

Characterisation of *Klebsiella pneumoniae* Xylanase and Increment of Its Activity in Heterologous Expression System

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ABSTRACT

A xylanase DNA sequence with a total length of 642 bp was previously isolated from a xylanolytic *Klebsiella pneumoniae*. Xylanase gene primers were designed with the addition of *Bam*H1 and *Eco*R1 restriction enzyme sites in order get a full xylanase gene that is in-frame with pSTAG expression vector. The isolated xylanase gene was amplified using the designed primers through PCR, then cloned and expressed in *E. coli* BL21 (DE3). *In-silico* characterization showed that the recombinant xylanase has a molecular weight of 23.9 kDa and a pI of 9.32. The signal peptide cleavage site for the recombinant xylanase was predicted to be between residues 61 and 62. The activity of the crude recombinant xylanase was 2.015 U/mL, which was higher than the crude native xylanase activity, with maximum at 0.642 U/mL. Staining of the birchwood xylan agar plate with Congo red showed a clearing zone around *E. coli* BL21 (DE3) colonies with recombinant pSTAG plasmid even without being induced with IPTG. This implied leaky expression of the *E. coli* BL21 (DE3) secretion system, which recognized the signal sequence of the recombinant xylanase, and proceeded to cleave and secreted out the mature protein into the culture medium. MALDI-TOF analysis of a 20 kDa protein present in the culture medium confirmed that the recombinant xylanase had been secreted into the culture medium.

Keywords: Heterologous expression, *Klebsiella pneumoniae*, recombinant xylanase

INTRODUCTION

Xylan consists of β -1,4-linked xylopyranosyl residues. It is the second most abundant polysaccharide and an important component that makes up the plant cell wall. Due to the heterogeneity and complexity of the xylan structure, the complete hydrolysis of xylan is carried out by a xylanolytic enzyme system. Endo-1,4- β -D-xylanase (EC 3.2.1.8) is part of this complex system (Singh *et al.*, 2007). Xylanase cleaves the xylan backbone at 1-4 carbon linkages to produce xylo-oligosaccharides and xylose (Kulkarni *et al.*, 1999). From a biotechnology perspective, xylanase is an important enzyme because of its thermal stability and its ability to work alone or in combination with other enzymes, making it suitable for a number of industrial applications. Examples of xylanase applications include its use in the kraft process and biobleaching in the paper and pulp industry (Helianti *et al.*, 2008; Kulkarni *et al.*, 1999; Te'o *et al.*, 2000).

Heterologous expression of a gene encoding β -1,4-endoxylanase in *E. coli* BL21 (DE3) was performed in this study. The xylanase gene was isolated from xylanolytic *Klebsiella pneumoniae*, a bacterium that had been locally isolated from soil from a sago plantation by Hussain *et al.* (2011). Here, we report on the expression of a xylanase gene from *K. pneumoniae* in *E. coli* BL21 (DE3) and to determine the characteristics of the recombinant xylanase expressed. To our knowledge, there are no prior publications that describe the isolation and heterologous expression of a xylanase gene from *K. pneumoniae*. Xylanase genes are commonly isolated from genus *Bacillus* which are abundantly found in soil (Gardener, 2004; Nakamura *et al.*, 1993; Touzel *et al.*, 2000). Thus the isolation of a xylanase gene from *K. pneumoniae* from agricultural soil suggests that xylanase might be naturally occurring among soil-inhabiting microbes.

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MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli JM109 strain was used as a host for plasmid amplification and *E. coli* BL21 (DE3) (Novagen) strain was used as a host for protein expression. Plasmids used in this experiment were pGEM-T Easy Vector (Promega) and pSTAG expression vector (Edwards *et al.*, 1999). The xylanase gene had been cloned into the pGEM-T Easy Vector in previous work by Hussain *et al.* (2011).

