XYLANASE GENE FROM A LOCALLY ISOLATED BACTERIUM

HUSSAIN, M.H.*, CHONG, N.F.M., CHAN, C.S.W., SAFARINA, A. and HUSAINI, A.

Proteomics Laboratory, Department of Molecular Biology, Faculty of Resource Science and Technology, University Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia
*E-mail: hhasnain@frst.unimas.my

ABSTRACT

A xylanolytic bacterium was isolated from sago plantation humus. Results from the morphological observation, biochemical tests and 16s rRNA sequencing suggested the bacterium to be *Klebsiella pneumoniae*. Due to the xylanolytic activity of this bacterium, isolation and characterization of the xylanase gene were attempted. A distinct fragment of about 650 bp was successfully amplified using PCR and cloned into *Escherichia coli* XL-1 Blue. A BLAST search confirmed that the DNA sequence from the amplified fragment was endo-1,4-beta-xylanase gene from the family 11 glycoside hydrolase. It showed 98% homology with *Bacillus subtilis* xylanase gene. In silico characterisation showed an open reading frame encoding a 213 amino acid sequence with a molecular weight of 23.3 kDa and theoretical isoelectric point (pI) at pH 9.42.

Key words: xylanase; sago; *Klebsiella pneumoniae*; humus

INTRODUCTION

Xylan is the second most abundant biopolymer in the world after cellulose. It is the major component of hemicellulose which is found abundantly in plant cell walls (Khanderparkar & Bhosle, 2006) and can consist up to 35% dry weight of higher plants (Silva et al., 1999). Xylan consists of a β-1,4-linked D-xylose backbone substituted to varying degrees with O-acetyl, α-L-arabinofuranosyl, 4-O-methylglucuronic acid groups or α-1,2-linked gluconic acids (Singh et al., 2003). Xylan is abundantly found in agro-industrial waste and in Malaysia, it can be found in sago pith waste, oil palm waste, paddy husks, and sugarcane bagasse. Degradation of xylan can be achieved by xylanolytic enzymes such as xylanase.

Using xylanases, xylan can be degraded to xylose, which can be fermented by bacteria and yeasts into ethanol or organic acids. Therefore, bioconversion of agricultural waste not only reduces pollution but also offers a renewable source of energy. Other potential uses of xylanases are in biobleaching (Morris et al., 1998; Viikari et al., 1994), production of oligosaccharides (Khandeparker & Numan, 2008), bakery industry (Romanowska et al., 2006), and in animal feed and fruit juice production (Beg et al., 2001). Thus, the aim of this work was to isolate and characterize the xylanase gene from locally isolated bacteria.

MATERIALS AND METHODS

Isolation of Xylanolytic Bacteria

Humus samples from a sago plantation in Mukah, Sarawak were inoculated into enriched broth...
sequences from different species. Primers for xylanase gene PCR were designed based on the endo-1,4-beta-xylanase gene sequences from different Bacillus species. DNA sequences of xylanases were obtained from GenBank (www.ncbi.nlm.nih.gov) and were aligned using ClustalW (Larkin et al., 1990), and incubated at 37°C for 24-48 hours. Qualitative screening was done by staining using Congo red dye (1%) for 30 minutes, and destaining with 1 M NaCl (Yang et al., 1995). The presence of halos indicated xylanolytic activity.

**Bacteria Identification**

The bacterium was identified using Gram staining, colony morphology observation, biochemical tests and 16s rRNA sequencing.

**Primers for xylanase gene PCR**

Primers to amplify the xylanase gene were designed based on the endo-1,4-beta-xylanase gene sequences from different Bacillus species. DNA sequences of xylanases were obtained from GenBank and were aligned using ClustalW (Larkin et al., 2007) and CLC sequence viewer (www.clcbio.com). Conserved regions were detected. The forward primer (5’-ATGGTTAAGTAAAAAGAATTTC-3’) and reverse primer (5’-TACACACTGTACGTTAG-3’) were then selected from these areas of high conservation and was predicted to amplify the full 642 bp endo-1,4-beta-xylanase gene.

**Xylanase Gene Isolation**

PCR was performed using genomic DNA isolated from the bacteria as a template as follows: 5 min at 95°C, followed by 35 cycles of 60 s at 95°C, 60 s at 42°C, 60 s at 72°C, and a final extension for 5 min at 72°C.

**Cloning and Transformation**

The PCR product was cloned into pGEM-T easy vector and transformed into E. coli XL-1 blue using the heatshock method. Successful transformants were selected using blue/white screening. Plasmids were extracted using a plasmid extraction kit (Vivantis), verified by restriction digest, and sent for sequencing.

