l\(^{-1}\) OD\(_{600}^{-1}\) on oat spelt xylan likewise 3665, 32900, 3579, 3678, 3800, 5400 U l\(^{-1}\) OD\(_{600}^{-1}\) on birchwood xylan, respectively. The activities of XynZ-BDC, XynZ-C, XynZ-BC, XynZ-CB, XynZ-BCB and XynZ-CBC against birchwood xylan on equimolar basis are 4740, 14820, 4120, 4160, 5092 and 19200 U µM\(^{-1}\), respectively.
Table 3.2: Expression levels and activity yields of the different variants of xylanase C of \textit{C. thermocellum} expressed in \textit{E. coli}, as determined against solublised substrates

<table>
<thead>
<tr>
<th>Xylanase variants (kDa)</th>
<th>Xylanase protein (mg l$^{-1}$ OD$^{600^{-1}}$)</th>
<th>Xylanase content (%)</th>
<th>Xylanase activities</th>
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<tbody>
<tr>
<td></td>
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<td></td>
<td>Birchwood xylan</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>U mg$^{-1}$ enzyme</td>
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<td></td>
<td>U µM$^{-1}$ enzyme</td>
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<td></td>
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<td></td>
<td>U l$^{-1}$ OD$_{600}^{-1}$ of culture</td>
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<td></td>
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<td></td>
<td>Oat spelt xylan</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>U mg$^{-1}$ enzyme</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>U µM$^{-1}$ enzyme</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>U l$^{-1}$ OD$_{600}^{-1}$ of culture</td>
</tr>
<tr>
<td>XynC-BC (58)</td>
<td>170</td>
<td>30</td>
<td>87</td>
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<td>89</td>
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<td>4550</td>
</tr>
<tr>
<td>XynC-C (38)</td>
<td>175</td>
<td>45</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3192</td>
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<td></td>
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<tr>
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<td></td>
<td></td>
<td>6700</td>
</tr>
<tr>
<td>XynC-CB (58)</td>
<td>165</td>
<td>30</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5220</td>
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<td>4500</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>92</td>
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<td></td>
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<td>5336</td>
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<td></td>
<td></td>
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<td>4580</td>
</tr>
<tr>
<td>XynC-BCB (76)</td>
<td>168</td>
<td>33</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>6150</td>
</tr>
<tr>
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<td></td>
<td>6232</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4520</td>
</tr>
</tbody>
</table>

Activities against oat spelt xylan are 4680, 14934, 4118, 4118, 4864 and 18432 U µM$^{-1}$, respectively. The specific activity was 79, 390, 68, 72, 67 and 200 against birchwood and 78, 393, 71, 71, 64 and 192 U mg$^{-1}$, against oat spelt xylan, respectively (Table 3.3).

The overall activities of XynZ-BDC, XynZ-C, XynZ-BC, XynZ-CB, XynZ-BCB and XynZ-CBC produced in the culture against solublised birchwood and oat spelt xylan were 5103, 34338, 5017, 5116, 5238, and 6838 U l$^{-1}$ OD$_{600}^{-1}$, likewise 5012, 34452, 5160, 5065, 5096, 6669 U l$^{-1}$ OD$_{600}^{-1}$, respectively. The activities of XynZ-BDC, XynZ-C, XynZ-BC, XynZ-CB, XynZ-BCB and XynZ-CBC against birchwood xylan on equimolar basis are 6660, 17404, 5510, 5800, 7020 and 24288 U µM$^{-1}$, respectively. Activities against oat spelt xylan are 6540, 17480, 5626, 5742, 6864 and 640224 U µM$^{-1}$, respectively. The specific activity was 111, 458, 95, 100, 90 and 253 against birchwood and 109, 460, 97, 99, 88 and 247 U mg$^{-1}$, against oat spelt xylan, respectively (Table 3.4).
Table 3.3: Expression levels and activity yields of the different variants of xylanase Z of *C. thermocellum* expressed in *E. coli*, as determined against insoluble substrates

<table>
<thead>
<tr>
<th>Xylanase variants (kDa)</th>
<th>Cell protein (mg l⁻¹ OD₆₀₀⁻¹)</th>
<th>Xylanase content (%)</th>
<th>Xylanase activities</th>
<th>Birchwood xylan</th>
<th>Oat spelt xylan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>U mg⁻¹</td>
<td>U µM⁻¹</td>
<td>U l⁻¹ OD₆₀₀⁻¹</td>
</tr>
<tr>
<td>XynZ-BDC (60)</td>
<td>185</td>
<td>25</td>
<td>79</td>
<td>4740</td>
<td>3665</td>
</tr>
<tr>
<td>XynZ-C (38)</td>
<td>187</td>
<td>40</td>
<td>390</td>
<td>14820</td>
<td>32900</td>
</tr>
<tr>
<td>XynZ-BC (58)</td>
<td>178</td>
<td>30</td>
<td>68</td>
<td>4120</td>
<td>3579</td>
</tr>
<tr>
<td>XynZ-CB (58)</td>
<td>170</td>
<td>30</td>
<td>72</td>
<td>4160</td>
<td>3678</td>
</tr>
<tr>
<td>XynZ-BCB (68)</td>
<td>175</td>
<td>33</td>
<td>67</td>
<td>5092</td>
<td>3800</td>
</tr>
<tr>
<td>XynZ-CBC (94)</td>
<td>180</td>
<td>15</td>
<td>200</td>
<td>19200</td>
<td>5400</td>
</tr>
<tr>
<td>XynZ-BC (58)</td>
<td>175</td>
<td>30</td>
<td>360</td>
<td>20880</td>
<td>19300</td>
</tr>
</tbody>
</table>

Table 3.4: Activity yields of the different variants of xylanase Z of *C. thermocellum* expressed in *E. coli*, as determined against soluble substrates

<table>
<thead>
<tr>
<th>Xylanase variants (kDa)</th>
<th>Cell protein (mg l⁻¹ OD₆₀₀⁻¹)</th>
<th>Xylanase content (mg l⁻¹ OD₆₀₀⁻¹)</th>
<th>Xylanase activities</th>
<th>Birchwood xylan</th>
<th>Oat spelt xylan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>U mg⁻¹</td>
<td>U µM⁻¹</td>
<td>U l⁻¹ OD₆₀₀⁻¹</td>
</tr>
<tr>
<td>XynZ-BDC (60)</td>
<td>185</td>
<td>46</td>
<td>111</td>
<td>6660</td>
<td>5103</td>
</tr>
<tr>
<td>XynZ-C (38)</td>
<td>187</td>
<td>75</td>
<td>458</td>
<td>17404</td>
<td>34338</td>
</tr>
<tr>
<td>XynZ-BC (58)</td>
<td>178</td>
<td>53</td>
<td>95</td>
<td>5510</td>
<td>5017</td>
</tr>
<tr>
<td>XynZ-CB (58)</td>
<td>170</td>
<td>51</td>
<td>100</td>
<td>5800</td>
<td>5116</td>
</tr>
<tr>
<td>XynZ-BCB (78)</td>
<td>175</td>
<td>58</td>
<td>90</td>
<td>7020</td>
<td>5238</td>
</tr>
<tr>
<td>XynZ-CBC (96)</td>
<td>180</td>
<td>27</td>
<td>253</td>
<td>24288</td>
<td>6838</td>
</tr>
</tbody>
</table>

3.7 Determination of optimum temperature for enzyme activity of different variants of XynC and XynZ of *C. thermocellum*

To determine the optimum temperature of activity, the birchwood xylan was used as substrate. The activity of xylanases was measured between 50°C to 80°C by DNS method as described in section 2.8.1.
The XynC-C, XynC-BC, XynC-CB and XynC-BCB retained 50, 55, 60 and 70% of activity at 50°C, respectively. The highest activity of XynC-C, XynC-BC, and XynC-CB was observed at 60°C while the XynC-BCB showed optimum activity at 65°C. There was gradual decrease in activity of all variants from 65 to 80°C (Fig 3.52).

![Fig. 3.52: Effect of temperature on activity of XynC-C (●), XynC-BC (◇), XynC-CB (△) and XynC-BCB (▲) variants of C. thermocellum expressed in E. coli after doing enzyme assay at 50, 55, 60, 65, 70, 75, and 80°C.](image)

The XynZ-C, XynZ-BC, XynZ-CB and XynZ-BCB retained more than 70% of activity at 50 and 55°C, respectively. The highest activity of all variants of XynZ was observed at 60°C. There was gradual decrease in activity of all variants from 65 to 80°C (Fig 3.53).
3.8 Determination of optimum pH for enzyme activity of different variants of XynC and XynZ of *C. thermocellum*

To determine the optimum pH of activity, the birchwood xylan was used as substrate. The activity of xylanases was measured between pH 4 and 9 and then enzyme activity was determined as method as described in material and method section 2.7. The XynC-C, XynC-BC, XynC-CB and XynC-BCB retained more than 60% and of activity at pH 4. The activity at pH 5 was found to be around 80%. The highest residual activity of XynC-C, XynC-BC and XynC-CB was observed at pH 6. All the enzyme variants retained around 80% of their activity up to pH 9 as shown in the Fig 3.54.
Similarly XynZ-C, XynZ-BDC, XynZ-BC, XynZ-BCB and XynC-CBC also showed maximum activity at pH 6. There was no significant difference in activity was observed at pH 6.5 to 9. All enzyme variants retained more than 90% of their activity up to the pH 9 (Fig 3.55).