Xylanase gene amplification and subcloning

The primers were designed based on the isolated xylanase gene from *K. pneumoniae* by Hussain *et al.* (2011). The designed primers were 5'-GCG GAT CCA TGT TTA AGT TTA AAA AGA ATT TC-3' as a forward primer and 5'-GCG AAT TCT ATT ACC ACA CTG TTA CGT TAG-3' as a reverse primer. The italic and underlined sequences show the restriction enzyme *Bam*H1 site (forward primer) and *Eco*R1 site (reverse primer) for subcloning into pSTAG expression vector. PCR amplification using the designed xylanase gene primers and template plasmid DNA was performed using *Pfu* DNA polymerase under the following conditions: 94°C for 3 min, then 30 cycles of 94°C for 45 s, 52°C for 1 min, and 72°C for 2 min for each cycle, and a final 72°C for 5 min. The PCR product matching the predicted size was purified using GF-1 PCR Clean Up Kit (Vivantis). The xylanase PCR product was digested using *Bam*H1 and *Eco*R1 restriction enzymes, ligated into linearised pSTAG expression vector with the same restriction enzymes, and transformed into *E. coli* JM109. Positive clones harbouring recombinant pSTAG plasmid were identified through colony PCR.

Bioinformatics analysis of xylanase gene

Automated sequencing of the recombinant pSTAG plasmid was done by First BASE Laboratories Sdn Bhd (Selangor, Malaysia). Translation of DNA sequence to amino acid sequence was performed using ExPASy Bioinformatics Resource Portal (<http://expasy.org/>).

Pairwise alignment was performed by using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). *In-silico* characterization for molecular weight and isoelectric point (pI) was predicted using ExPASy Bioinformatics Resource Portal (<http://expasy.org/>), while signal peptide was predicted using PrediSi Bioinformatics Tool (<http://www.predisi.de/>).

Transformation into *E. coli* BL21 (DE3)

Recombinant pSTAG plasmid and empty pSTAG plasmid were transformed into *E. coli* BL21 (DE3) using the heat shock method. The empty pSTAG plasmid would act as a negative control.

Confirmation of xylanase activity through Congo red agar staining

M9 minimal agar containing ampicillin (50 µg/mL) and 1% birchwood xylan (Sigma) was used to grow the *E. coli* BL21 (DE3). A single colony of *E. coli* BL21 (DE3) harboring the recombinant pSTAG plasmid was picked and streaked onto the agar plate, side by side with the negative control. After incubation at 37°C overnight, the colonies were induced with a drop of IPTG to a final concentration of 0.4 mM. Replicate plates were also prepared, but IPTG was not added to the replicate plates. Following overnight incubation, all plates were stained with 1% Congo red solution and destained by washing with 1 M NaCl.

Expression of xylanase in liquid medium

The *E. coli* BL21 (DE3) harboring the recombinant pSTAG plasmid was cultured in LB broth supplemented with ampicillin (50 µg/mL) at 30°C for 10 hours with shaking at 180 rpm under two conditions; with and without the addition of 1% glucose. Protein expression was induced with addition of IPTG to a final concentration of 0.4 mM. Cells were harvested by centrifugation at 5000 x g for 5 min at 4°C.

Protein extraction

Freeze and thaw method using liquid nitrogen was carried out to obtain the soluble and insoluble protein fraction (Wanarska *et al.*, 2007). To extract the soluble fraction, the

frozen cell pellet was resuspended in a soluble protein buffer (20 mM sodium phosphate, 0.5 M NaCl and 20-60 mM imidazole at pH 7.4) and the sample was frozen and thawed several times. The sample was centrifuged at 4000 x g for 40 min at 4°C, and the supernatant (soluble fraction) was collected. To extract the insoluble fraction, the pellet was resuspended in insoluble protein buffer (6 M urea, 20 mM sodium phosphate, 0.5 M NaCl and 20 mM imidazole at pH 7.4), incubated overnight at 4°C and centrifuged at 4000 x g for 40 min at 4°C. For the media fraction, culture media was precipitated overnight at -20°C using 0.1 M ammonium acetate in methanol.

