

XYLANASE GENE FROM A LOCALLY ISOLATED BACTERIUM

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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.

ABSTRAK

Bakteria pengurai xilan telah dipencilkan daripada sampel humus yang diambil di ladang sago. Keputusan pemerhatian morfologi, ujian biokimia dan jujukan rRNA 16s mencadangkan bahawa bakteria ini ialah *Klebsiella pneumoniae*. Kajian juga dijalankan untuk memencil dan mencirikan gen xylanase daripada bakteria pengurai xilan ini. Produk bersaiz 650 pasangan bes telah berjaya diperolehi melalui proses PCR dan diklonkan ke dalam *Escherichia coli* XL-1 blue.. Analisis BLAST telah menunjukkan bahawa jujukan DNA produk tersebut merupakan gen endo-1,4-beta-xilanase daripada keluarga 11 glikosida hidrolase. Ia menunjukkan 98% kepadanan dengan gen xylanase *Bacillus subtilis*. Pencirian secara *in silico* menunjukkan ia mempunyai rangka bacaan terbuka (ORF) yang mengekodkan rantai peptida bersaiz 213 asid amino dengan berat molekul 23.3 kDa dan ramalan titik isoelektrik (pI) pada 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 (Khanderparker & 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 with varying degrees with *O*-acetyl, α -L-arabinofuranosyl, 4-*O*-methylglucuronic acid groups or α -1,2-linked glucuronic 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

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containing xylan for two days at room temperature. This broth was serially diluted and plated on xylan agar consisting of 0.1 % yeast extract, 1 % xylan from corncobs, 2 % agar, 0.4 % KH_2PO_4 , 0.2 % NaCl, 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.005 % MnSO_4 (Nanmori *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 (www.ncbi.nlm.nih.gov) and were aligned using ClustalW (Larkin *et al.*, 2007) and CLC sequence viewer (www.clcbio.com). Conserved regions were detected. The forward primer (5'-ATGTTTAAGTTTAAAAAGAATTTC-3') and reverse primer (5'TTACCACACTGTTACGTTAG-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, 60s 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 four 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 H_2S 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 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).