---

**Fig. 3.54**: Effect of pH on activity of XynC-C (●), XynC-BC ( ◊ ), XynC-BC ( △ ) and XynC-BCB ( ▲ ) variants of *C. thermocellum* expressed in *E. coli*.

**Fig. 3.55**: Effect of pH on activity of XynZ-C (●), XynZ-BDC ( ◊ ), XynZ-BC ( △ ), XynZ-BCB ( ▲ ) and XynC-CBC ( ◊ ) variants of *C. thermocellum* expressed in *E. coli*. 
3.9 Determination of thermostability of different variants of XynC and XynZ of *C. thermocellum*

Thermal stability of the xylanases was determined by incubating an aliquot of the sample at 50, 55, 60, 65, 70, and 75°C for different periods up to 120 min and determining the residual activity at 60°C as described in section 2.8.

The XynC-C, XynC-BC, and XynC-BCB retained more than 80% of its activity when incubated at 50, 55, 60, and 65°C. The XynC-C retained 80% and 40% of its activity after incubation at 70°C for 90 and 120 min, respectively. However, it retained only 10% of its activity after incubation at 75°C for 10 min no residual activity was observed on further incubation on 75°C (Fig. 3.56 A)

The XynC-BC retained more than 70% of its activity after incubation at 75°C up to 120 min, respectively. However, XynC-BC retained only 58%, 37%, 20%, and 5% of its activity after incubation at 75°C for 10, 30, 60, 90, and 120 min, respectively (Fig. 3.56 B)
The XynC-CB retained more than 80% of its activity when incubated at 50, 55, and 60°C, and 70% of its activity was retained after incubation at 65 °C for 120 min, while incubation at 70 °C it retained 70, 60, 58 and 56% of its activity after 30, 60, 90, and 120 min, respectively. After incubation at 75°C it retained 58, 20, 8 and 5% of its activity, respectively. However no activity was observed after 120 min (Fig. 3.57 A).

The XynC-BCB retained more than 70% of its activity after incubation at 50, 55, 60, 65, and 70°C for 120 min. The XynC-BCB retained more than 50% of its activity after 10 min of incubation at 75°C. However, the XynC-BCB retained 43, 38, 20 and 10% of its activity after 20, 30, 60, 90, and 120 min of incubation at 75°C.

Fig. 3.56: Residual xylanase activity of XynC-C (A) and XynC-BC (B), after incubation at 50°C (●), 55°C (□), 60°C (■), 65°C (△), 70°C (▲), and 75°C (○) for different time periods.
Fig. 3.57: Residual xylanase activity of XynC-CB (A) and XynC-BCB (B) variants of *C. thermocellum* expressed in *E. coli* after incubation at 50°C (○), 55°C (●), 60°C (□), 65°C (■), 70°C (○), 75°C (●).
The XynZ-C retained more than 80% of its activity when incubated at 50, 55, 60, and 65°C for 120 min. However, it retained only 10% of its activity after incubation at 70°C for 10 min no residual activity was observed on further incubation on 70°C (Fig. 3.58 A).

Fig. 3.58: Residual xylanase activity of XynZ-C (A) and, XynZ-BDC (B), after incubation at 50°C ( ), 55°C ( ), 60°C ( ), 65°C ( ), and 70°C ( ) for different time periods.
The XynZ-BDC retained more than 80% of its activity when incubated at 50, 55, and 60°C for 120 min. It retained 50 and 10% of its activity after incubation at 65°C for 60 min and 70°C for 10 min, respectively (Fig. 3.58 B). Similarly XynZ-BC, XynZ-CB retained 50%, while XynZ-BCB, and CBC XynZ-CB retained 40 and 38% of its activity after incubation at 65°C for 60 min, respectively (Fig. 3.59).

Fig. 3.59: Residual xylanase activity of XynZ variants after incubation at 65°C for 60 min.

3.10 \( K_m \) and \( V_{max} \) values

Kinetic parameters \( K_m \) and \( V_{max} \) of XynC and XynZ derivatives were determined using Lineweaver-Burk plot. \( K_m \) values for XynC-C, XynC-BC, XynC-CB and XynC-BCB were found to be 3.57, 3.125, 3.1 and 1.47 U mg\(^{-1}\) ml\(^{-1}\), respectively, and the \( V_{max} \) values were found to be 200, 125, 125 and 384 U.min\(^{-1}\).mg\(^{-1}\) protein, respectively (Table 3.5).

Table 3.5. Kinetic parameters of different variants of xylanase XynC of \textit{C. thermocellum}

<table>
<thead>
<tr>
<th>Xylanase variants</th>
<th>Birchwood Xylan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_m ) (mg/ml)</td>
</tr>
<tr>
<td>XynC-BC</td>
<td>3.12</td>
</tr>
<tr>
<td>XynC-C</td>
<td>3.57</td>
</tr>
<tr>
<td>XynC-CB</td>
<td>3.1</td>
</tr>
<tr>
<td>XynC-BCB</td>
<td>1.47</td>
</tr>
</tbody>
</table>
Km values for XynZ-BDC, XynZ-C, XynZ-BC, XynZ-CB, XynZ-BCB, XynZ-CBC and XynZ-BC were found to be 33, 15, 32, 33, 32 28 and 3.3 U mg\(^{-1}\) ml\(^{-1}\), respectively, and the V\(_{\text{max}}\) values were found to be 50, 333, 51, 51, 50 110 and 142 U.min\(^{-1}\).mg\(^{-1}\) protein, respectively (Table 3.6).

Table 3.6. Kinetic parameters of different variants of xylanase XynZ of \textit{C. thermocellum}

<table>
<thead>
<tr>
<th>Xylanase variants</th>
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<td>Km(mg/ml)</td>
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</tr>
<tr>
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<tr>
<td>XynZ-BC</td>
<td>3.3</td>
</tr>
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3.11 Substrate binding

Studies on xylanase binding to its substrate showed that in the case of XynC-C 50\% of the activity was left unbound when incubated with oat spelt or Birchwood xylans (Table 3.7). However, in the cases of XynC-BC, XynC-CB and XynC-BCB the unbound activities were 34, 31 and 13\% on oat spelt xylan, while 33, 32, and 12\% on birchwood xylan, respectively. Thus only one third activities remain unbound with each of the two substrates in case of XynC-BC and XynC-CB.
Table 3.7. Levels of unbound activities of different variants of xylanase XynC of *C. thermocellum* on incubation with insoluble substrates.

<table>
<thead>
<tr>
<th>Xylanase variants</th>
<th>Xylanase unbound activity (%age)</th>
</tr>
</thead>
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<td>Birchwood xylan</td>
</tr>
<tr>
<td>XynC-BC</td>
<td>33</td>
</tr>
<tr>
<td>XynC-C</td>
<td>51</td>
</tr>
<tr>
<td>XynC-CB</td>
<td>32</td>
</tr>
<tr>
<td>XynC-BCB</td>
<td>12</td>
</tr>
</tbody>
</table>

Similarly the binding essay for different variants of XynZ showed that in the case of XynZ-C 61% of the activity was left unbound when incubated with oat spelt or Birchwood xylans (Table 3.8). However, in the cases XynZ-BDC, XynZ-BC, XynZ-CB, XynZ-BCB XynZ-CBC and XynZ-BC the unbound activities were 52, 51, 52, 53 50 and 39% on birchwood xylan, while 51, 50, 51, 55, 49 and 40 % on oat spelt xylan, respectively.

Table 3.8. Levels of unbound activities of different variants of xylanase XynZ of *C. thermocellum* on incubation with insoluble substrates.

<table>
<thead>
<tr>
<th>Xylanase variants</th>
<th>Xylanase unbound activity (%age)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Birchwood xylan</td>
</tr>
<tr>
<td>XynZ-BDC</td>
<td>52</td>
</tr>
<tr>
<td>XynZ-C</td>
<td>61</td>
</tr>
<tr>
<td>XynZ-CB</td>
<td>51</td>
</tr>
<tr>
<td>XynZ-BC</td>
<td>52</td>
</tr>
<tr>
<td>XynZ-BCB</td>
<td>53</td>
</tr>
<tr>
<td>XynZ-CBC</td>
<td>50</td>
</tr>
<tr>
<td>XynZ-BC</td>
<td>39</td>
</tr>
</tbody>
</table>

### 3.12 Structural characterization

The molecular modeling and docking methods were used to characterize the structures of CBM6, CBM22, XynZ-BC (CBM6-l-XYNZ) and XynZ-B'C (CBMB22-l-XYNZ) complexes. The structure of CBM6 and CBM22 is comprised on the β-sandwich fold. There are two β-sheets and each sheet consists of 3-6 anti-parallel β-pleated sheets. These secondary structural elements form a small cleft for the binding of substrate. Three amino residues tyrosine tryptophane and
asparagine ($Y_{36}$, $W_{92}$ and $N_{120}$) were identified as substrate binding for CBM6 and two tyrosine tryptophane and phenylalanine ($Y_{77}$, $Y_{106}$, $W_{452}$, $F_{457}$) were identified as substrate binding residues for CBM22. Similarly the catalytic domains of both Xylanases (XynZ and XynC) belong to GH10 family and thy have two glutamate which are present at the active site of these enzymes (Fig 3.60).

