산업기술연구(강원대학교 산업기술연구소 논문집), 제29권 A호, 2009.
Journal of Industrial Technology, Kangwon Natl. Univ., Korea, No. 29 A, 2009.

Construction of bifunctional xylanase-cellulase fusion protein from *Bacillus licheniformis* NBL420 and its expression in *E. coli*

Bacillus licheniformis NBL420 유래의 Xylanase-Cellulase 활성을 갖는 융합단백질 제작과 대장균에서의 발현

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Abstract

The bifunctional Xylanase-Cellulase hybrid protein was constructed by gene fusion. Two genes corresponding to endoxylanase gene (*xylS*) and endocellulase gene (*celA*) were amplified by PCR from *Bacillus licleniformis* NBL420. It was then linked through splicing by overlap extension (SOE) by PCR method. The two resulting fused hybrids, *xyl/cel* and *cel/xyl*, which differ by its orientation, were confirmed by its nucleotide sequencings. One of two fusion genes, *xyl/cel* was successfully expressed into pET22b(+) vector (p*xyl/cel*) with bifunctional xylanase-cellulase activity. On the contrary, the other *cel/xyl* fusion protein showed only cellulase activity with much decreased xylanase activity. Enzymatic properties of Xyl/Cel fusion protein were investigated regarding optimum pH, optimum temp, thermostability, and pH stability. It was revealed that Xyl/Cel fusion protein retained the bifunctional xylanase-cellulase activities eventhough two enzymes were connected with each other directly. These informations could be useful for construction of other hybrid proteins as well as increased range of substrate utilization.

키워드 : *Bacillus licheniformis* NBL420, *xylanase-cellulase 융합단백질*
Keywords : *Bacillus licheniformis* NBL420, *xylanase-cellulase fusion protein*

1. INTRODUCTION

Considering the recent increase in fuel prices and the environmental pollution caused due its excessive use, there is a growing interest among many research groups in converting biomass into ethanol by fermentation. It can be used as fuel or gasoline enhancer. In the fermentation process efficiency of biomass conversion into fermentable sugar is very important. Therefore, the engineering of the enzymes involved in this process such as, cellulase, hemicellulases, lignin-degrading enzyme group, amylases, amylopectinases, β-glucosidase, and xylanase etc can open new frontiers in this field. [8, 10, 12]

Engineering enzymes to improve their properties is one of the obvious goals of biotechnology. This point of view, combining structural elements belonging to different proteins is powerful method for generating proteins with new properties [3, 6, 8, 10, 13]. One of the ways of recombinant enzyme's exhibiting better activity and increased substrate

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range has been obtained as a result of gene fusion. Fusion proteins produced in *E. coli* have been studied extensively. The artificial bi- or polyfunctional enzymes, created through gene fusion techniques, have great potential in enzyme technology [4]. Close proximity between different enzymes or active sites encoded in one polypeptide, often enhances both the specificity and rate of the coupled reactions since intermediates can be transferred more efficiently to the desired second enzyme instead of going to a competing enzyme.

Standard methods for generating recombinant DNA constructs are sequence-dependent in that they rely on the use of restriction enzymes to cut DNA into specific fragments, which can then be rejoined in new combinations [9, 11]. But this method is sometime involving the generation and subsequent removal of restriction sites [1]. Horton and colleagues [6] described the technique of splicing by overlap extension (SOE) by PCR. A technique which was not limited by the presence of restriction sites at appropriate locations [11]. Synthetic multienzymes consisting of cellulolytic and xylanolytic enzyme domains have been prepared successfully by fusing the *Cellulomonas fimi* gene *cenA* to both the *C. fimi cex* gene and a *Bacillus subtilis* xyn gene fragment [12]. The genes of an endocellulase and endoxylanase (CelA, XylS) have been isolated from a genomic library of polymer-producing alkali-tolerant *Bacillus licheniformis* NBL420 [5]. Orientation effects as well as existence of linker regions between two different subunits are still challenging area in construction of fusion proteins. This paper describes the construction and characterization of a bifunctional hybrid between a *B. licheniformis* xylanase (*XylS*, Genbank #AF441773) and *B. licheniformis* cellulase (*CelA*, Genbank #AY039744) [5] by SOE method.