**Crude Enzyme Activity**

Cultures were incubated in minimal medium at 37°C for 24 hours, and centrifuged at 9000 X g for 5 minutes at 25°C. The supernatant was used as crude enzyme. Xylanase assay was performed according to Miller (1959). The reaction mixture contained 0.5 mL of 2% xylan suspension in 0.1 M acetate buffer at pH 6.0 together with 0.5 mL of crude enzyme. The mixture was incubated at 55°C for 30 minutes. After 30 minutes, the mixture was left to cool and centrifuged, and 0.5 mL of supernatant was added to 1 mL of dinitrosalicylic acid (DNS) solution and boiled. After boiling for 10 minutes, the mixture was left to cool and the absorbance at 530 nm was read. The assay was carried out at 4 different incubation times: 6 hours, 24 hours, 30 hours and 42 hours to determine the time for the highest production of enzyme. One unit of xylanase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar per min under the assay condition.

**In Silico Characterization**

Molecular weight and isoelectric point (pI) was predicted using ExPASy ProtParam tool (http://www.expasy.ch/tools/protparam.html; Gasteiger et al., 2005) Signal peptide was predicted using SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/; Bendtsen et al., 2004). Catalytic domain was predicted using MyHits motif scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan; Pagni et al., 2007). Tertiary structure was predicted using Swiss-Model (http://swissmodel.expasy.org; Guex et al., 1997; Schwede et al., 2003; Arnold et al., 2006) and molecular modelling was displayed using Rasmol (http://rasmol.org; Sayle & Milner, 1995; Bernstein, 2000).

**RESULTS AND DISCUSSION**

**Qualitative Screening**

Out of 53 isolates screened, only one was found to have xylanolytic activity. Staining of the xylan agar plate with Congo red showed formation of halo zones indicating xylanolytic activity of the isolate.

**Bacterium Identification**

The bacterium was identified as Gram negative rods, with milky, circular, convex colonies. The bacterium was non-motile, tested negative for H2S production, positive for Voges-Proskauer test and positive for citrate utilization test as shown in Table 1. Morphological and biochemical tests match that of genus Klebsiella (Brisse et al., 2006). BLAST performed on the sequenced 16s rRNA gene showed that the closest match was K.pneumoniae 342 (Accession no: CP000964) with 85% homology. Results from the morphological, biochemical and 16s rRNA sequencing suggest the bacteria to be K.pneumoniae. K.pneumoniae is commonly known as a pathogen but is also found in soil (Brisse et al., 2006).

Analysis using data from NCBI database showed that the available number of deposited xylanase sequence from all types bacteria species was more than 8500 (as of February 2011) while search on Klebsiella containing xylanase returned with only 8 sequences, all of which are hypothetical xylanase.
This showed the very low prevalence of xylanolytic gene in *Klebsiella*. When compared to other bacteria species containing xylanase gene, *Bacillus* showed a total of 1600 sequence deposited in the database.

**Xylanase Gene Isolation**

The target xylanase was a commonly occurring endo-1,4-beta-xylanase gene found in *Bacillus* spp. The genus *Bacillus* was chosen to compare with the locally isolated bacterium due to higher prevalence of xylanase in this group of bacteria. In addition, the locally isolated bacterium was yet to be identified prior to the attempt to isolate the xylanase gene. Amplification of the gene using PCR produced a bright fragment of about 650 bp, which matched the expected size of 642 bp of xylanase gene from *Bacillus* (Fig. 1A).

**Sequencing Results**

The cloned 650 bp sequence was successfully sequenced. The full sequence was shown to be exactly 642 bp, as predicted. The full sequence with primer annealing sites is shown in Fig. 1B.

BLAST search (megablast) for highly similar sequences confirmed that the 642 bp sequence was the xylanase gene, with all 44 BLAST hits related to xylanase, with identities ranging from 77% to 98%. The closest match with 98% identities were *Bacillus subtilis* Xyl gene for xylanase (Accession number AB457186.1), *Bacillus subtilis* subsp. *subtilis* str. 168 complete genome (Accession number AL009126.3), *Bacillus subtilis* MW10 endo-1,4-beta-xylanase (Accession number DQ100307.1), *Bacillus subtilis* chromosome region between terC and odhAB (Accession number AF027868.1), *B. subtilis* xylanase gene (Accession number M36648.1), *Bacillus subtilis* 168 trpC2 xyna gene encoding xylanase (Accession number Z34519.1), *B. circulans* xlnA gene for xylanase (Accession number X07723.1). The close match confirms that a family 11 endo-1,4-beta-xylanase gene was successfully isolated. In addition to the megablast search, sequence alignment was also done to compare the xylanase gene obtained from this work with hypothetical xylanase gene sequence obtained from *Klebsiella*. The pairwise alignment did not show any similarity between xylanase genes from these two species. The number of peptide units for hypothetical xylanase from *Klebsiella* in the NCBI database is 388 amino acid while the peptide units for *Bacillus* is 213–228 amino acids. Therefore, the sequencing results showed that this locally isolated bacterium shows similarity of xylanase gene to *Bacillus* sp. although the 16s rRNA analysis showed that the bacterium is more similar to *Klebsiella*.