Fig. 3.60: Structure of binding module (cyan) and catalytic domain (green) of XynZ-BC (A) and XynZ-B'C (B). (AI, AII) shows the model for XynZ-BC where the substrate binding domain showing a cleft and substrate (red color) is situated in the pocket like a tunnel, whereas (BI, BII) shows the model for XynZ-B'C where the substrate binding domain showing an open structure for substrate binding. Oligosaccharide of xylan shown in red.
In case of XynZ-BC, CBM6 and XynZ catalytic domain molecules bind along binding residues and thus forming a tunnel shaped pocket for xylan binding. The GOLD docking placed the xylan molecule just outside the tunnel as it was not able to place the molecule inside the pocket. However superimposing the crystal structure of PDB ID 1UQY (that includes a docked xylan molecule) on the XynZ-BC complex places the xylan molecule inside this pocket (Fig. 3.61 A).

Fig. 3.61: Relative position of binding module (cyan) and catalytic domain (green) of XynZ-BC (A) and XynZ-B’C (B). (A1) shows the section of the model for XynZ-BC involved in substrate binding making a tunnel, wherese (AII) shows the relative position of the binding (blue) and catalytic (yellow) residues of CBM6 and catalytic domain, respectively. (B1 and BII) shows the same section of XynZ-B’C, which is open pocket with binding (blue) and catalytic (yellow) residues of CBM22 and catalytic domain, respectively. Oligosachride of xylan shown in red.
In case of XynZ-B'C, CBM22 and XynZ catalytic domain bind the substrate through a new combination of amino acid residues Tyr\textsuperscript{77}, Tyr\textsuperscript{106} from CBM22 and Trp\textsuperscript{452}, Phe\textsuperscript{457} from the C-terminal of XynZ. This new bind site is open and is present on the surface of the protein in contrast to the tunnel shape structure showed by XynZ-BC (Fig. 3.61 B).
4.1. Cloning and relative expression of the xylanase genes

The primers for the construction of truncated and engineered derivatives of XynC and XynZ were designed according to their structural organization. The Multidomain XynC contains a family 22 CBM (20 kDa) followed by a catalytic domain of GH10 (38 kDa) and a dockerin domain. A short linker peptide is present between CBM and catalytic domain. The structural organization of XynZ is different from XynC it contains an esterase domain which is linked to family 6 CBM (435 bp/16 kDa) through a linker region (78 bp/3 kDa). The family 6 CBM is attached to a dockerin domain which is followed by a catalytic domain (38 kDa). The Linker region between dockerin and catalytic domain is 3.5 kDa. Thus in order to avoid the influence of linker region the primers were designed in such a way that they amplify the linker regions.

For in-frame insertion of xylanases in the expression vector, primers were designed with NdeI restriction sites in the forward primers for xynC and NcoI restriction sites in the forward primers for amplification of xynZ. These choices were imposed as xynC contained NcoI and xynZ contained an NdeI sites which cut them internally. The cloning of a gene at the NdeI and NcoI sites in pET22b(+) and pET28a(+), respectively, reduces the chances of frame shifting. Therefore the expression vectors pET22b(+) and pET28a(+) were selected for cloning and production of different variants of XynC and XynZ, respectively.

The amplified products were purified and cloned into pTZ57R/T. Xylanase genes were then sub-cloned from pTZ57R to expression vectors, pET22b(+) and pET28a(+). The correctness of the plasmids isolated from transformed cells of the overnight grown colonies was confirmed by colony-pick PCR and restriction analysis.

The PCR products xynC-BC, xynC-C, and xynC-B were analyzed on agarose gel electrophoresis and they were found to be of the expected size, i.e., 1.5, 1.0, and 0.5 kb,
respectively. The constructs pXynC-CB and pXynC-BCB, made by sequential cloning of \textit{xynC-C} and \textit{xynC-BC} upstream to \textit{xynC-B} in pXynC-B.

The PCR products \textit{xynZ-BDC}, \textit{XynZ-BC}, \textit{XynZ-C}, and \textit{XynZ-B} were analyzed on agarose gel electrophoresis and they were found to be of the expected size, i.e., 1.6, 1.5, 1.0, and 0.5 kb, respectively. The constructs pXynZ-CB, pXynZ-BC, pXynZ-BCB and pXynZ-CBC were made by sequential cloning of the individual fragments as reported previously (Fan \textit{et al.}, 2009). Their confirmation was done by colony pick PCR as well as by restriction digestion which showed \textit{XynZ-CB}, \textit{XynZ-BC}, \textit{XynZ-BCB} and \textit{XynZ-CBC} of 1.5 kb, 1.5 kb, 2 kb and 2.5 kb, respectively and \textit{xynC-CB}, \textit{xynC-BC} and \textit{xynC-BCB} of 1.5 kb, 1.5 kb and 2 kb, respectively.

Formation of secondary structure in the sequence in or around 5´-end of mRNA has been reported to play a significant role on expression, more stable the structure, lower the expression level (Khan \textit{et al.}, 2007). The free energy values of nucleotide sequences from the ribosomal binding site to +10 codon in both (XynC-BC and XynC-BCB) the constructs showed almost the same value (Annex IV). Therefore this factor was likely to have similar effect on the expression of the xylanase variants and it was evident, as the expression level of XynC-CB, XynC-BC and XynC-BCB was nearly the same.

However the XynC-C showed higher level of expression than that of all other variants of XynC. As energy values of nucleotide sequences from ribosomal binding site to +10 codons of the xylanases showed that those with a non-catalytic domain sequences have lower free energy values as compared to those in the case of xylanase sequences without non-catalytic domains (Annex IV). It appears that the factors other then the free energy values are more dominant in determining a higher expression of xylanase without CBM.
Similarly in case of XynZ the expression level of XynZ-C was higher than all other variant insipid of having higher free energy value. Thus xylanases consisting of only catalytic domains were expressed to a higher level than those having CBM. Removal of CBM to a smaller size protein seems to make translation process more efficient. Synthesis of a smaller size protein would not only reduce the burden on availability of amino acids but also time required for completion of a chain elongation. The equimolar yields of smaller size protein in a definite period would therefore be greater.

Heat treatment of the cell lysate supernatant to eliminate most of the *E. coli* proteins resulted in prominent appearance of three major protein bands in case of XynZ-BDC (Fig. 3.46). The size of these bands seems to correspond with the sizes of the intact enzyme molecule, the catalytic domain only and binding module. Previously, a xylanase from a *Bacillus* sp. when expressed in *E. coli* showed two activity bands in the cell lysate supernatant, which was proposed to be the result of proteolytic degradation (Blanco *et al.*, 1999).

### 4.2. Activity yields on soluble and insoluble substrates

All the variants of XynC whether having binding domain at N or C or both terminus when expressed in *E. coli* showed similar level of specific activity in cell lysate supernatant when assayed against solublised birch wood xylan. However, the activity yield was directly proportional to the expression level. The expression level of XynC-C was higher than all other variant as the activity yield, a similar pattern was observed in case of XynZ-C (Table 3.2).

On the basis of equimolar amount of enzyme the activity of XynC-BC and XynC-CB was almost same, while the activity of XynC-BCB was 52% higher than XynC-C and 82% higher than the XynC-BC and XynC-CB. The overall activity produced in culture supernatant of XynC-
C was 67% higher than XynC-BC, XynC-CB and XynC-BCB, respectively. Similar results were obtained against oat spelt xylan (Table 3.2).

The specific activity of different variants of XynZ showed interesting results. The XynZ-C showed 24%, 20%, 21%, 19% and 55% higher specific activity than XynZ-BDC, XynZ-BC, XynZ-CB, XynZ-BCB and XynZ-CBC, respectively. The activity on equimolar amount of XynZ-C showed 38, 31, 33 and 40% higher activity than XynZ-BDC, XynZ-BC, XynZ-CB and XynZ-BCB, respectively. However, the XynZ-CBC showed more than 70% higher activity than XynZ-C. Thus these result show that the presence of two catalytic domains has enhanced the activity yield of XynZ. The overall activity yield in culture supernatant of XynZ-C was 86% higher than XynZ-BDC, XynZ-BC, XynZ-CB and XynZ-BCB, respectively, while 81% higher than XynZ-CBC (Table 3.4).

The comparison of activity yield against insoluble substrates showed that XynC-C showed less than 30% but XynC-CB and XynC-BCB showed 13 and 46% higher specific activity as compared to that of XynC-BC the native form of enzyme on birchwoodxylan. Although, in general similar patterns of relative activities of these enzymes were observed against oat spelt xylan and pretreated bagasse, but against pretreated biomass the specific activity of XynC-BCB was three fold higher as compared to that of XynC-BC.

On the basis of equimolar amounts the XynC-BCB showed 2, 11 and 2 fold higher activity than XynC-BC, XynC-C and XynC-CB, respectively. The overall activity yield in culture supernatant of XynC-BCB was 2, 4, 1.4 fold higher than XynC-BC, XynC-C and XynC-CB, respectively (Table 3.1).

Whereas XynZ-C and XynZ-BCB showed 5 and 2.5 fold higher specific activity, while XynZ-BC, XynZ-CB, and XynZ-BCB showed comparable specific activity as compared to
XynZ-BDC, the native form, on birch wood xylan, respectively. Although in general similar patterns of relative activities of these enzymes were observed against oat spelt xylan.

The activity of the variant XynZ-CBC increased nearly four fold on equimolar basis as compared to that of native form of the enzyme. However, overall yield of XynZ-C remained manifold higher as compared to all the other variants due to combined result of its expression level and specific activity.