2. MATERIALS AND METHODS

2.1 Bacterial Strains, Plasmids, and Growth conditions

Bacillus licheniformis. NBL420 was grown aerobically in a medium at 30℃, as previously described by Lee *et. al.* [7]. The culture medium was comprised of 1.0% (w/v) glucose, 0.5% (w/v) polypeptone, 0.5% (w/v) yeast extract, 0.1% (w/v) KH_2PO_4, 0.02% (w/v) $MgSO_4 \cdot 7H_2O$, and a 1% (w/v) Na_2CO_3, pH 10.0 buffered basal medium (pH 10.0). *E. coli* DH5α was used as the host strain for the plasmid maintenance and gene cloning. Ampicillin (Sigma) was added to the media at a final concentration of 50 ㎍/㎖. Plasmid pBluescriptⅡKS(-) (Stratagene), pUC18 (Gibco BRL) and pET22b (Novagen) were used for the cloning, subcloning and heterologous expressions vector.

2.2 Cloning of CelA and XylS genes

Genomic library of *B. licheniformis* NBL420 were constructed by partial Sau3AI digestion and its ligation into alkaline phosphatase-treated BamHI site of pUC18. From extensive activity screening, two positive clones were selected and they showed cellulase and xylanase activity, respectively. Both *CelA* and *XylS* genes were confirmed by DNA sequencing and deposited into Genbank database with an accession numbers of AY039744 and AF441773, respectively. A 1.3 kb of cellulase gene (CelA) and a 0.8 kb of xylanase gene (XylS) were PCR amplified from subcloned plasmids and used for fusion experiments.

2.3 Construction of Fusion Gene

Two fusion genes, *Xyl/Cel* and *Cel/Xyl,* were constructed by a modification of the Splicing by overlap extension (SOE) by PCR (Fig. 1.). In first stage PCR, XylS gene and CelA gene were amplified by specific four pairs of primers, Xyl22F (*BamH*I)-XylRCel, XylFCel-Cel22R (*Xho*I), Cel22F (*BamH*I)-CelRXyl and CelFXyl-Xyl22R (*Xho*I) (Table 1.) with *B. licheniformis* NBL420 genomic DNA as the template. The two DNA fragments were purified by QIAquick® PCR purification kit (QIAGEN, Germany). Second stage PCR was performed to amplify the fusion gene. Two pairs of the flaking primers, Xyl22F (*BamH*I)-Cel22R (*Xho*I) and Cel22F (*BamH*I)-Xyl22R (*Xho*I), were used to amplify fusion genes (*Xyl/Cel* and *Cel/Xyl*) with first stage PCR product as the template. Target genes were purified from 0.8% agarose gels by QIAquick® gel elution kit (Qiagen, Germany) and directly ligated into pGEM® T easy vector system I (Promega, USA). The ligated DNA was

used to transform the *E.coli*. DH5α.

2.4 Subcloning

The resulting recombinant plasmids which carry two hybrid genes were analyzed by

Table 1. Primers used in this study

Primer	Sequence
For *Xyl/Cel*	
Xyl22F(*BamH* I)	5'-CGGGATCCGGCTAGTCCAGACTACT-3'
XylRcel	5'-GGGTGTTTGAGAAGCCCACACTGTTACGTTAGA-3'
XylFcel	5'-CTAACGTAACAGTGTGGGCTTCTCAAACACCCGTT-3'
Cel22R(*Xho* I)	5'-CCGCTCGAGATTGGACGAATTTGT-3'
For *Cel/Xyl*	
Cel22F(*BamH* I)	5'-CGGGATCCGATGTCATACATGAAA-3'
CelRXyl	5'-CCAGTAGTCTGGACTAGCATTGGACGAATTTGTGCGT-3'
CelFXyl	5'-CGCACAAATTCGTCCAATGCTAGTCCAGACTACT -3'
Xyl22R(*Xho* I)	5'-CCGCTCGAGCCACACTGTTACGT-3'