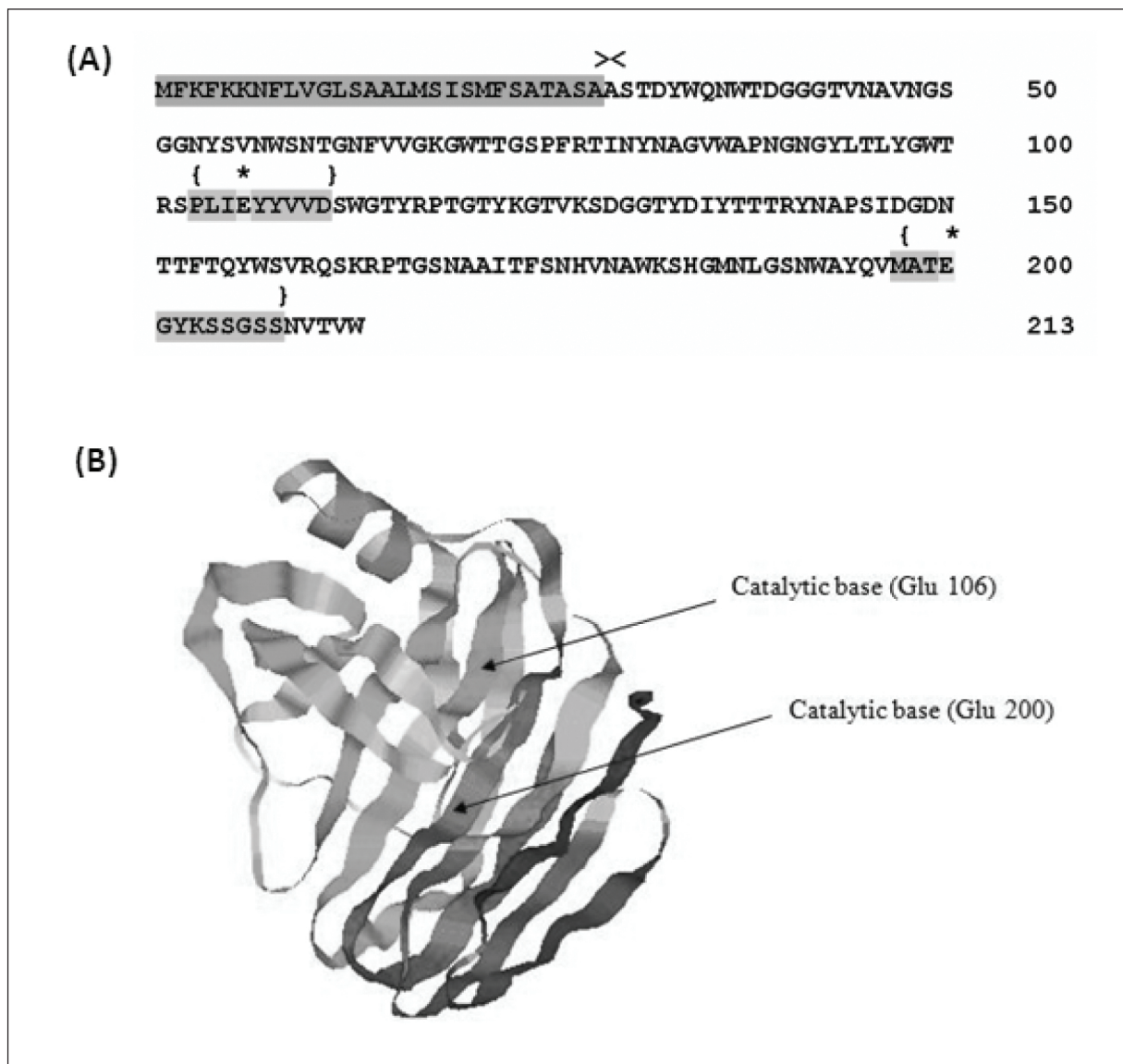


Fig. 2. (A) Full amino acid sequence of xylanase gene. Signal peptide cleavage site is marked by “×”. Catalytic core is enclosed by “{” and “}” while catalytic base is marked by “*”; (B) Molecular model of 642 bp endo-1,4-beta-xylanase predicted using Swiss-Model and displayed using Rasmol programme.

Table 1: Summary of morphological and biochemical characterization for *Klebsiella pneumoniae*.

Characterization	<i>Klebsiella pneumoniae</i>
Morphology:	
Form	circular
Elevation	convex
Margin	entire
Colour	milky
Gram staining (colour and shape)	Pink, rod
Biochemical tests	
Motility	Non-motile
H ₂ S production tests	-
Vogues Proskauer test	+
Citrate test	+

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. pneumoniae*

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

- Arnold, K., Bordoli, L., Kopp, J. & Schwede, T. 2006. The SWISS-MODEL Workspace: A web-based environment for protein structure homology modelling. *Bioinformatics*. **22**: 195-201.
- Beg, Q.K., Kapoor, M., Mahajan, L. & Hoondal, G.S. 2001. Microbial xylanases and their industrial applications: a review. *Applied Microbiology and Biotechnology*. **56**:326-338.
- Bendtsen, J.D., Nielsen, H., von Heijne, G. & Brunak, S. 2004. Improved prediction of signal peptides: SignalP 3.0. *J. Mol. Biol.* **340**:783-795.
- Bernstein, H.J. 2000. Recent changes to RasMol, recombining the variants. *Trends in Biochemical Sciences*. **25**: 453-455.
- Flores, M.E., Perez, R. & Huitron, C. 1997. Beta-xylanase and xylanase characterization and production by *Streptomyces* sp. CH-M-1035. *Lett. Appl. Microbiol.* **24**: 410-416.
- Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M.R., Appel, R.D. & Bairoch, A. 2005. *Protein Identification and Analysis Tools on the ExPASy Server*. In Walker, J. M. (Ed.), *The Proteomics Protocols Handbook*. pp. 571-607. USA: Humana Press.
- Guex, N. & Peitsch, M.C. 1997. SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modelling. *Electrophoresis*, **18**: 2714-2723.
- Helianti, I., Nurhayati, N. & Wahyuntari, B. 2008. Cloning, sequencing, and expression of a β -1,4-endoxylanase gene from Indonesian *Bacillus licheniformis* Strain I5 in *Escherichia coli*. *World Journal of Microbiology Biotechnology*. **24**:1273-1279.
- Jalal, A., Rashid, N., Rasool, N. & Akhtar, M. 2009. Gene cloning and characterization of a xylanase from a newly isolated *Bacillus subtilis* strain R5. *J Biosci Bioeng.* **107**: 360-365.