Activities of XynZ-BC and XynZ-CB, determined against birchwood xylan, were 4,120 and 4,160 U/µM and these are close to that of XynZ-BDC (Table 3.3). Overall yields of both XynZ-BC and XynZ-CB activities were ~3,600 U/l/OD\textsubscript{600} of the culture, which again is similar to that of XynZ-BDC. It appears that CBM6 has a common effect of lowering the activities of the three variants carrying this binding module. However, when CBM22, which occurs in association with XynC of \textit{C. thermocellum}, when attached with XynZ to produce XynZ-B'C, its specific activity increased approximately five fold than that of the XynZ variants containing CBM6. Activity of XynZ- B'C at 20,880 U/µM was even higher than that of XynZ-C, which is 14,820 U/µM (Table 3.3).

These results show that the binding module whether attached to the N- or C-terminal of the catalytic domain enhances activity to the same level and presence or absence of dockerin domain has no effect, whereas the catalytic domain at both the termini results in further increase in activity. The role of the binding module belonging to family 6 in facilitating the catalytic domain to bind to the substrate seems to have no importance for the activity on xylan as the presence of family 22 enhances the activity as reported previously (Khan \textit{et al.}, 2010).

Involvement of family 22 CBMs in xylan-binding have been reported in several studies (Meissner \textit{et al.}, 2000, Shin \textit{et al.}, 2002). However, not all the members of family 22 display
affinity for xylan; some show higher affinity towards insoluble cellulose while other bind more strongly with β-glucans (Ali et al., 2005). In the CBM of Xyn10B (formerly known as XynY) from C. thermocellum, three aromatic and two polar amino acid residues (i.e., Trp$^{53}$, Tyr$^{103}$, Tyr$^{136}$ and Arg$^{25}$, Glu$^{138}$, respectively) were identified to be critical in maintaining the structural integrity of the binding cleft and/or the interaction of catalytic module with the xylose polymer (Xie et al., 2001). When the primary sequence of family 22 CBM, used in this study, was compared with Xyn10B CBM using the Clustal 2.1 multiple alignment program, the five xylan-binding residues could be traced at the conserved positions despite the otherwise low alignment score of the two modules. This suggests that because of its higher affinity towards xylan, the family 22 CBM might have concentrated enough substrate at the active site of its appended catalytic module, which resulted in manifold increase in the activity yields of XynZ-B'C chimera as compared to the others.

4.3 Enzyme characteristics

Studies on xylanase binding to its substrate showed that in the case of XynC-C 50% of the activity was left unbound when incubated with oat spelt or Birchwood xylan. However, in the cases of both XynC-BC and XynC-CB only one third activities remain unbound with each of the two substrates. The incorporation of additional binding domain, as in case of XynC-BCB, resulted in even greater binding leaving only slightly more than 10% of the activity unbound (Table 3.7). The varying binding levels of the different enzyme variants were found to be generally proportional to their level of activities. These results are in agreement with the results on binding of XynX of C. thermocellum, with and without binding domain, reported previously (Shin et al., 2002)
However, in the cases XynZ-BDC, XynZ-BC, XynZ-CB, XynZ-BCB and XynZ-CBC only half of activities remain unbound with each of the two substrates. The incorporation of additional binding domain, as in case of XynZ-BCB, showed no significant difference in binding to xylan as compared to XynZ-BDC (Table 3.8). The varying level of binding of the enzyme is due to the binding module associated with catalytic domain as the family 22 CBM has xylan binding characteristics: hence its presence increases the binding of the enzyme to xylan (Ali et al., 2005).

All variants of XynC and XynZ, with and without CBM showed a broad pH optimum with only a little activity variation over pH 5.0 – 9.0. Each of the enzymes in both the forms was also stable over a broad pH range retaining most of the activity when incubated at pH 4.0 - 9.0 for 2 hrs at room temperature (30°C).

The optimum temperature for all forms of each enzyme was found to be 60 ºC except for XynC-BCB, which showed optimum temperature at 65ºC under assay conditions used. XynC-BCB when incubated at 70ºC for 120 min retained almost 80% of its activity, while XynC-CB retained less than 60% of its activity. Incubation at 75ºC showed loss of >80% activity of XynC-CB after only 30 min while XynC-BCB retained >40% activity at the same temperature. In case of XynC-BC only 20% of the activity was lost while the XynC-C lost nearly all of its activity when incubated at 70ºC for 120 min (Sajjad et al., 2010).

XynZ-BC, XynZ-CB when incubated at 70ºC for 120 min retained almost 50% of its activity, while XynZ-BCB and XynZ-CBC retained less than 40% of their activity. However, XynZ-C when incubated at 70ºC for 120 min retained almost 90% of its activity. Thus the catalytic domain of xylanase Z on its own is more thermostable and fusion of an additional
binding or catalytic module at either terminus seems to have no effect on preventing thermal
denaturation of the enzyme

Shin et al., 2002 also showed that transposition of xylan binding module of XynX showed little effect on substrate binding but led to lower thermostability, when the binding module was shifted from the native N to C terminal. Fusion of an additional binding module at C-terminal seems to further reduce thermal denaturation of the enzyme in case of XynC. However, the fusion of an additional binding and catalytic domain at either terminus seems to have no effect on thermostability.

Dissimilar roles of binding modules on enzyme stability have been reported previously. Leskinen et al. (2005) also reported 2-4 fold increased thermal stability at 80°C of derivatives of xylanase (Xyn11A) of actinomycete Nonomuraea flexuosa with truncation of carbohydrate binding module located at C-terminal. Similarly, Wang et al. (2009) reported three-fold increase in thermal stability of truncated endoglucanase without carbohydrate binding module at 65°C. Greater stability of XynC with CBM22 is also consistent with previous reports (Hayashi et al., 1997; Ali et al., 2005) describing the carbohydrate binding module belonging to family 22 as a thermo-stabilizing domain. Blanco et al. (1999) also reported decrease in thermal stability of a xylanase of Bacillus sp. with truncation of thermo-stabilizing domain.

Kinetic parameters $K_m$ and $V_{max}$ of XynC and XynZ derivatives were determined using Lineweaver-Burk plot. The $K_m$ value XynC-C, XynC-CB and XynC-BC (Sajjad et al., 2010) remained the same while $V_{max}$ value of XynC-CB was slightly higher. The presence of binding domain at either of the termini of XynC did not seem to have an effect on substrate binding. However, lower $K_m$ and high $V_{max}$ value for XynC-BCB shows increased affinity for the substrate (Table 3.5).
In case of XynZ the $K_m$ value of XynZ-BDC, XynZ-BC, XynZ-CB, and XynZ-BCB remained the same while $V_{\text{max}}$ value of XynZ-CBC was higher. The $k_m$ value of XynZ-C was less than half than that of the intact enzyme. The presence of binding module at either of the termini of XynZ did not seem to have an effect on substrate binding. However, lower $K_m$ and high $V_{\text{max}}$ value for XynZ-C shows increased affinity for the substrate.

Fusion of family 22 CBM, however, greatly improves the binding of XynZ catalytic module with the substrate as is reflected by the lower unbound activity (39 %) of XynZ-B’C, as compared to XynZ-BC or XynZ-CB. The $K_m$ value of XynZ-B’C chimera is much lower confirming its stronger affinity with the birchwood xylan. The $V_{\text{max}}$ of XynZ-B’C is also nearly three-fold higher as compared to the enzyme variants having CBM6. The higher affinity of the substrate for XynZ-B’C could be due to the intrinsic property of CBM22 or a favourable structural arrangement between the binding module and the catalytic domain or both.

The variable behavior of the two enzymes, XynC and XynZ with respect to increase in activities, stabilities and $K_m$ values seem to be due to the carbohydrate binding modules associated with the enzyme. The catalytic domains of the enzymes have a good level of homology as analyzed by sequence alignment using ClustalW at EMBL-EBI. Sequence alignment of the two carbohydrate binding domains however, showed low level of homology between the two domains (13.5%).

4.4 Structural Analysis

To characterize the structure of protein in order to find an explanation for the higher affinity of the substrate and thus higher activity of XynZ-B’C than XynZ-BC, molecular modeling and docking methods were used. It revealed that the CBM22 molecule has a binding site for its natural substrate, comprised of Trp53, Tyr$^{103}$ and Tyr$^{134}$ (Charnock et al., 2001) while
CBM6 comprised of Y^{36}, W^{92} and N^{120} (Pires et al., 2004). However in XynZ-B’C complex, a new binding mode for CBM22 was observed which formed an inverted binding site conformation possibly because of the short length linker used in this study. The amino acid residues Tyr^{77} and Tyr^{106} from CBM22 and the Trp^{452} and Phe^{457} from the C-terminal of XynZ (numbering as in sequence of XynZ-B’C), involved in this new binding mode, lined up the space of active site which could stack the xylan molecule in place. Proposed binding site had an excellent affinity for hydrophobic atoms in its vicinity. In contrast to the tunnel shaped pocket seen in XynZ-BC, a side pocket was seen in XynZ-B’C which was comparatively open and exposed to solvent (Fig. 3.61). GOLD docked the Xylan molecule in this pocket with a high docking score. This position is comparable to the position of Xylan in PDB Id: 1UQY (Pell et al., 2004), when superimpose the crystal structure on XynZ-B’C. The deep tunnel shape pocket in XynZ-BC perhaps is slow to release the xylan molecule while the side facing and solvent exposed pocket will release it quickly after metabolism; hence there is higher activity yield. The results presented here show that a binding module, depending upon its 3-dimensional arrangement along with the specific catalytic domain, may have different effects on the activity of xylanases.
4.5 Conclusion

In this study various truncated and engineered derivatives of xylanase C and xylanase Z were produced and expressed in *E. coli*. The comparison of expression, activity yield, stability and substrate binding affinity showed that the nature of binding module effects the activity of the enzyme. The results showing higher production and some of the improved characteristics of the enzyme with (XynC-BCB and XynZ-B´C) and without CBM (XynZ-C) would be advantageous in scaling up the production and their applications. Following conclusions are drawn from this study.