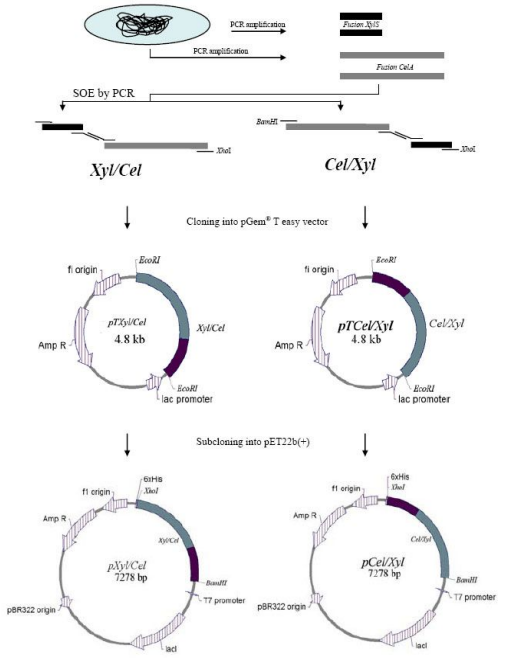


Fig. 1. Overview of the bifunctional hybrid protein construction

restriction analysis and DNA sequencing. The positive clons, pT-Xyl/Cel and pT-Cel/Xyl, were digested with *Bam*HI and *Xho*I. A 1.7 kb fusion gene fragment were eluted, and ligated into pET22b(+) plasmid (Novagen, Germany), previously treated with same restriction enzymes. *E.coli* BL21(DE3) transformed with ligation mixture was plated on LB agar ampicillin (50 ㎍/㎖) plates. Recombinant clones (pXyl/Cel and pCel/Xyl) were tested on a LB agar ampicillin (50 ㎍/㎖) containing 0.1% (w/v) CMC for cellulase activity or LB agar ampicillin (50 ㎍/㎖) containing 0.1%(w/v) RBB dyed xylan plate for xylanase activity. After incubation at 37℃ for 12 hr, production of cellulase activity was detected by congo red dye staining method. Xylanase activity was detected by the appearance of a clearing zone around active colonies. For T7 promoter induction, 4㎕ of IPTG (200 ㎎/㎖) was previously spread on the plates before cell plating.

2.5 Hybrid Enzyme Purification

To purify hybrid protein (Xyl/Cel), *E. coli* BL21(DE3) harboring pXylCel was grown in 50 ml LB containing 50㎍/㎖ ampicillin to an absorbance of approximately 0.6 at 600 nm. The culture was then adjusted to 0.4 mM IPTG, and incubation continued at 30°C for 8 hours. Cells were harvested by centrifugation at 15,000 rpm for 5 min, resuspended in 20 mM sodium phosphate buffer (pH 7.0) containing 1 mM PMSF (phenyl-methanesulphonyl fluoride or phenyl-methylsulphonyl fluoride). After sonication, the sonicated cells were then centrifuged at 15,000 rpm for 10 min, and the supernatant was used to purify the protein. CM sepharose CL-6B (Sigma, USA) was equilibrated with a same buffer and packed in the column. The sample was then applied to the column. The column was washed several times with 20 ㎖ of the same buffer. The protein was eluted with a gradient of NaCl (0~1 M) in the same buffer. The fractions exhibiting cellulase activity were analyzed on a SDS-PAGE gel and used for the enzymatic characterizations.

2.6 Enzymatic Characterizations of Recombinant Proteins

The CMC hydrolyzing activity of recombinant proteins were determined by measuring the amount of reducing sugar ends in the reaction

mixture. The reaction mixture consisted of 1.0 % CMC in 100 ㎕ of a 20 mM sodium phosphate buffer (pH 7.0) and 10 ㎕ of the enzyme solution. The reaction was stopped after 30 min of incubation at 55℃ by boiling for 5 min, then 1 ㎖ of a DNS reagent (0.8 M NaOH, 4 mM dinitrosalisylic acid, and 1.5 M potassium sodium tartrate) was added. After boiling for 10 min, the absorbance was measured at 570 nm using a Shimadzu UV mini-1240 spectrophotometer. The enzyme activity was obtained from a calibration curve prepared following the same procedure with D-glucose as the standard. One unit (U) of enzymatic activity was defined as the amount of enzyme that produced 1.0 ㎛㏖ of reducing sugar as glucose per minute under the reaction conditions.