SDS-PAGE and Western blot immunodetection

The total cell protein, soluble fraction, insoluble fraction, and media fraction were checked for xylanase expression using 15% SDS-PAGE gels stained with Coomassie blue. For Western blot analysis, the proteins were transferred onto a nitrocellulose membrane according to standard procedure (Bio-Rad Laboratories) using the tank transfer method. S•Tag Western Blot Kit was used according to the S•Tag™ System (Novagen) instruction manual. The transferred membrane was blocked briefly with gelatin, and then incubated with S-protein alkaline phosphatase conjugate. NBT/BCIP substrate was used to visualize target bands.

MALDI-TOF analysis

Several spots from a Coomassie stained SDS-PAGE gel were excised, pooled together, and air dried. MALDI-TOF mass spectrometry and analysis of the MALDI-TOF spectra using Mascot sequence matching software [Matrix Science] with Ludwig NR Database was performed by Proteomics International (Australia).

Crude recombinant enzyme activity

The activity of the crude recombinant xylanase enzyme using the soluble protein fraction was determined using the Bicinchoninic Acid (BCA) assay, which was carried out based on the protocol described in Hussain *et al.* (2003). The amount of reducing sugar released was

determined based on a glucose standard curve. 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.

RESULTS AND DISCUSSION

The isolated xylanase gene is from family 11 glycoside hydrolase and showed similarity to *Bacillus* spp., although 16S rRNA showed that the bacterium closely resembled *Klebsiella*. *In-silico* characterization of the isolated xylanase gene showed that it has an open reading frame (ORF) of 642 bp encoding 213 amino acids, and is predicted to code for a xylanase with a molecular weight of 23.3 kDa and an isoelectric point of 9.44, which is quite similar to the xylanase isolated by Jalal *et al.* (2009) from *B. subtilis*, as well as Helianti *et al.* (2008) and Lee *et al.* (2008) from *B. licheniformis*.

The PCR amplification using the designed primers produced a fragment of about 650 bp (Figure 1), which matched the expected size of 642 bp of the xylanase gene from *Bacillus*. The PCR product was then subcloned into pSTAG expression vector, and then transformed into *E. coli* JM109. Positive clones harbouring recombinant pSTAG plasmid were chosen through colony PCR. Plasmids were extracted and sent for sequencing.

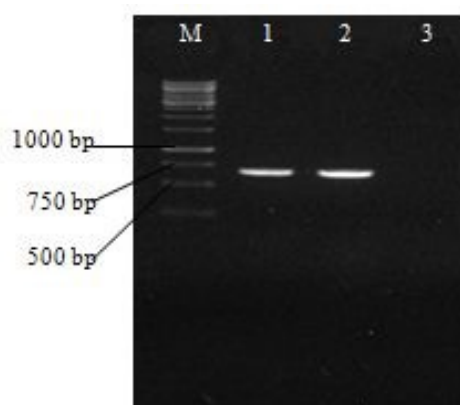


Figure 1. PCR amplification of xylanase gene visualised on 1% agarose gel. Lane 1 and 2: xylanase PCR product; Lane 3: negative control. M is a GeneRuler™ 1 kb DNA Ladder (Fermentas).

Analysis of the cloned xylanase DNA sequence showed it to be in-frame with the S-tag, having an ORF of 738 nucleotides with an initiation and a stop codon. Based on the translated amino acid sequence, the calculated molecular weight including the the S-tag and signal peptide was predicted to be 26.86 kDa, and the isoelectric point (pI) was predicted at pH 9.28. With the S-tag removed, the recombinant xylanase is predicted to have 219 amino acids with a molecular weight of 23.9 kDa and a pI of 9.32. The recombinant xylanase was predicted to have a signal peptide cleavage site between amino acid residues 61 and 62 (sequence not shown) due to presence of the S-tag peptides, while the native xylanase signal peptide cleavage site was predicted to be between residues 28 and 29 (Hussain *et al.*, 2011). The signal peptide was left intact to direct the xylanase into the secretory pathway of *E. coli*. The recombinant mature xylanase was predicted to have a molecular weight of 20.37 kDa and a pI of 9.29. For recombinant gene expression, signal peptide cleavage site can be used as a cleavage position to obtain shorter active proteins for further research and development. This has been shown in isoamylase proteins obtained from pea and potato in which the active isoamylase showed native properties even when expressed as truncated recombinant proteins (Hussain *et al.*, 2003; Hussain & Martin, 2009).