- Khanderparkar, R.D.S. & Bhosle, N.B. 2006. Isolation, purification and characterization of the xylanase produced by *Arthrobacter* sp. MTCC 5214 when grown in solid-state fermentation. *Enzyme and Microbial Technology*. **39**: 732-742.
- Khanderparker, R. & Numan, M.T. 2008. Bifunctional xylanases and their potential use in biotechnology. *J Ind Microbiol Biotechnol*. **35**: 635-644.
- Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J. & Higgins, D.G. 2007. ClustalW and ClustalX version 2. *Bioinformatics*. **23**: 2947-2948.
- Lee, C.C., Kibblewhite-Accinelli, R.E., Smith, M.R., Wagschal, K., Orts, W.J. & Wong, D.W.S. 2008. Cloning of *Bacillus licheniformis* xylanase gene and characterization of recombinant enzyme. *Current Microbiology*, **57**: 301-305.
- Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31**: 426-428.
- Morris, D.D., Gibbs, M.D., Chin, C.W.J., Koh, M.H., Wong, K.K.Y., Allison, R.W., Nelson, P.J. & Bergquist, P.L. 1998. Cloning of the xynB Gene from *Dictyoglomus thermophilum* Rt46B.1 and action of the gene product on kraft pulp. *Applied and Environmental Microbiology*. **64**: 1759-1765.
- Nanmori, T., Watanabe, T., Shinke, R., Kohno, A., and Kawamura, Y. 1990. Purification and properties of thermostable xylanase nad beta-xylosidase produced by a newly isolated *Bacillus stearothermophilus* strains. *Journal of Bacteriology*. **172**: 6669-6672.
- Pagni, M., Ioannidis, V., Cerutti, L., Zahn-Zabal, M., Jongeneel, C.V., Hau, J., Martin, O., Kuznetsov, D. & Falquet, L. 2007. MyHits: improvements to an interactive resource for analyzing protein sequences. *Nucleic Acids Res.* **35**: W433-7.
- Romanowska, I., Polak, J. & Bielecki, S. 2006. Isolation and properties of *Aspergillus niger* IBT-90 xylanase for bakery. *Applied Microbiology and Biotechnology*. **69**: 665-671.
- Sayle, R. & Milner, E.J. 1995. RasMol: Biomolecular graphics for all. *Trends in Biochemical Sciences*; **20**: 374.
- Schwede, T., Kopp, J., Guex, N. & Peitsch, M.C. 2003. SWISS-MODEL: an automated protein homology-modeling server. *Nucleic Acids Research*. **31**: 3381-3385.
- Silva, C.H.C., Puls, J., de Sousa, M.V. & Filho, E.X.F. 1999. Purification and characterization of a low molecular weight xylanase from solid-state cultures of *Aspergillus fumigatus* Fresenius. *Revista de Microbiologia*. **30**: 114-119.
- Singh, S., Madlala, A.M. & Prior, B.A. 2003. *Thermomyces lanuginosus*: properties of strains and their hemicellulases. *FEMS Microbiology Reviews*. **27**: 3-16.
- Viikari, L., Kantelinen, A., Sundquist, J. & Linko, M. 1994. Xylanases in bleaching: from an idea to the industry. *FEMS Microbiology Reviews*. **13**: 335-350.
- Yang, V.W., Zhuang, Z., Elegir, G. & Jefferies, T.W. 1995. Alkaline-active xylanase produced by alkaliphilic *Bacillus* sp isolated from kraft pulp. *Journal of Industrial Microbiology*. **15**: 434-441.