The xylanase activity of recombinant proteins were determined by measuring the amount of released reducing sugar using the dinitrosalicylic acid method. The reaction mixtures containing 100 ㎕ 1%(w/v) of soluble xylan in 20 mM sodium phosphate buffer, pH6.0, and 10㎕ enzyme solution, were incubated at 50℃ for 20 min. One unit of the enzyme activity was defined as the amount of enzyme that produced 1 ㎛㏖ of reducing sugar per min. The activity was measured by incubating the reaction mixture at different pHs and temperatures for 30 min, as described in the enzyme assay. To measure the pH stability and thermostability, the enzyme was incubated at room temperature for 30 min. in different buffers. The residual activity was then measured, as described in the enzyme assay. The buffer solutions used were a 20 mM citric acid-Na_2HPO_4 buffer (pH 5~7), 20 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 7~8) and 20 mM Glycine-NaOH buffer (pH 8~10).

3. RESULTS AND DISCUSSION

3.1 Construction of Fusion Gene and Purification

The design of the hybrid gene is shown in Fig. 1. A 639 bp of *XylS* DNA fragment and 1321bp of CelA DNA fragment were engineered by construction of fusion gene. For Xyl/Cel fusion gene construction, fusion-XylS (558bp) and fusion-CelA (1227bp) were prepared by amplification of the mature *XylS* and *CelA* protein coding sequence from *B. licheniformis* NBL420 chromosomal DNA using the primers Xyl22F (*BamH* Ⅰ)-XylRCel and XylFCel-Cel22R(*Xho* Ⅰ). The XylRCel primer includes 5' overlapping sequence of fusion-*CelA*, and did not encoding stop codon of *XylS*. For Cel/Xyl fusion gene construction, fusion-*CelA* and fusion-*XylS* were prepared by the similar method described as before. The CelRXyl primer includes 5' overlapping sequence of fusion-*XylS*, and did not encoding stop codon of CelA. In second stage PCR, fusion-*CelA* and fusion-*XylS* were mixed in the ratio of 1:1(100 ng each DNA fragment). The product of the PCR and the SOE reaction described above are shown on an Et-Br stained agarose gel in fig. 2. Fig. 2A represent the fusion-*XylS* and fusion-*CelA*. Fig. 2B represent the SOE reaction product.(0.5 kb, 1.2 kb and 1.7 kb) The target product, Xyl/Cel and Cel/Xyl(1.7kb) were eluted from the agarose gel, ligated into pGEM® T easy vector system. The recombinant plasmids were analyzed by cutting the *EcoR* Ⅰ (Fig. 2C) and DNA sequencing.

3.2 Bifunctional Behavior of Hybrid Protein

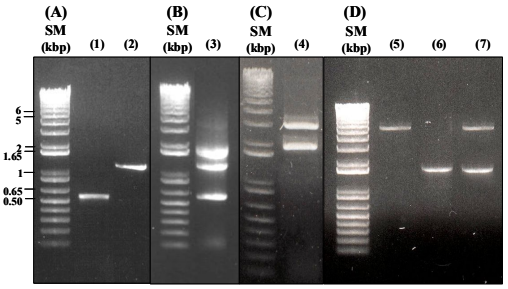


Fig. 2. Agarose gel electrophoresis pattern of hybrid Xyl/Cel DNA. (A) Lane 1: PCR product of fusion CelA (558bp), Lane 2: PCR product of fusion XylS (1227bp), (B) Lane 3: SOE by PCR Products (0.5 kb,1.2 kb, and 1.7 kb), Lane 4: *EcoR*Ⅰ-digested pTXylCel, Lane 5: *Xho* Ⅰ, *BamH*Ⅰ-digested pET22b (5493bp), Lane 6: *Xho* Ⅰ and *BamH*Ⅰ-digested Xyl/Cel (1785bp), Lane 7: *Xho* Ⅰ and *BamH*Ⅰ-digested pXyl/Cel (5493bp and 1785bp)

The *BamH* Ⅰ and *Xho* Ⅰ restricted *Xyl/Cel*

and *CelXyl* gene fragment were subcloned into pET22b(+) vector. (pXyl/Cel and pCel/Xyl) (Fig. 2D) The Hybrid enzyme Xyl/Cel and Cel/Xyl were expressed by IPTG with poly-histidin (6xHis) tail under the control of T7 promoter. The hybrid proteins were active against CMC. But CelXyl did not show activity against RBB dyed Xylan and soluble xylan (Fig. 3.).