From Figure 2, both media plates showed a clear zone on the medium around *E. coli* BL21 (DE3) transformed with recombinant pSTAG plasmid carrying the xylanase insert. The *E. coli* BL21 (DE3) with empty pSTAG plasmid did not show any clear zone on the medium after Congo red staining. Plate X in which the *E. coli* was not induced with IPTG, showed the presence of a clear zone. Similar observation was made on plate Y induced with IPTG, with presence of clear zone. However, the clear zone on plate Y is much more pronounced than on plate X. The addition of IPTG had induced bacteria colonies to produce more protein, hence the reason the clearing zone of plate Y is much more pronounced than of plate X.

As described in Hussain *et al.* (2011), *in-silico* characterization of the native xylanase from *K. pneumoniae* showed 99.5% identity to the isolated xylanase from *B. subtilis* (Jalal *et al.*, 2009). Jalal *et al.* (2011) reported that in heterologous expression, the *E. coli* secretion system had recognized the signal sequence of the native xylanase and proceeded to cleave it, then secreted out the mature protein into the culture medium. The signal peptide cleavage site for native xylanase of *E. coli* described by Jalal *et al.* (2011) was reported to be between Ala 28 and Ala 29. This is identical to the signal peptide cleavage site predicted for the native xylanase found in *K. pneumoniae* and heterologously expressed in *E. coli* BL21

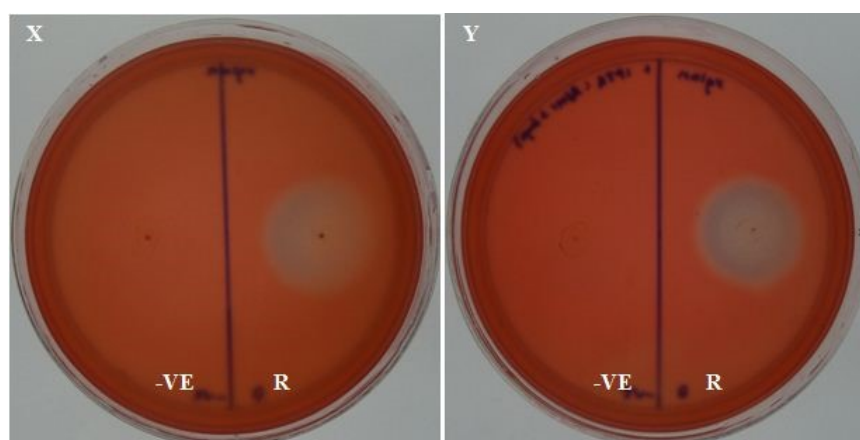


Figure 2. 1% Congo red staining of agar plates. *E. coli* BL21 (DE3) with empty pSTAG plasmid (-VE) or recombinant pSTAG plasmid (R) were grown on M9 minimal media plates containing ampicillin and 1% birchwood xylan. The bacteria colonies were uninduced (X) or induced with IPTG (Y) after overnight incubation. For *E. coli* BL21 (DE3) with recombinant pSTAG plasmid (R), both uninduced and induced bacteria colonies showed clearing zones.



Figure 3. Signal peptide of the xylanase. Positively charged lysine residues are marked ^, highly hydrophobic residues are marked *, and slightly hydrophobic residues are marked !. (-3 -1) indicates the -3 and -1 position relative to cleavage site.

(DE3) described here. The leaky expression followed by secretion of the recombinant mature xylanase into the culture medium would explain the observation of the clearing zone on the medium even without IPTG induction since the secreted recombinant mature xylanase would degrade the xylan in the medium.