**Crude Enzyme Activity**

The isolate showed a standard pattern with low enzyme production at earlier stages, increasing steadily to the maximal level at 30 hours. Enzyme activity at different incubation time was as follows: 0.470 U/mL at 6 hours, 0.579 U/mL at 24 hours, 0.642 U/mL at 30 hours and 0.504 U/mL at 42 hours. At 42 hours, enzyme production was lowered, and

![Fig. 1. (A) Gel photo of PCR results showing dominant band with the estimated size of 650 bp. Lane M: Vivantis 1KB Ladder. Lane 1: PCR product; (B) Full sequence of 642bp DNA sequence. Forward primer is marked by “>” and reverse primer is marked by “<”.](image-url)
this could be attributed to saturation of culture, depletion of nutrient availability or due to proteolysis as suggested by Flores et al. (1997). Although the crude enzyme activity is relatively low, it is expected that the recombinant enzyme would have a multi-fold increase in activity once expressed and purified. This assumption is based on the observation by Helianti et al. (2008) of recombinant xylanase that showed more than twenty times increase in enzyme activity compared to the native crude enzyme activity.

In Silico Characterization

The 642 bp sequence was found to have an open reading frame (ORF) of 642 bp, encoding a 213 amino acid sequence. The xylanase was predicted to have a molecular weight of 23.3 kDa and the theoretical isoelectric point (pI) is at pH 9.44. The signal peptide cleavage site was predicted with >95% probability, to be between residues 28 and 29. Two catalytic cores were predicted, with both catalytic bases being glutamic acid residues. The full amino acid sequence with predicted signal peptide cleavage site and catalytic cores are shown in Fig. 2A.

The molecular model of the endo-1,4-beta-xylanase is described as consisting of an alpha helix and several beta pleated sheets with a compact structure and is shown in Fig. 2B.

The amino acid sequence of the xylanase was most similar to a xylanase from B. subtilis isolated in Japan, with 99.5 % identity (Jalal et al., 2009).

---

**Fig. 2.** (A) Full amino acid sequence of xylanase gene. Signal peptide cleavage site is marked by “<>”. Catalytic core is enclosed by “{“ and “}” while catalytic base is marked by “*”; (B) Molecular model of 642 bp endo-1,4-beta-xylanase predicted using Swiss-Model and displayed using Rasmol programme.
Table 1: Summary of morphological and biochemical characterization for *Klebsiella pneumoniae*.

<table>
<thead>
<tr>
<th>Characterization</th>
<th><em>Klebsiella pneumoniae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morphology:</strong></td>
<td></td>
</tr>
<tr>
<td>Form</td>
<td>circular</td>
</tr>
<tr>
<td>Elevation</td>
<td>convex</td>
</tr>
<tr>
<td>Margin</td>
<td>entire</td>
</tr>
<tr>
<td>Colour</td>
<td>milky</td>
</tr>
<tr>
<td><strong>Gram staining</strong> (colour and shape)</td>
<td>Pink, rod</td>
</tr>
<tr>
<td><strong>Biochemical tests</strong></td>
<td></td>
</tr>
<tr>
<td>Motility</td>
<td>Non-motile</td>
</tr>
<tr>
<td>H₂S production tests</td>
<td>-</td>
</tr>
<tr>
<td>Vogues Proskauer test</td>
<td>+</td>
</tr>
<tr>
<td>Citrate test</td>
<td>+</td>
</tr>
</tbody>
</table>

Amino acid sequence comparison with xylanase isolated from thermophilic bacterium (Helianti et al., 2008; Lee et al., 2008) showed lower identity (91%), accounting for the thermophilic adaptations of those thermostable xylanases.

**Xylanase from *K. pneumonia***

The xylanase matched most closely with the highly conserved 23 kDa endo-1,4-beta-xylanases from genus *Bacillus*. This form of xylanase is said to be ubiquitous among *Bacillus* spp (Helianti et al., 2008). To the author’s knowledge, this is the first time this xylanase had been isolated from bacteria not from genus *Bacillus*, suggesting that this xylanase could be commonly occurring among soil-inhabiting microbes, not just limited to genus *Bacillus*. The high conservation indicates that this xylanase plays an important role in the degradation of xylan in the natural environment. Furthermore, this form of xylanase has no cellulase activity (Lee et al., 2008). Coupled with the small compact structure of this enzyme allows it to degrade the xylan without damaging the cellulose fibers in the cellulose-hemicellulose matrix. Thus, this enzyme can potentially be used in applications requiring the removal of hemicelluloses but requiring intact cellulose such as in biobleaching of paper pulp.

**CONCLUSION**

An endo-1,4-beta-xylanase gene from a locally isolated bacterium was successfully isolated and characterized. The bacterium was putatively identified as *K. pneumoniae* based on the morphological, biochemical characterisation and 16s rRNA sequence. The xylanase gene of this bacterium, however, showed higher similarity to *Bacillus* sp. than the hypothetical xylanase from *Klebsiella* sp. deposited in the NCBI database.

**REFERENCES**