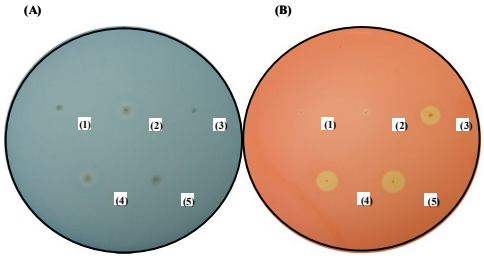


Fig. 3. Detection of bifunctional activities of *E.coli* BL21(DE3) containing hybrid gene (pXyl/Cel and pCel/Xyl) and recombinant *E. coli* clones. Activities were monitored by the appearance of a clearing zone of LB ampicillin plates containing RBB dyed xylan (A) and by Congo-red staining of LB ampicillin plates containing CMC (B). (1) *E. coli* DH5α containing as a negative control ; (2) *E. coli* DH5α containing pXylS ; (3) *E. coli* BL21(DE3) containing pCelA ; (4) *E. coli* BL21(DE3) containing pXyl/Cel ; (5) *E. coli* BL21(DE3) containing pCel/Xyl

Furthermore, activity staining analysis goes to show that, CelXyl was expressed with incorrect molecular size (data not shown). Therefore, active bifunctional xylanase-cellulase fusion protein, Xyl/Cel was further studied. On the contrary, Cel/Xyl was expressed with correct molecular size (Fig. 4)

Purification of a hybrid protein (Xyl/Cel) with Ni-NTA column were attempted, but it failed. Alternatively, Xyl/Cel was purified by cation exchanger chromatography. Purification to homogeneity was confirmed by SDS PAGE analysis. The bifunctional characteristics of cellulase and xylanase in hybrid protein were further confirmed by activity staining analysis (Fig. 5). The molecular mass of purified hybrid protein was estimated as 65.8 kDa by SDS PAGE which was identical to calculated size.

3.3 Enzymatic Properties of Xyl/Cel Hybrid Protein on Cellulase Activity

The optimum temperature of the purified enzyme was found to be 40℃ (Fig. 5A), as compared to an optimum of 50℃ for CelA. Thermostability profiling illustrates that the Xyl/Cel is less thermostable than CelA. After 30

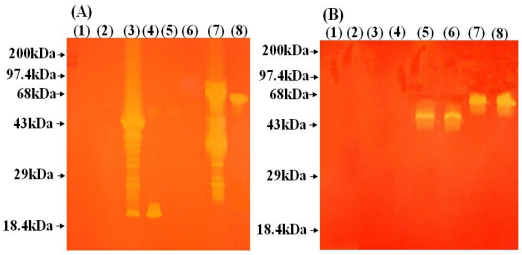


Fig. 4. Detection of bifunctional activities of hybrid enzyme (Xyl/Cel), cloned endocellulase(CelA), and cloned endoxylanase (XylS) by activity staining. (A) 0.1% Soluble xylan+12% SDS PAGE gel, (B) 0.25% CMC+12%SDS PAGE gel, (1) *E. coli* DH5α containing pUC19 as a negative control; (2) 80℃, 5 min-treated lane 1; (3) *E. coli* DH5α containing pXylS; (4) 80℃, 5 min-treated lane 3; (5) *E. coli* DH5α containing pCelA; (6) 80℃, 5 min-treated lane 5; (7) *E. coli* DH5α containing pXyl/Cel and (8) 80℃, 5 min-treated lane 7

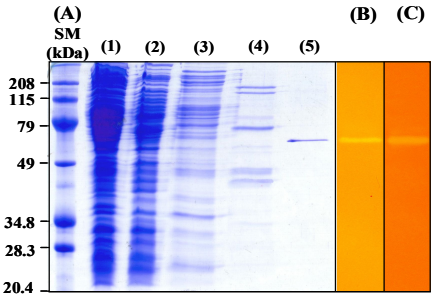


Fig. 5. Purification of Xyl/Cel with CM sepharose CL-6B cation-exchanger chromatography. (A) protein band profile, (1) ; crude extract of *E. coli* BL21(DE3) containing pXyl/Cel, (2); column flow-through, (3) : and (4) ; column washes (5); Purified Xyl/Cel (concentrated)