Despite the addition of the S-tag to the N-terminal, the signal peptide was still recognized by the *E. coli* secretory pathway. The signal peptide matches the model for bacterial signal peptides targeting the protein into the SEC pathway and does not match any other pathway as described by Paetzel *et al.* (2002). The signal peptide contains a positively charged N-domain and hydrophobic H-domain (Figure 3). In the carboxyl-domain, residues in positions -3 and -1 relative to cleavage site Alanine, which is non-polar and small, thus matching the (-3,-1) rule for cleavage by signal peptidase (Nielsen *et al.*, 1997).

E. coli BL21 (DE3) is simple to transform, produce high yield, and have a T7-based expression system, which is one of the most efficient expression system known for *E. coli*. However, the *lacUV5* promoter is leaky, and causes relatively high level of basal expression of the recombinant genes even before induction. As such, a more tightly regulated version of the strain, BL21 (DE3) pLysS, is more favoured since it limits the level of uninduced recombinant protein expression (Field *et al.*, 2007; Suvorov, 2008; Zhou *et al.*, 2004).

The expression of the xylanase gene was done at 30°C in two ways; with and without the addition of 1% glucose. The addition of glucose in the media was to prevent leaky expression (Novagen, 1999). The result was checked using SDS-PAGE and Western blot. Analysis of total cell protein extracts showed that the molecular weight of crude recombinant xylanase in lane 1 and lane 3 (Figure 4) matched the expected molecular weight of 25 kDa predicted from *in-silico* characterization.

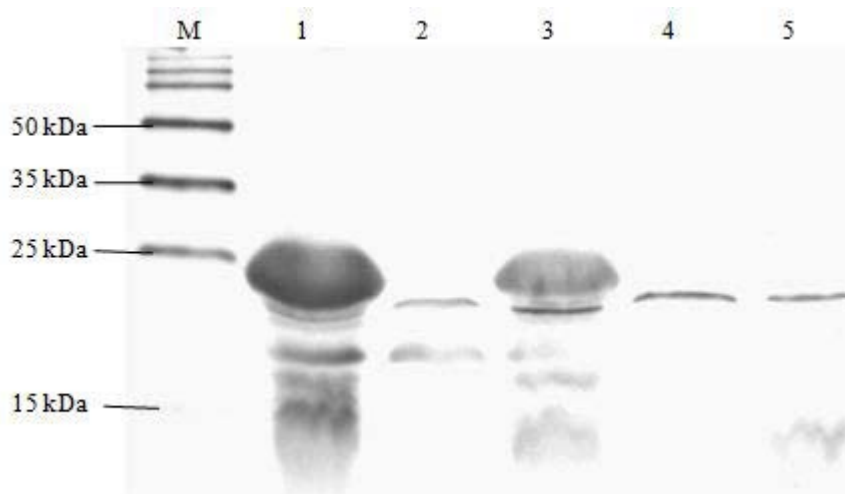


Figure 4. Western blot analysis for xylanase gene expression. Media without glucose (lane 1 and 2) showed higher protein expression compared to media with glucose (lane 3 and 4). Same sample volumes were loaded onto SDS-PAGE. Lane 1: induced recombinant pSTAG plasmid at 30°C without glucose; Lane 2: uninduced recombinant pSTAG plasmid at 30°C without glucose; Lane 3: induced recombinant pSTAG plasmid at 30°C with 1% glucose; Lane 4: uninduced recombinant pSTAG plasmid at 30°C with 1% glucose; Lane 5: induced empty pSTAG plasmid at 30°C with 1% glucose. M is a Perfect Protein™ Western Markers (Novagen).

The expression without the presence of glucose showed the thickest protein band compared to expression in the presence of glucose, although the same volume of extracted proteins were loaded on the SDS-PAGE gel. Both uninduced recombinant pSTAG plasmid with xylanase insert and induced empty pSTAG plasmid that acted as a control did not show the presence of a protein band at the predicted molecular weight.

The insoluble, soluble and media fractions were analysed through SDS-PAGE (Figure 5). A 25 kDa protein band, matching the predicted size was detected in the insoluble fraction and media fraction. Western blot analysis of this 25 kDa band identified it as the full-length recombinant xylanase with S-tag attached. Furthermore, another thick protein band, estimated at 20 kDa was detected in all fractions. The size of this protein band matched the size of the predicted recombinant mature xylanase after signal peptide cleavage, which is 20.37 kDa.

