การคัดแยกเอนไซม์ไซแลนเนสจากกลุ่มเชื้อแบคทีเรียที่ย่อยสลายกากซานอ้อยเพื่อนำไปใช้ประโยชน์ทางด้านเทคโนโลยีชีวภาพ

Identification of A Novel Xylanase Enzyme from A Sugarcane Bagasse-Degrading Microbial Consortium

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บทคัดย่อ

เอนไซม์ไซแลนเนสพบได้ตามธรรมชาติในกลุ่มจุลินทรีย์ที่สามารถย่อยชีวมวลจากพืชได้ เอนไซม์กลุ่มนี้ถูกนำไปใช้ประโยชน์ในอุตสาหกรรมหลายชนิด โดยเฉพาะอย่างยิ่งในกระบวนการการแปรสภาพชีวมวลเป็นเชื้อเพลิงชีวภาพ กลุ่มเชื้อจุลินทรีย์ที่ย่อยสลายประกอบด้วยเชื้อจุลินทรีย์ที่ย่อยและไม่ย่อยลิกโนเซลลูโลสอยู่ร่วมกัน และทำงานร่วมกันเพื่อให้การย่อยลิกโนเซลลูโลสสมบูรณ์ การศึกษานี้ได้ทำการโคลนและแยกยีนไซแลนเนสจากกลุ่มเชื้อจุลินทรีย์ดังกล่าว ด้วยวิธี PCR ผลการศึกษาพบบางส่วนของลำดับนิวคลีโอไทด์ 150 คู่เบส เมื่อเปรียบเทียบกับฐานข้อมูลพบว่ามีลำดับกรดอะมิโนที่คล้ายยีนของเอนไซม์ไซแลนเนสจาก *Clostridium phytofermentans* ISDg และ *Butyrivibrio fibrisolvens* 78% และ 66% ตามลำดับ แล้วนำลำดับเบสจากชิ้นยีนมาทำการโคลนต่อโดยวิธี genome-walking PCR จากการศึกษาพบว่าสามารถโคลนยีนไซแลนเนสทั้งชิ้นได้และมีความยาว 1,587 คู่เบส ซึ่งถอดรหัสให้โปรตีนที่มีความยาว 528 กรดอะมิโน เมื่อเทียบกับฐานข้อมูลพบว่าโปรตีนมีความคล้ายคลึง 77% ในลำดับกรดอะมิโนกับเอนไซม์ไซแลนเนสของ *Clostridium phytofermentans* ISDg ในการศึกษาการแสดงออกของ mature ไซแลนเนสยีนโคลน XYN12 ใน *Escherichia coli* Rosetta-gami พบว่า สามารถผลิตโปรตีนขนาดประมาณ 55 kDa ได้ และเมื่อทดสอบกิจกรรมของโปรตีนดังกล่าวนี้บน AZCL-xylan พบว่า สามารถแสดงคุณสมบัติของเอนไซม์ต่อซับสเตรทที่จำเพาะต่อยีนไซแลนเนสได้

คำสำคัญ : ไซแลนเนส กลุ่มเชื้อจุลินทรีย์ที่ย่อยชีวมวล เมตาจีโนมิก กากซานอ้อย genome-walking, *Clostridium phytofermentans* ISDg

ABSTRACT

Xylanases are found naturally in lignocellulose-degrading microorganisms and are used in various industries, particularly in the biomass conversion process for ethanol production. Degradation of lignocellulosic biomass involves the cooperative action of many microorganisms -- cellulolytic microorganisms work synergistically with noncellulolytic microorganisms as a consortium to enhance

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cellulolytic activity. In this study, we have cloned and identified a xylanase gene using PCR-based approach from sugarcane bagasse-degrading microbial consortium. The obtained partial sequence of 150 bp showed 78% and 66% amino acid sequence identity to xylanases of *Clostridium phytofermentans* ISDg and *Butyrivibrio fibrisolvens*, respectively. Using genome-walking methods, the 5' and 3'-end of the xylannase gene was obtained. The result showed the obtained full-length of 1,587 bp encoding 528 amino acid residues. Sequence analysis of the full-length xylanase exhibited 77% identity to xylanase gene of *Clostridium phytofermentans* ISDg. The gene was successfully expressed in *Escherichia coli* Rosetta-gami as a 55 kDa protein whose xylanase activities was detected on AZCL-xylan agar plate.