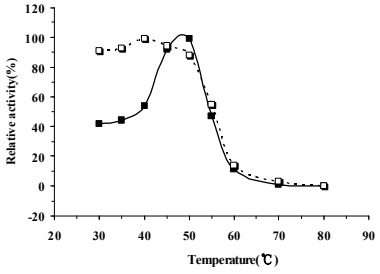
min. at 50℃ the Xyl/Cel remained 7%

remaining activity, while comparative values for the CelA was 95% after its incubation for 30 min. at 50℃ (Fig. 5C). Both enzymes displayed below 10% remaining activity. Optimum enzyme activity of Xyl/Cel was reported at pH 7 which was similar to CelA. This activity was stable (more than 60%) after incubation for 12 hrs at pH 5~8 (Fig. 5B and 5D).

3.4 Enzymatic Properties of Xyl/Cel Hybrid Protein on Xylanase Activity

Xylanase activity produced by Xyl/Cel was detected over a wide range of assay pH(4~10), and the activity of the enzyme was optimal when the assay was carried out at pH 6 (Fig.6B and 6D). During the assay period, the optimal temperature for retention of xylanase activity (80% of remaining activity) from Xyl/Cel was 30℃. At 55℃ for 30 min, the enzyme was completely inactivated (Fig. 6A and 6C).

(A)



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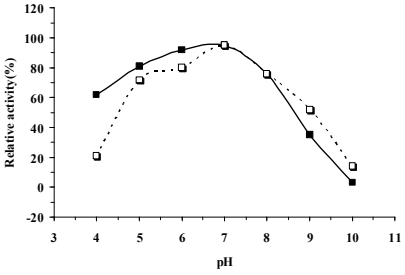

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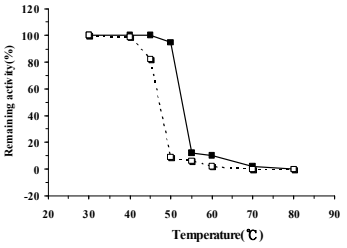


(D)

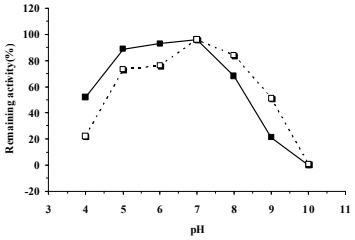
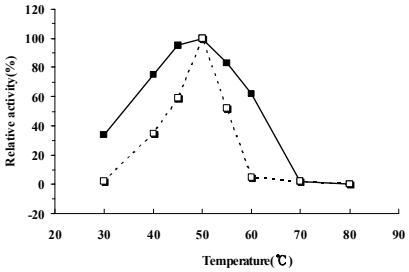


Fig. 6. Comparisons of cellulase activity of XylS and Xyl/Cel ; (A) Temperature, (B) pH, (C) Thermo-stability and (D) pH stability on cellulase activity. (…□… Xyl/Cel, —■— CelA)

(A)



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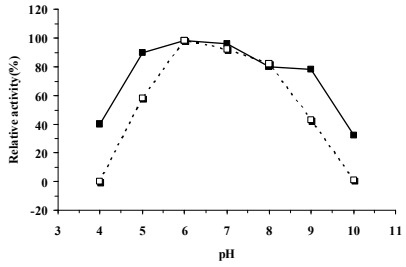
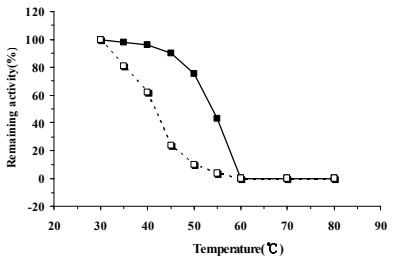


(C)



(D)

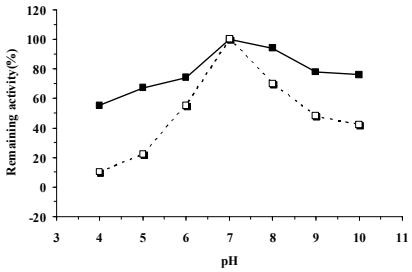


Fig. 7. Comparisons of xylanase activity of XylS and Xyl/Cel ; (A) Temperature, (B) pH, (C) Thermo-stability and (D) pH stability on xylanase activity. (…□… Xyl/Cel, —■— XylS)

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