- The expression level of proteins consisting only on catalytic domain was higher than the proteins containing CBM.
- The activity yield of XynZ-C (catalytic domain) was higher than other variant because of higher expression level.
- Transposition of CBM from N to C- terminus influences the activity at same extent.
- Presence of CBM at both terminus of catalytic domain of XynC enhances activity, substrate affinity and thermostability
- Fusion of Family 22 CBM at the N- terminus of catalytic domain of XynZ (XynZ-B´C) generates a new bind site for the substrate thus aggregating the enzyme on the substrate and hence enhances the activity.


Annex I

Restriction map of pTZ57R/T vector. The vector was predigested with Eco321 and contains 3'-ddT overhangs to facilitate T/A cloning and prevent self ligation. It was used for blue and white colony selection. Lac Z encodes β-glactosidase that hydrolyzes X-gal into lactose and 4 chloro 3-bromo indigo that gives blue color. Cloning of insert in the multiple cloning site of this vector disrupts Lac Z, therefore X-gal is not hydrolyzed thus showing white color of bacterial colony.
Annex II

Map of pET 28a (+) expression vector and region of multiple cloning site. The necessary elements for the regulation of transcription and translation are present in the vector i.e., the origin of replication (Ori), fI origin of replication, gene for the resistance against kanamycine, proficient T7lac promoter, lac operator sequence and lac I gene for repressor. A ribosomal binding site (rbs) and pelB leader sequence (ss) for secretory expression of recombinant protein. His-tag for the production of fusion proteins (either at N or C terminus) and site for the thrombin.
Annex III

Map of pET 22b (+) expression vector and region of multiple cloning site. The necessary elements for the regulation of transcription and translation are present in the vector i.e., the origin of replication (Ori), Fl origin of replication, gene for the resistance against ampicilline, proficient T7lac promoter, lac operator sequence and lac I gene for repressor. A ribosomal binding site (rbs), pelB leader sequence (ss) for secretory expression of recombinant proteins optional his tag at the C- terminus.
Annex IV

Free energy values for the secondary structures over the sequence from the ribosomal binding site to the +10 codon of xylanase C and xylanase Z variants.

<table>
<thead>
<tr>
<th>Xylanase variants</th>
<th>Sequence (5'-end)</th>
<th>( \Delta G ) (kcal/mol)</th>
<th>Size (kDa)</th>
</tr>
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<tbody>
<tr>
<td>xynC-C</td>
<td>GAAGGAGATACATATG AGC TTG AAA GAT GTC TTT GCC GGT TAT M S L K D V F A G Y</td>
<td>-4.0</td>
<td>38</td>
</tr>
<tr>
<td>xynC-BC</td>
<td>GAAGGAGATACATATG GCA GCT CTG ATT TAC GAT GAT TTT GAA M A A L I Y D D F E</td>
<td>-1.5</td>
<td>58</td>
</tr>
<tr>
<td>xynZ-C</td>
<td>GAAGGAGATACCCTG GAT CCG TCT GTT ACT CCG ACA CAA AAC M D P S V T P T Q T</td>
<td>-6.5</td>
<td>38</td>
</tr>
<tr>
<td>xynZ-BDC</td>
<td>GAAGGAGATACCCTG GCA AAG CCG GCT AAC ACA CGT ATT GAA M A K P A N T R I E</td>
<td>-3.1</td>
<td>60</td>
</tr>
</tbody>
</table>
The secondary structures at the 5’-end of xylanase C (A, B) and xylanase Z (C, D) gene sequence starting from ribosomal binding site to the +10 codon as analyzed by Zuker mfold web server.
Annex V

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 (xynC) 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 thetic protein with the binding domain at C-terminal (XynC-CB), and the one with the binding domain at both N- and C-terminal (XynC-RBC) were expressed in E. coli. Thermobacterium phoenicis, phycocyanin E. coli and phycocyanin B could be constructed by inserting the corresponding gene in pET22b (+) XynC-CB and XynC-RBC were expressed at levels around 30% and 51% of the total E. coli cell proteins, respectively, while losing 50% and 28% of their activities at 70 °C for 120 min, respectively. The specific activities of XynC-CB and XynC-RBC were 76 and 98 U mg⁻¹, while the activities on equimolar basis were 11.19 and 7.450 U mg⁻¹ against birchwood xylan, respectively. Their overall activities produced in the culture were 3660 and 5430 U l⁻¹. Substrate binding studies showed that in case of XynC-C 51% of the activity remained bound to birchwood xylan whereas in the case of XynC-BC XynC-CB and XynC-RBC the activities lost bound were 33%, 32% and 12%, respectively, under the assay conditions used. Similar binding values were obtained in the case of oat spelt xylan. Kₘ values for XynC-C,B and XynC-RBC 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.

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1. Introduction

Xylan, a major component of hemicelluloses, contains backbone of xylopyranose residues linked by β-1→4 glycosidic bond, which is mainly hydrolysed by xylanases (E.C.3.2.1.8) (Shaham and Shoham, 2002). Clostridium thermocellum is an anaerobic thermophile, which produces a cellulolytic and xylanolytic enzymes in a multienzyme complex called cellulosome. The hydrolytic enzymes associated with cellulosome are usually multidomain 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 Thermus thermoacidophilum xylanase A (Ive et al., 1993), Thermotoga maritima xylanase A (Meissner et al., 2000), Cellulosamia fimii 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 Cellulosamia cellulolytica (Summa 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 (Hope 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
Table 1  
Nucleotide sequence of PCR primers used for amplifications.

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer</th>
<th>Sequence</th>
<th>Restriction site</th>
<th><em>ΔG</em> (kcal mol⁻¹)</th>
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<tr>
<td>1</td>
<td>yunc-C-F1</td>
<td>5′-GATCGATATCAAGCCACAGACACGTTG-3′</td>
<td>BamHI</td>
<td>-1.5</td>
</tr>
<tr>
<td>2</td>
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<td>5′-GATGCTGCTTAATGAAAGGCATATGAC-3′</td>
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<tr>
<td>3</td>
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<td>yunc-C-R2</td>
<td>5′-GATGCTGCTTAATGAAAGGCATATGAC-3′</td>
<td>BamHI</td>
<td>-1.5</td>
</tr>
</tbody>
</table>

4 Restriction sites are underlined in the sequences.

no. D98188). 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). Insta/Clone PCR product cloning kit was obtained from Fermentas (Ontario, Canada). QiAquick gel extraction kit was from Qiagen Inc. (USA). Strains were grown in JM109 MONIC media (Sukaf et al., 2007).

2.2. PCR amplification and production of constructs

Oligonucleotide primers used for amplification of xylanase genes were designed using NEBuffer (Vince et al., 2003), Primer 3.0 (Rozen and Skalnikov, 2000) and OligoCalc (Kilbo, 2007). These primers, designed on the basis of domain organization given at NCBI (YuncC: GenBank accession no. BA215161), 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 Yunc-C, Yunc-C-B and Yunc-C-BC were PCR amplified by initial denaturation at 95°C for 5 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 yunc-C-BC and yunc-CBC were produced following the scheme shown in Fig. 1. The fragment encoding the binding domain (yunc-C-B) was amplified using F1 and R1 as forward and reverse primers, respectively. For fragments encoding Yunc-C and Yunc-C-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 yunc-C-B obtained by restriction of pTZ-B with BamHI and EcoRI was purified and then sub-cloned into expression vector pET22b (+) at the same restriction sites, producing pYunc-C-B. Similarly, the inverted after digestion of pTZ-BC and pTZ-C, with NdeI and BamHI were purified and then sub-cloned into pYunc-C upstream to yunc-C-B, to produce pYunc-C-BC and pYunc-C-BCB, 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 OD₆₀₀ reached 0.5–0.6. Expression of xylanases was induced with 0.5 mM IPTG or 10 mM lactose when M9Nc medium (Sukaf 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 0.05M phosphate buffer (pH 6.0) to an OD₆₀₀ of 50. The cells were lysed in a French Press cell disruptor (Thermo Electron Corporation) and the lysate supernatant was obtained after centrifuging at 3000 rpm for 15 min at 4°C. Purified xylanases from the supernatant of the lysed cells was incubated at 80°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 200 μg substat and 1.0 ml xylanase sample suitably diluted in 0.05 M phosphate buffer.
buffer (pH 6.0), and incubating the mixture in a shaking water bath at 60°C for 30 min. The reducing sugars thus liberated were determined by DNS method (Ghose, 1987). The substrates used were birchwood and oat spelt xylans (Sigma, Germany) or pre-treated bagasse, prepared by autoclaving ground bagasse powder (100 mesh) with 0.5 M H₂SO₄ at 150°C for 24 h, followed by washing with water to neutrality and drying.

One unit of xylanase activity is defined as the amount of enzyme that released 1 μmol of xylose equivalent per minute under the assay conditions. Protein concentration was determined by dye binding method using bovine serum albumin as a standard (Bradford, 1976). All the assays were done in triplicate.

2.5. Binding assays

The binding of the xylanase variants to insoluble birchwood and oat spelt xylans were determined by mixing 200 μg of the insoluble substrate with 1.0 ml of the enzyme sample, suitably diluted in 0.05 M phosphate buffer (pH 6.0), and incubating the mixture in a shaking water bath at 4°C for 20 min (Irwin et al., 1994). The mixture was then centrifuged at 12,000 rpm for 10 min and the unbound xylanase activity in the supernatant was determined against the corresponding insoluble substrate.