Keywords : xylanase, lignocellulose-degrading microorganisms, metagenomics, sugarcane bagasse, genome-walking, *Clostridium phytofermentans* ISDg

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INTRODUCTION

Xylan is the most abundant biopolymer after cellulose and is the major component of hemicelluloses in plant cell wall. It is a heteropolymer with β-1, 4-linked D-xylose backbones with arabinose, 4-O-methyl-D-gluculonic acid, and acetic acid substituents. Xylanases are generally belong to the families 10 and 11 of the glycosyl hydrolases, which randomly cleave the β-1,4-backbone of this complex plant cell wall to xylooligosaccharides and xylose (Collins *et al*., 2002). These enzymes are required in many applications such as prebleaching in the paper and pulp industry (Medeiros *et al*., 2007), reducing viscosity of the intestinal contents in the animal feed industry (Zyla *et al*., 1999) and to break down the hemicelluloses fraction of biomass in ethanol production (Burchhardt *et al*., 1992). However, current xylanases employed in the various industries are from culturable microorganisms, which represent less than 1% of microorganisms existed in the environment. To access and exploit the xylanases from the remaining microorganisms, metagenomic approach is used to recover novel xylanase from environmental sample. One such environment where xylanase must be produced is from sugarcane bagasse compose. Indeed, previously we have constructed a stable sugarcane bagasse-degrading microbial consortium which was originally obtained from prolonged subcultivation of microflora originated from sugarcane bagasse compose. This microbial consortium is used as a source for discovery of lignocelluloses-degrading enzyme – especially xylanase.

MATERIALS AND METHODS

1. Genomic DNA extraction and partial amplification of xylanase gene

Total DNA was extracted from the stable sugarcane bagasse microbial consortium by benzyl chloride method with SDS and DNA extraction buffer (Zhu *et al*., 1993). The DNA was purified by β-

agarase and then used as template in PCR reaction using DyNAzyme II polymerase (Finnzymes, USA) and xylanase degenerate primers, XYNFR and XylF23 (Table 1) in a total volume of 50 μl. PCR was performed for 35 cycle of denaturation at 94°C for 30 sec, annealing at 46°C for 1 min and extension at 72°C for 1 min. The PCR product was electrophoresed on 0.8% agarose gel and the expected size was gel-purified and cloned into pGEM-T Easy vector and then transformed into *E. coli* DH5α. The plasmid was extracted and sequenced.

2. **Amplification of 5' and 3' ends of xylanase (XYN12) gene**

To obtain the full-length xylanase gene, the genome-walking method (Clontech) was employed. Briefly, the genomic DNA (gDNA) was digested with *Eco*RV and purified using Wizard DNA Clean-Up system (Promega, USA). The digested gDNA was then ligated to GenomeWalker Adaptor to construct the GenomeWalker library. The GenomeWalker library was used as template in nested-PCR reaction in a total volume of 50 μl using 50x Advantage 2 polymerase mix. For the 5' end, the primary PCR was performed using the outer adaptor primer 1 (AP1) and an outer, gene-specific primer (xyn1_up1). 1 μl of 1:50 dilution of primary PCR mixture was used as template for a secondary PCR with a nested adaptor primer (AP2) and a gene-specific primer (xyn1_up2). For 3'end, the primary PCR was performed using the outer adaptor primer 1 (AP1) and an outer, gene-specific primer (xyn1_Dn1). The second PCR was performed using adaptor primer 2 (AP2) and nested gene-specific primer (xyn1_Dn2) and 1 μl of 1:50 dilution of primary PCR products as template. The PCR product was then analyzed on 0.8% agarose gel, purified, subcloned into pGEM-T Easy vector and sequenced.

3. **Amplification of a full-length xylanase (XYN12) gene**

To verify that the obtained xylanase gene is correct, nested-PCR was performed to obtain the full-length gene. Briefly, gDNA was used as template in nested-PCR using XYNF10_F1 and XYNF10_R1 primers in the first PCR and pETXYNF10_F2 and pETXYNF10_R2 primers in secondary PCR with 1X Mg^{2+}-free DyNazyme EXT buffer, 2 mM $MgCl_2$, 5% DMSO, and DyNazyme EXT polymerase in a total volume of 50 μl. PCR was performed for 35 cycle of denaturation at 94°C for 1 min, annealing at 53°C for 1 min and extension at 72°C for 2 min; followed by a final extension at 72°C for 15 min. The PCR product was then analyzed on 0.8% agarose gel, purified, subcloned and sequenced.

4. **Construction of XYN12 in *E.coli* expression vector**

In order to express the xylanase gene in *E. coli* system, nested PCR was performed as mentioned in method #3 except that the second PCR was performed using pETXYNF10_F3 and pETXYNF10_R2 primers. The mature genes were constructed in an *E. coli* expression vector, pET-28a, using *Nde*I and *BamH*I sites. Transformation was performed using *E. coli* Rosetta-gami as an expression host. The presence of recombinant plasmid harboring XYN12 full-length gene was analyzed by colony PCR and double digestions (*Nde*I and *BamH*I).

5. **Expression of recombinant XYN12 in *E. coli***

Single colony of *E. coli* transformant harboring XYN12 gene was inoculated into LB broth containing kanamycin and chloramphenicol and cultivated with aeration at 30°C. When $O.D._{6000}$ reached 0.6, cell culture was induced with 0.5 mM IPTG for 3 h. The culture was placed on ice for 5 min and then harvested. Cells were lysed by using Enhance lysis buffer (BIOTEC). The cell lysate was centrifuged to separate soluble and pellet fractions. All fractions were analyzed by SDS-PAGE to determine the presence of the target protein.

6. **Xylanase activity assay using AZCL-xylan agar plate**

AZCL-xylan agar (0.05% AZCL-xylan, 0.7% bacteriology agar, 0.1M phosphate buffer pH 5.5) was poured into Petri dish. After the agar is solidified, small wells were made using sterile 5 mm diameter straw. Then 20 µl of the recombinant cell supernatant was applied into each well. The plate was incubated at 37°C for 3 h. Xylanase activity was detected by blue zone surrounding the well.

Table 1 Xylanase degenerate primers used in this study

Primer names	Sequence 5′⟶3′	T_m(°C)
XylFR	T(AC)GTT(GT)AC(AC)AC(AG)TCCCA	44
XYNF23	MGNGGICAYACNYTIGTITGGCA	55
xyn1_up1	GACTGACTTACATAGGCACCATTTGAAC	57.5
xyn_up2	TGTCCTAAAGAACCAGTCAGGTGTCTGTGC	64.3
xyn_Dn1	GTCAGTCTGTAATGGATGCCAGAATGG	60.7
xyn_Dn2	TTTAGGTAACTACGGCAGTGTGGTTTATGC	61.5
XYNF10_F1	CATAAATCGGCAAAAAAGCAGG	56.5
XYNF10_R1	CTGGTATCAGACACAGCAGAAAAC	61.0
pETXYNF10_F2	GGCATATGCTACATAAGAGGGTTGGCAAACA	66.8
pETXYNF10_F3	GGCATATGGCTACCGAAAAGTACTATAACTTTAATAATC	66.3
pETXYNF10_R2	GCGGATCCTTACTGTATCGTATAACCAGCATCTG	69.5

Note: Abbreviations used, N= A, T, G, C; M= A, C; K= G, T; Y= C, T; R= A, G; W= A, T; S= C, G; H= A, C, T; D= A, G, T; V=A, C, G; I= Inosine.

RESULTS AND DISCUSSION

1. **Identification of a partial xylanase gene from purified sugarcane bagasse microbial consortium gDNA**

The size of the purified gDNA was larger than 23 Kb and its quality was sufficient for the downstream steps (Fig. 1A). To obtain the partial xylanase gene from such microbial consortium, PCR was performed using degenerate primers, XYNFR and XylF23. An amplicon of 150 bp which was the expected size was obtained (Fig. 1B). This band was purified and cloned. Five recombinant clones

were randomly chosen for sequencing. The results showed that all 5 clones contained the same sequence to one another. This is unexpected that only a single xylanase gene was found. This may be because we have only sequence a small fraction of transformants (5 out of 44). BlastX analysis showed 78% and 66% amino acid identity to xylanase genes of *Clostridium phytofermentans* ISDg and *Butyrivibrio fibrisolvens*, respectively (data not shown).

2. Amplification of 5' and 3' ends of xylanase (XYN12) gene using genome walking method

To retrieve the 5' and 3' ends of xylanase gene, the genome-walking method was employed. For the 5' end of this xylanase gene, a prominent PCR product of approximately 1.3 kb was obtained (Fig. 2A). This band was extracted, purified, subcloned and sequenced. Sequence analysis showed 73% amino acid identity to *C. phytofermentans* xylanase. For the 3'end, the PCR product of approximately 750 bp was obtained (Fig. 2B). Sequence analysis showed 84% amino acid identity to xylanase gene of *C. phytofermentans*. Moreover, a stop codón (TAA) was found in the sequence. Finally, the 3 overlapping xylanase fragments obtained were assembled *in silico* using vector NTI program.