2.6. Effect of pH and temperature on stability

For determining optimum pH for activity the enzyme samples were suitably diluted with 0.05 M acetate (pH 3.0–5.0), 0.05 M phosphate (pH 5.5–7.5) and 0.05 M Tris-Cl (pH 8.0–10.0) buffers. For determining pH stability, the enzyme sample was incubated at room temperature (30°C) at pH 3–10, for 120 min and the residual activity was assayed using solubilised birchwood xylan, obtained as described previously (Sajjad et al., 2010).

The thermal stability of the xylanases was determined by incubating an aliquot of the sample at 50, 55, 60, 65, 70, and 75°C for different periods up to 120 min and determining the residual activity at 60°C.

2.7. Determination of $K_M$ and $V_{max}$

To determine the $K_M$ and $V_{max}$ of recombinant xylanases, activities were determined using different concentrations of solubilised birchwood xylan ranging from 1 to 50 mg ml⁻¹ in 0.05 M phosphate buffer (pH 6.0), Lineweaver–Burk plot was used to obtain the $K_M$ and $V_{max}$.

3. Results and discussion

3.1. Production of the constructs

The PCR products xynC-B, xynC-C and xynC-B were analyzed on agarose gel electrophoresis and they were found to be of the expected size, i.e., 1.5, 1.0, and 0.5 kb, respectively. The constructs pXynC-CB and pXynC-BCB, made by sequential cloning of xynC-C and xynC-BC upstream to xynC-B in pXynC-B. Their confirmation was done by colony pick PCR as well as by restriction digestion which showed xynC-CB and xynC-BCB of 1.5 and 2 kb, respectively.

3.2. Xylanase expression and activity yields

The expression of transpositional xylanase genes xynC-CB and xynC-BC was determined by transforming E. coli BL21 (Codon-Plus) with their constructs. After induction with IPTG or lactose, the cell proteins were analyzed by SDS-PAGE. Xylanases XynC-CB (76 kDa) and XynC-CB (58 kDa) showed production of about 33% and 30% of the total E. coli cellular proteins, respectively (Figs. 2 and 3). Formation of secondary structure in the sequence in or around 5’-end of mRNA has been reported to play a significant role in expression, more stable the structure, lower the expression level (Khan et al., 2007). The free energy values of nucleotide sequences from the ribosomal binding site to +10 codon in both the constructs showed almost the same value (Table 1). Therefore this factor was likely to have similar effect on the expression of the two variants.

<table>
<thead>
<tr>
<th>Xylanase variants (kDa)</th>
<th>Cell protein (mg ml⁻¹ OD600⁻¹)</th>
<th>Xylanase content (mg ml⁻¹ OD600⁻¹)</th>
<th>Xylanase activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>Birthwood xylan</td>
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<tr>
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<td>100 µg enzyme</td>
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<tr>
<td>XynC-C (38)</td>
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<td>XynC-C (38)</td>
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<tr>
<td>XynC-C (58)</td>
<td>100</td>
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<td>76</td>
</tr>
<tr>
<td>XynC-C (76)</td>
<td>140</td>
<td>58</td>
<td>88</td>
</tr>
</tbody>
</table>

Table 2: Expression levels and activity yields of the different variants of xylanase C of C. thermocellum expressed in E. coli, as determined against insoluble substrates.

![Fig. 2. SDS-PAGE analysis of proteins of E. coli cells expressing XynC-CB after induction with 10 mM lactose. M: protein markers, 1: uninduced sample, 2: total cell protein, 3: cell lysate supernatant, 4: insoluble pellet, 5: cell lysate supernatant after heat treatment at 65°C for 30 min.](image-url)
Previously, we have reported that XynC having binding domain at the N-terminal (XynC-BC) and the one without a binding domain (XynC-C) when expressed in E. coli showed similar levels of specific activities in the cell lysis supernatant when assayed against cellulosed birchwood xylan (Sajjad et al., 2010). In this study, activities of these two enzymes and the two newly produced variants, i.e., XynC-CB and XynC-BCB were compared against the insoluble substrate. Whereas XynC-C showed less than 30%, but XynC-BC and XynC-BCB showed 13% and 48% higher specific activities as compared to that of XynC BC, the native form, on birchwood xylan, respectively. Although in general similar patterns of relative activities of these enzymes were observed against oat spelt xylan and pretreated bagasse, but against the pretreated biomass the specific activity of XynC-BCB was three fold higher as compared to that of XynC-BC (Table 2).

More significant variations in relative activities are observed when calculated on the basis of equal mass amounts of the enzymes. Thus activities of XynC-BC, XynC-C, XynC-BC and XynC-BCB against birchwood xylan are 38900, 720, 4440 and 7450 µM⁻¹, respectively. Activities against oat spelt xylan are 4000, 720, 4640 and 7830, while against pretreated bagasse these are 2440, 420, 2840 and 9120 µM⁻¹, respectively.

The overall activities of XynC-BC, XynC-C, XynC-CB and XynC-BCB produced in the culture were 2150, 650, 2370, 6640 U L⁻¹ O₆₆₅₃⁻¹ on pretreated bagasse while 3400, 1490, 3560, 5730 U L⁻¹ O₆₆₅₃⁻¹ on oat spelt xylan likewise 3400, 1530, 3660, 5430 U L⁻¹ O₆₆₅₃⁻¹ on birchwood xylan, respectively.

These results show that the binding domain whether attached to the N- or C-terminal of the catalytic domain enhances activity to the same level, whereas the binding domain at both the termini results in further increase in activity. The role of the binding domain belonging to family 22 in facilitating the catalytic domain to bind to the substrate seems to be important for activity as reported previously (Zhao et al., 2005).

2.2. Substrate binding

Studies on xylanase binding to its substrate showed that in the case of XynC-C 50% of the activity was left unbound when incubated with oat spelt or birchwood xylan (Table 2). However, in the cases of both XynC-BC and XynC-BCB only one-third activities remain unbound with each of the two substrates. The incorporation of additional binding domain, in case of XynC-BCB, resulted in even greater binding leaving only slightly more than 10% of the activity unbound. The varying binding levels of the different enzyme variants were found to be generally proportional to their level of activities. These results are in agreement with the results on binding of XynX of C. thermocellum, with and without binding domain, reported previously (Shin et al., 2002).

3.4. Enzyme characteristics

Both XynC-CB and XynC-BCB showed broad pH optima with only small differences in activities at pH between 5.0 and 9.0. Both the derivatives were also stable over broad pH range retaining most of their activity when incubated at pH 4.0-9.0 for 120 min at room temperature.

The optimum temperature for activities of XynC-CB and XynC-BCB was found to be 60 and 85°C under the assay conditions used, respectively. XynC-BCB when incubated at 70°C for 120 min retained almost 80% of its activity, while XynC-CB retained less than 80% of its activity. Incubation at 75°C showed loss of over 80% activity of XynC-CB after only 30 min while XynC-BCB retained ~40% activity at the same temperature (Fig. 4). We have reported previously that the presence of binding domain at N-terminus, as in XynC-BC only 20% of the activity was lost when incubated at 70°C for 120 min (Sajjad et al., 2010). Shin et al., 2002 also showed that transposition of xylan binding domain of XynX showed little effect on substrate binding but led to lower thermostability, when the binding domain was shifted from the native N- to C-terminal. Fusion of an additional binding domain at C-terminal seems to further reduce thermal denaturation of the enzyme.

3.5. Km and Vmax of recombinant xylanases

Kinetic parameters Km and Vmax of XynC derivatives were determined using Lineweaver–Burk plot. The Km values for XynC-BC and XynC-BCB were found to be 3.1 and 1.47 mg mL⁻¹ and Vmax values were 125 and 384 U mg⁻¹ min⁻¹, respectively (Fig. 5). The Km value XynC-C and XynC-BC (Sajjad et al., 2010) remained the
same while $V_{max}$ value of XynC-CB was slightly higher. The presence of binding domain at either of the termini of XynC did not seem to have an effect on substrate binding. However, lower $K_M$ and high $V_{max}$ value for XynC-BCB shows increased affinity for the substrate.

This study shows that the addition of a carbohydrate binding domain at the C-terminal of XynC-BC to produce XynC-BCB not only enhances thermal stability of the enzyme but also the overall activity yield is increased 3–4 fold against the different substrates used, making it more attractive for biotechnological applications.