3. Amplification of a full-length and mature xylanase (XYN12) gene

After the 5' and 3' end of XYNE12 sequence were identified. Nested PCR was performed to obtain the full-length gene of XYNE12. The result showed an amplicon of approximately 1,600 bp (Fig. 3A). This fragment was purified and cloned into pGEM-T easy vector. Sequence analysis showed that the complete xylanase gene (1587 bp) from the sugarcane bagasse-degrading microbial consortium encoding xylanase protein of 528 amino acid residues with a calculated mass of 59 kDa (data not shown) and showed 77% identity to xylanase gene of *Clostridium phytofermentans*.

4. Construction of XYN12 in *E.coli* expression vector

To express the XYN12 in *E. coli*, the mature xylanase gene was used instead of the full-length gene. The mature xylanase gene was obtained using nested PCR. The result showed a PCR product of approximately 1,500 bp (Fig. 3B). This fragment was purified and first cloned into pGEM-T Easy vector and then subcloned into pET-28a using *Nde*I and *BamH*I sites. Recombinant clones were randomly selected for sequencing. Sequence analysis revealed that the target fragment was inserted at the expected site. Moreover, no mutation was observed in the sequence of the mature XYN12. This recombinant plasmid was designated as XYNF10_E12.

5. Expression of recombinant XYN12 in *E. coli*

E. coli transformant harboring XYNF10_E12 was inoculated into LB broth. After IPTG induction, cells were harvested and lysed. The cell lysate was centrifuged to separate soluble and cell pellet fractions and analyzed by 12% SDS-PAGE. The expected protein of 55 kDa was detected in both the soluble and pellet fractions (Fig. 4).

6. Xylanase activity assay using AZCL-xylan

To determine xylanase activity, both soluble and pellet fractions were applied into each well of AZCL-xylan agar plate. The result showed that blue zone was developed from both fractions (Fig. 5).

The soluble fraction showed higher xylanase activity as it has larger blue zone. The blue zone was detected on pellet fraction may be because the soluble protein still remained. The results suggested that a xylanase gene was successfully obtained from sugarcane bagasse-degrading microbial consortium and functionally expressed in *E. coli*.

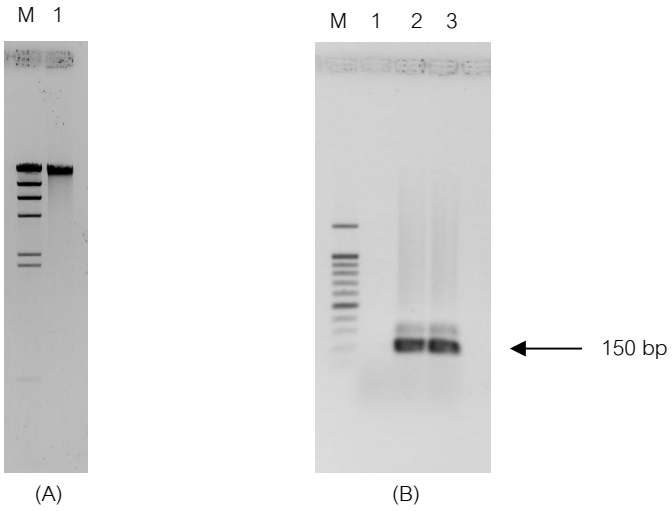

Figure 1 PCR amplification of a partial xylanase gene from sugarcane bagasse microbial consortium

(A) gDNA from sugarcane bagasse microbial consortium was analyzed on 0.8% agarose gel. Lane M: Lamda DNA/*Hin*dIII marker, lane 1: purified DNA. (B) PCR amplification of partial xylanase gene using XYNFR and XylF23 primers. Arrow indicates the size of the expected PCR product of approximately 150 bp. PCR product was analyzed on 0.8% agarose gel. Lane M: 100 bp ladder with 1.5 kb plus marker. Lane 1: Negative control (water). Lanes 2-3: The amplified PCR product using XYNFR and XylF23 primers.

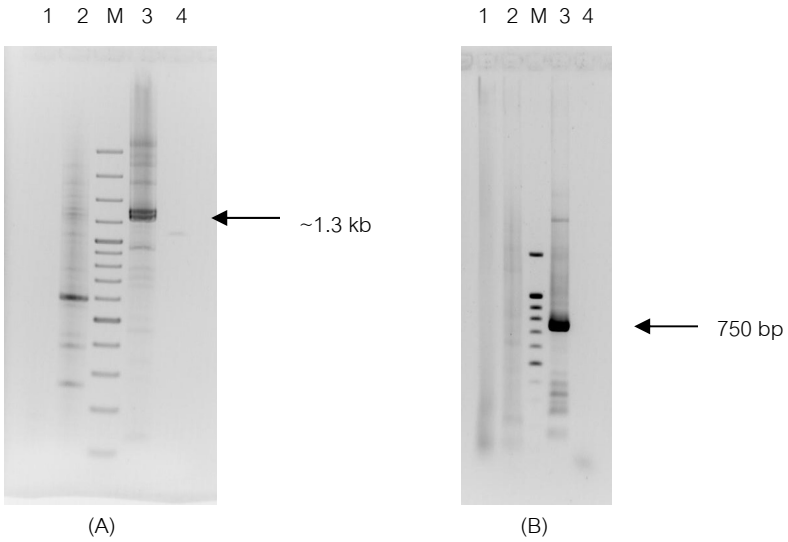

Figure 2 Genome-walking of xylanase gene

(A) 0.8% agarose gel electrophoresis of the 5' end xylanase gene using genome walking method. Lane M is Gene Ruler 100 bp ladder plus marker (B) 0.8% agarose gel electrophoresis of the 3' end xylanase gene using genome walking method. Lane M is 100 bp ladder plus 1.5 kb marker. Lane 1 and lane 4 are negative control of primary and secondary PCR. Lanes 2 and 3 are PCR product of the primary and secondary PCR, respectively (arrow indicated the size of the expected PCR product).

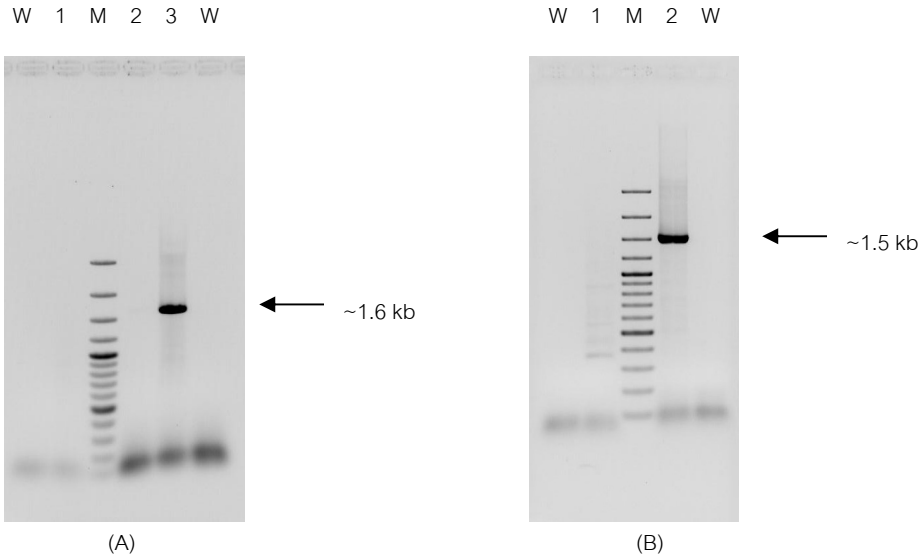

Figure 3 0.8% agarose gel electrophoresis of full-length and mature xylanase gene

Full-length and mature xylanase gene was amplified from the gDNA of sugarcane bagasse-degrading microbial consortium using nested PCR and gene-specific primers. (A) Lanes 2 and 3 are secondary PCR products using pETXYNF10_F2 and pETXYNF10_R2 primers. 1U DyNazyme EXT polymerase was used in lane 2, whereas, 2U was used in lane 3. (B) Lanes 2 is PCR product using pETXYNF10_F3 and pETXYNF10_R2 primers and 1U DyNazyme EXT polymerase. Lanes W are negative control (water). Lane 1 is the primary PCR product using XYNF10_F1 and XYNF10_R1 primers. Lane M is Gene Ruler 100 bp ladder plus marker (arrow indicates the expected size of the PCR product