Acknowledgement

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References

Messner, K., Wassenberg, L., Liebl, W., 2000. The thermostabilising domain of the modular xylanase XynA of Thermotoga maritima represents a novel type of binding domain with affinity for soluble xylan and mixed-linkage [β-(1,3)-β-(1,4)-glucan]. Mol. Microbiol. 37, 969–981.
Enhanced expression and activity yields of *Clostridium thermocellum* xylanases without non-catalytic domains

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**A R T I C L E** **I N F O**

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*Clostridium thermocellum*
Xylanases
Over-expression
Thermal-stability
Specific activity

**A B S T R A C T**

Two major xylanase components, XynC and XynZ, from the anaerobic thermophilic bacterium, *Clostridium thermocellum*, were cloned and expressed with and without non-catalytic domain. E. coli. Two constructs of XynC, one with its cellulose binding domain and the catalytic domain (pxynC-BC) and the other with only the catalytic domain (pxynC-C) were produced. For XynZ, the constructs produced were pxynZ-BC, which included the dockerin domain, and pxynZ-C which did not. E. coli cells transformed with pxynC-BC or pxynZ-BC gave xylanase expression of 30% and 23% total cell protein, respectively. Transformation of E. coli cells with the constructs carrying only the catalytic domains gave expression levels of ~45% in each case. The specific activities of XynC with and without the non-catalytic domains were similar but for XynZ the specific activity of the enzyme without the non-catalytic domains was ~5-fold greater than that of the intact enzyme. The total activity increased from 18.25 (U mg\(^{-1}\)) for XynC-BC to 3000 (U mg\(^{-1}\)) for XynC-C. However, the overall increase in activity was ~9-fold higher for XynZ-BC (32,400 (U mg\(^{-1}\)) and 3650 U mg\(^{-1}\)) for XynZ-C. Both the enzymes with and without non-catalytic domains were found to be quite stable under a broad pH range (pH 4–9). XynZ-C was more thermostable than XynZ-BC as it retained 87% of xylanase activity when incubated at 70°C for 7 h as compared to 2% for XynZ-BC. However, XynZ-BC retained its activity on incubation at 70°C for 2 h but XynC-C lost all activity under the same conditions. K\(_m\) values for XynC-BC and XynZ-C determined on soluble xylan were 3.1 and 3.6 mg ml\(^{-1}\), respectively, whereas these values for XynC-BC and XynZ-C were 33.3 and 15.4 mg ml\(^{-1}\), respectively. Thus the production of xylanase activity by expressing only the catalytic domains of XynC and XynZ is significantly enhanced.

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1. Introduction

Hemicelluloses are the second most abundant renewable biomass component on earth, which accounts for 20–40% of lignocellulosic biomass (Lee et al., 2007). These are heterogeneous polymers composed of xylan, mannans, galactans, and arabinans. The dominant component of hemicellulose is xylan, which contains a backbone of xylose residues linked by β-1,4-glycosidic bond (Shalom and Shoham, 2003). Xylan along with cellulose and lignin makes the plant cell wall, where all these constituents interact covalently and non-covalently imparting strength and integrity (Beg et al., 2001). It varies in degree of polymerization in hardwood and softwood, generally consisting of 150 200 and 70 120 β-xylanopyranose residues, respectively (Kilkenny et al., 1999).

*Clostridium thermocellum* is gram-positive, anaerobic, thermophilic bacterium producing lignocellulose hydrolyzing enzymes in the form of multi-enzyme complexes known as cellulosomes. The enzymes in cellulosomes are usually multi-domain, having a catalytic domain, a dockerin domain and occasionally a carbohydrate binding module, which are linked through short linker peptides, generally PI-rich. Eight xylanase genes have been characterized from *C. thermocellum*. XynC and XynZ are the major endo-xylanases associated with the cellulosome. XynC contains a family 22 CBD, a glycoside hydrolase family 10 domain and a dockerin domain (Hayashi et al., 1997) while XynZ consists of a N-terminus feruloyl esterase domain, a family 6 carbohydrate binding domain, a dockerin and a glycoside hydrolase family 10 domain (Crepyhet et al., 1988; Blum et al., 2000).

In the present study XynC and XynZ from *C. thermocellum* were expressed in E. coli with and without non-catalytic domains. Their expression levels, specific activities and characteristics of the products, and overall yields of the enzymes were compared.
Table 1

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer</th>
<th>Sequence</th>
<th>Restriction Sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>yxyC-L-F</td>
<td>5'-CAATATATGGTTAATACGACGCTG-3'</td>
<td>NdeI</td>
</tr>
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<td>2</td>
<td>yxyC-L-R</td>
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<td>yxyC-B-F</td>
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<td>4</td>
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<td>5'-CTAGAAGCTTTCGCGGCTACGTCGTAACG-3'</td>
<td>BamHI</td>
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</tbody>
</table>

* Restriction sites are shown in bold in the sequences.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth media

Chromosomal DNA of C. thermocellum (ATCC 27403D), kindly provided by Prof. D. B. Wilson, Cornell University, Ithaca, NY, USA, was used as a source of the xylanase genes (yxyC: GenBank Ac. No. D84188 and yxyZ: GenBank Ac. No. M22624). pT757R/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 CodonPlus (RILP) and vectors, pET28(+)- and pT757R/T used for over-expression, were obtained from Novagen (Madison, USA). InstAclone PCR product cloning kit was obtained from Fermentas (Ontario, Canada). QiAquick gel extraction kit was obtained from Qiagen Inc. (USA). E. coli strains were grown in LB or M9NG Media (Sadaf et al., 2007).

2.2. PCR amplification of xylanase genes

Oligonucleotide primers used for amplification of xylanase genes were designed using NEBuffer (Vincenzi et al., 2003), Primer 3.0 (Rozen and Skaletsky, 2000) and in-house (Kibbe) and are listed in Table 1. Primers were designed on the basis of domain organization given at NCBI (yxyC: GenBank Ac. No. BA025165 and yxyZ: GenBank Ac. No. AAA22346) and further checked with Pfam web-server (Fluhr et al., 2005). Schematic diagrams of different forms of the two genes are shown in Fig. 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).

For xynZ derivatives PCI was done with initial denaturation at 95°C for 4 min, then 30 cycles of denaturation at 95°C for 45s, annealing at 50°C for 30s and extension at 72°C for 1 min 45s.

The reactions for xynZ derivatives were same except for annealing, which was carried at 65°C for 30 s. Final extension was done for 25 min in both the cases. PCR amplified products were run on 1% agarose gel, purified by the QiAquick gel extraction kit (Qiagen Inc., USA) and cloned into pT757R/T by InstAclone PCR product cloning kit (Fermentas, USA). Competent cells of E. coli DH5α were prepared and transformed with the recombinant vectors according to the standard protocol (Sambrook and Russell, 2001).

2.3. Cloning of xylanases in expression plasmids

Recombinant pT757R/Ts were isolated and purified by alkaline lysis with SDS (Sambrook and Russell, 2001). The xynC recombinants and pET28(+) were restricted with NdeI and EcoRI, while xynZ recombinants and pET28(+) were cut with NcoI and BamHI. The inserts and linearized pET28(+) and pT757R/T were purified using the QiAquick gel extraction kit (Qiagen Inc., USA). Purified xynC fragments were ligated with linearized pET28(+), while those from xynZ were ligated with linearized pET28(+) competent cells of E. coli DH5α were prepared, transformed with the recombinant vectors and plated on LB-agar plates containing either 50 µg/ml ampicillin or 60 µg/ml kanamycin. After incubation at 37°C overnight, positive transformants were screened by colony PCR. Recombinant plasmids of xynC thus produced were named pXynC-B (with binding and catalytic domains) and pXynC-C (with catalytic domain only). Similarly, recombinant plasmids of xynZ were named pXynZ-BDC (with binding, dockerin and catalytic domain) and pXynZ-C (with catalytic domain only). Recombinant plasmids were purified and used to transform competent cells of E. coli BL21 CodonPlus (RILP). Transformed cells were then plated on LB-agar plates containing either 50 µg/ml ampicillin or 60 µg/ml kanamycin depending upon the expression plasmid, and incubated at 37°C overnight.

2.4. Expression and analysis of recombinant xylanases

10 ml of LB broth, each containing 100 µg ml⁻¹ ampicillin for XynC constructs or 60 µg ml⁻¹ kanamycin for XynZ constructs were inoculated with cells from a single colony of transformed E. coli and incubated at 37°C overnight. 1ml of this culture was then added to 100 ml LB broth containing antibiotic and incubated at 37°C until OD600 of 0.5-0.6. Expression of xylanases was induced with 0.5 mM IPTG. In a parallel set, the enzymes were also induced with 10 mM lactose in M9NG medium (Sadaf et al., 2007). Samples were drawn at regular intervals and analyzed by SDS-PAGE.

For preparative experiments, 400 ml M9NG medium in 21 baffled flasks were inoculated with an overnight culture of transformed E. coli and induced with 10 mM lactose. The cells were harvested after induction for 15 h by centrifugation at 6500 rpm for 15 min at 4°C. The cell pellet was resuspended in 0.05 M phosphate buffer (pH 6.0) to OD600 of 50, and lysed in French Press cell disrupor (Thermo Electron Corporation). Cell lysis supernatant was obtained after centrifuging at 6500 rpm for 15 min at 4°C and further incubated at 65°C for 30 min. Supernatant after heat treatment was used for stability assays and Km determination.
2.5. Xylanase activity and protein assays

Xylanase activity was determined by incubating 0.5 ml of suitably diluted enzyme with 0.5 ml of 1% (w/v) 0.1 M NaOH treated birchwood xylan, as substrate in 0.05 M phosphate buffer (pH 6.0) and the liberated reducing sugars were determined by DNS method (Ghose, 1987). For assays on insoluble substrates the enzyme was incubated with 10 mg ml⁻¹ untreated birchwood xylan. All the assays were performed in duplicates. One unit of xylanase activity is defined as the amount of enzyme that released 1 μmol of xylose equivalent per minute under the assay conditions.

Protein concentration was determined by a dye binding method using bovine serum albumin as a standard (Bradford, 1976).

2.6. Effect of pH and temperature on enzyme stability

For determining the optimum pH for activity the enzyme samples were suitably diluted with 0.05 M acetate (pH 3.0–5.0), 0.05 M phosphate (pH 5.5, 7.5) and 0.05 M Tris·Cl (pH 8.0, 10.0) buffers. The substrate pretreated birchwood xylan was dissolved in the corresponding buffer. For determining pH stability, the enzyme sample was incubated at room temperature (25°C) for 3–120 min and residual activity was assayed at regular intervals.

Thermal stability of the xylanases was determined by incubating an aliquot of the sample at 50, 55, 60, 65, 70 and 75°C for different periods up to 120 min and determining residual activity at 60°C.

2.7. Determination of Kₘ and Vₘₐₓ of recombinant xylanases

To determine the Kₘ and Vₘₐₓ of recombinant xylanases, different substrate concentrations ranging from 1 to 50 mg ml⁻¹ in 0.05 M phosphate buffer (pH 6.0) were used and the activities were determined. A Lineweaver–Burk plot was used to find the Kₘ and Vₘₐₓ.

3. Results and discussion

3.1. Cloning of the xylanase genes

For in-frame insertion of xylanases in the expression vector, primers were designed with NdeI|EcoRI restriction sites in case of xyc and NcoI|RsaI restriction sites in case of xynZ. These choices were used as xynC contained an NcoI site and xynZ contained an NdeI site. PCR products were analyzed by agarose gel electrophoresis and were found to be of the expected size, which were ~1.0 kb for xynC, and ~1.5 kb for xynC-BC and ~1.5 kb for xynZ-BC. The amplified products were purified and cloned into pTZ57R/T. Xylanase genes were then sub-cloned from pTZ57R into expression vectors, pET22b(+) and pET28a(+). The plasmids isolated from transformed cells of overnight grown colonies were confirmed by colony PCR and restriction analysis.

3.2. Relative expression and activity levels

SDS-PAGE analysis of the proteins in the IPTG as well as lactose induced cells showed successful expression of all the recombinant xylanases. The XynC-C (38 kDa) and XynC-BC (38 kDa) showed expression of about 45% and 30% of the total cellular protein of E. coli, respectively after induction (Fig. 2). For XynZ, induced E. coli cells expressed XynZ-C (38 kDa) and XynZ-BC (20 kDa) proteins at 45% and 25% of the total cell proteins, respectively (Fig. 3). Heat treatment of the cell lysate supernatant to eliminate most of the E. coli proteins resulted in the appearance of three major protein bands. The size of these bands corresponded with the sizes of the intact enzyme, the catalytic domain and the non-catalytic domains. Previously, a xylanase from a Bacillus sp. when expressed in E. coli

![Image of SDS-PAGE analysis of total proteins of E. coli cells expressing XynC-C and XynC-BC after induction with 10 mM lactose lane M: protein markers lane 1: uninduced samples lanes 2 and 6: total cell protein; lanes 3 and 7: cell lysate supernatant; lanes 4 and 8: insoluble pellet; lanes 5 and 9: cell lysate supernatant after heat treatment at 65°C for 30 min.](image-url)

![Image of SDS-PAGE analysis of total proteins of E. coli cells expressing XynZ-C and XynZ-BC after induction with 10 mM lactose lane M: protein markers lane 1: uninduced samples lanes 2 and 6: total cell protein; lanes 3 and 7: cell lysate supernatant; lanes 4 and 8: insoluble pellet; lanes 5 and 9: cell lysate supernatant after heat treatment at 65°C for 30 min.](image-url)
Table 2
Free energy values for the secondary structures of the sequences from the ribosomal binding site to the +10 codon of xylanase C and xylanase Z variants.

<table>
<thead>
<tr>
<th>Xylanase variants</th>
<th>Sequence (5'-3')</th>
<th>ΔG (Kcal/mol)</th>
<th>Size (KDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>xynC-C</td>
<td>GAGGAGATACATAGC CAC GCC GTT GTT GCC TGT TGT</td>
<td>-4.0</td>
<td>38</td>
</tr>
<tr>
<td>xynC-BC</td>
<td>GAGGAGATACATAGC GCA GCT CTG ATT TAC GAT TGT TGT GAA</td>
<td>-15</td>
<td>58</td>
</tr>
<tr>
<td>xynZ-C</td>
<td>GAGGAGATACATAGC GAT CGG TCT GCT CCT CGT CCC AAG GAA AAG</td>
<td>-65</td>
<td>38</td>
</tr>
<tr>
<td>xynZ-BDC</td>
<td>GAGGAGATACATAGC GAA CGG CCT AAC ACA CTA ATT GAA</td>
<td>-31</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 3
Yields of XynC and XynZ proteins with and without non-catalytic domains expressed in E. coli and their activities against insoluble birchwood xylan.

<table>
<thead>
<tr>
<th>Xylanase variants</th>
<th>Cell soluble protein (mg^{-1} OD_{600}^{-1})</th>
<th>Xylanase yield (mg^{-1} OD_{600}^{-1})</th>
<th>Specific activity (U/mg^{-1})</th>
<th>Enzyme activity (U/100 OD_{600}^{-1})</th>
<th>Relative proportion of total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>XynC-BC</td>
<td>179</td>
<td>20</td>
<td>51</td>
<td>35</td>
<td>1025</td>
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<tr>
<td>XynC-C</td>
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<td>35</td>
<td>70</td>
<td>90</td>
<td>2050</td>
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<tr>
<td>XynC-BDC</td>
<td>185</td>
<td>40</td>
<td>84</td>
<td>70</td>
<td>2050</td>
</tr>
<tr>
<td>XynZ-C</td>
<td>188</td>
<td>40</td>
<td>84</td>
<td>300</td>
<td>32000</td>
</tr>
</tbody>
</table>

* Activity was measured in duplicates.

3.2. Effect of pH and temperature on enzyme stability

Both XynC and XynZ, with and without non-catalytic domains, showed broad pH optima with only a small variation between pH 5.0 and 9.0. Both forms of the enzymes were stable over a broad pH range retaining most of the activity when incubated at pH 4.0-9.0 for 2 h at room temperature (30 °C).

The optimum temperature for both forms of each enzyme was found to be 60 °C under the assay conditions and XynC-C less stable than XynC-BC. The former lost nearly all activity when incubated at 70 °C for 15 min but the latter lost only ~ 10% under the same conditions (Fig. 4). However, XynZ-C was found to be more stable than XynZ-BDC, losing ~ 10% and ~ 50% activities, respectively when incubated at 70 °C for 2 h (Fig. 5).

Dissimilar roles of non-catalytic domains on enzyme stability have been reported previously. Leskinen et al. (2005) reported 2-4-fold increased thermal stability at 80 °C of derivatives of xylanase (Xyn11A) from the actinomycete Nonomuraea flavus with trun
cation of a carbohydrate binding domain located at C-terminal. Similarly, Wang et al. (2009) reported a 3-fold increase in thermal stability of a truncated endoglucanase at 65°C. Greater stability of XynC with non-catalytic domains is also consistent with previous reports (Hayashi et al. 1997; Ali et al., 2005) describing the carbohydrate binding domain belonging to family 22 as a thermoinsulating domain. Blanco et al. (1999) also reported a decrease in thermal stability of a xylanase of Bacillus sp. with truncation of a thermoinsulating domain.

3.4. $K_m$ and $V_{max}$ values

$K_m$ values for XynC-C, XynC-BC, XynZ-C and XynZ-BDC were found to be 2.6, 2.1, 15.4 and 22.3 mg ml$^{-1}$, respectively and the $V_{max}$ values were found to be 200, 125, 323 and 50 U min$^{-1}$ mg$^{-1}$ protein, respectively (Fig. 6). The $K_m$ of xynC with and without noncatalytic domains changed only a little, and the $K_m$ value for XynZ-C was less than half of that of the intact enzyme showing greater substrate affinity in the former case. A similar $K_m$ value for C. thermocellum XynC was previously reported to be 3.9 mg ml$^{-1}$ (Hayashi et al., 1997).

The variable behavior of the two enzymes, XynC and XynZ with respect to increase in activities, stability and $K_m$ values seem to be due to the different nature of their carbohydrate binding domains as the catalytic domains of the enzymes have a high level of homology. Sequence alignment of the two carbohydrate binding domains however, showed a very low level of homology between the two domains (13%).

The results showing higher production and some of the improved characteristics of the enzyme without non-catalytic domains would be advantageous in scaling up their production.

Acknowledgements

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References


ANNEX VI

12.1gm Tris base in 100 ml H₂O, pH adjusted with HCl

30% Acrylamide solution
Acrylamide  29.2gm
Bisacrylamide  0.8gm
Total volume 100ml with H₂O

5X Tris-glycine-SDS buffer (SDS-PAGE)
Tris base  15.1gm
Glycine  72.0gm
Total volume 1000ml with H₂O
SDS  5gm

2X SDS sample buffer
0.1M Tris-Cl, pH = 6.8  7ml
Glycerol  2ml
SDS  0.5gm
2-mercaptoethanol  0.25ml  OR
Dithiothreitol  (0.308gm)
Bromophenol blue  4mg
Total volume 10 ml with H₂O, (stored as 1 ml aliquots)

10X Phosphate Buffered Saline
NaCl  8gm
KCl  0.2gm
Na₂HPO₄  1.44gm
KH₂PO₄  0.27gm
Total volume 100ml with H₂O, (pH adjusted with HCl)

Staining solution
Coomassie b. blue R250  0.5gm
Methanol  500ml
Glacial acetic acid  100ml
Total volume 1000ml with H₂O

Destaining solution
Methanol  50ml
Glacial acetic acid  70ml
Total volume 1000ml with H₂O

DNS reagent
NaOH  9.9gm
Dinitro-salicylic acid  5.3gm
H₂O  708ml
Sod. Pot. Tartarate  15.3gm
Sod. Metabisulphite  4.15gm
Phenol 3.8ml
Filter the whole solution