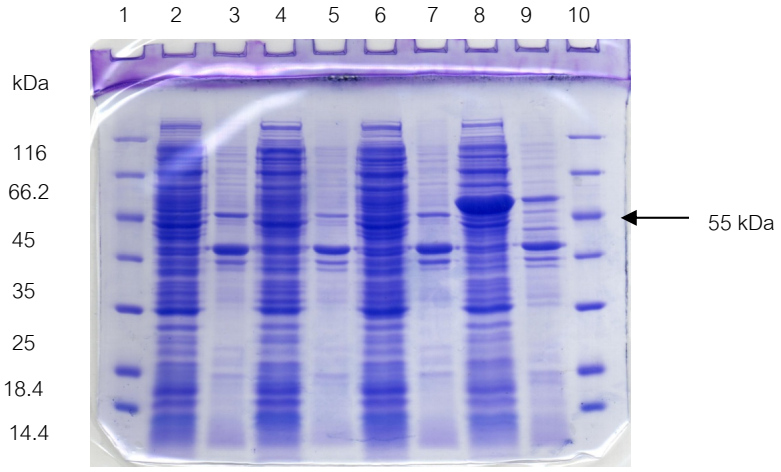

Figure 4 SDS-PAGE analysis of XYN12 expression in *E. coli*

XYN12 was expressed in *E. coli*. The soluble and cell pellet fractions were analyzed on 12% SDS-PAGE. Lanes 1 & 10: protein molecular weight marker (Fermentas). Lanes 2 & 3: soluble and pellet fractions from uninduced *E. coli* containing pET-28a. Lanes 4 & 5: soluble and pellet fractions from IPTG-induced *E. coli* containing pET-28a. Lanes 6 & 7: soluble and pellet fractions from uninduced *E. coli* containing XYNF10_E12. Lanes 8 & 9: soluble and pellet fractions from IPTG-induced *E. coli* containing XYNF10_E12.

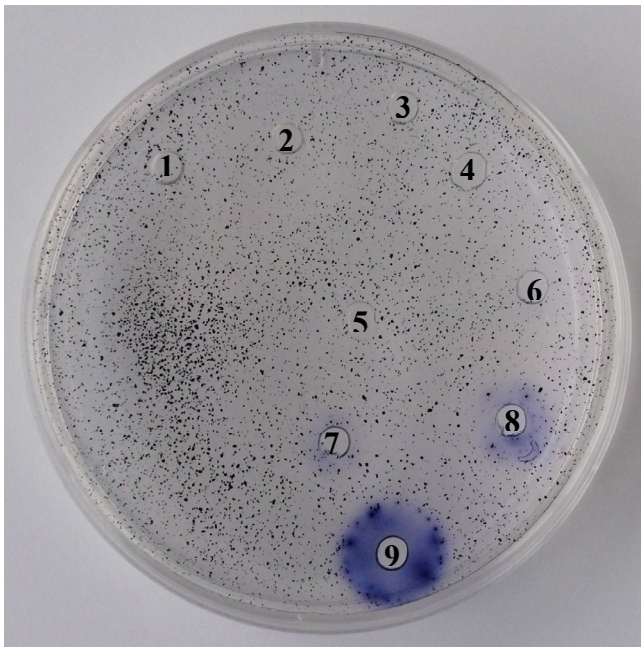

Figure 5 Xylanase activity analysis by AZCL-xylan

XYN12 was expressed in *E.coli* using pET-28a. A 20 μl of soluble and pellet fractions were used to assay for xylanase activity on AZCL-xylan plate. Wells 1, 2: the pellet and soluble fractions of uninduced *E. coli* containing pET-28a, respectively. Wells 3, 4: the pellet and soluble fractions of IPTG-induced *E. coli* containing pET-28a, respectively. Wells 5, 6: the pellet and soluble fractions of uninduced *E. coli* containing XYNF10_E12, respectively. Wells 7, 8: the pellet and soluble fractions of IPTG-induced *E. coli* containing XYNF10_E12, respectively. Well 9: positive xylanase control (Fluka Biochemika, Switzerland)

CONCLUSION

In this study, xylanase gene was recovered from sugarcane bagasse-degrading microbial consortium. A full-length xylanase gene (XYN12) from the sugarcane bagasse-degrading microbial consortium is 1,587 bp encoding 528 amino acids with a calculated mass of 59 kDa. The mature xylanase gene containing 1,485 bp open reading frame encoding 494 amino acid residues with a calculated mass of 55 kDa was successfully expressed in *E.coli*.

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