This 20 kDa was not detected on the Western blot because the S-tag had been cleaved off by the *E. coli* secretory system. Instead, the identity of this 20 kDa protein was supported by the MALDI-TOF spectra analysis, where the 20 kDa protein was matched to an endo-1,4-beta-xylanase from *Bacillus subtilis* (AEP91071) with a score of 392.

This result supports the qualitative assay where xylanase was secreted out and degraded the xylan in the agar. The recombinant mature xylanase was found mainly in the culture medium. It is likely that the 25 kDa xylanase, abundant in the insoluble fraction consists of inclusion bodies which accumulate in the cell because the *E. coli* secretory machinery cannot cope with the large amounts of polypeptide produced by the expression mechanism under the control of the T7 promoter (Novagen, 1999).

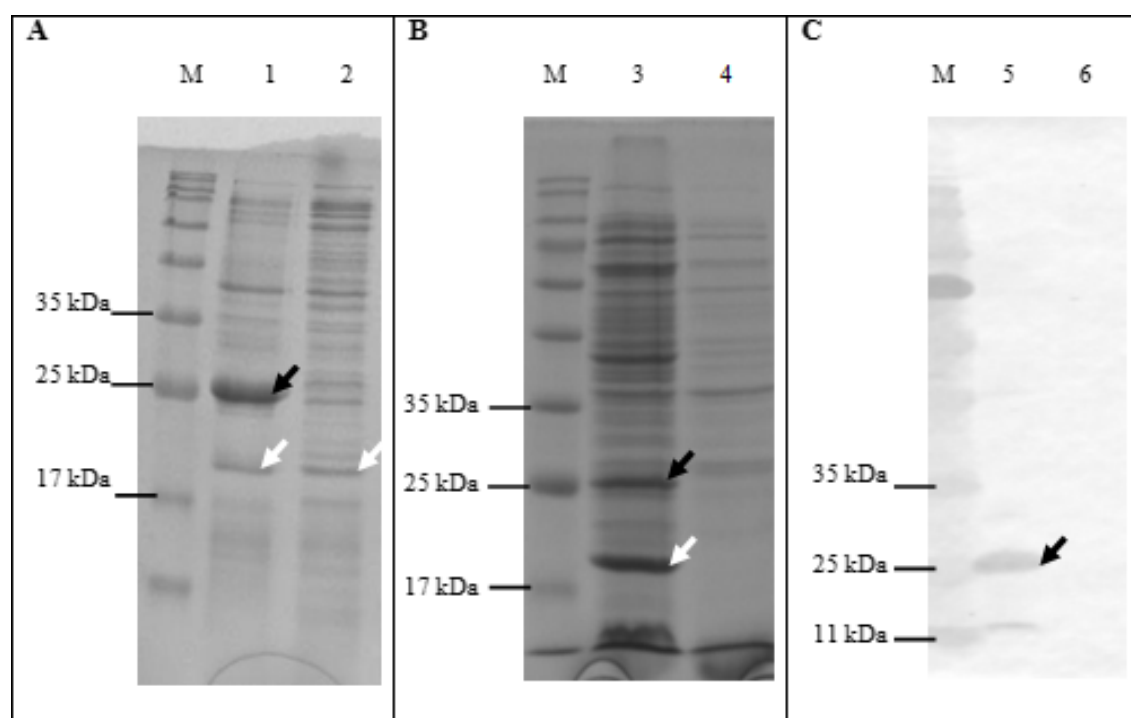


Figure 5. SDS-PAGE and Western blot analysis for proteins extracted from the insoluble, soluble and media fractions. Protein expression was done at 30°C with the addition of glucose. The 25 kDa full length recombinant xylanase is indicated by black arrows while the secreted 20 kDa recombinant mature xylanase is indicated by the white arrows. M is a BlueRAY Prestained Protein Ladder (GeneDireX). A. 15% SDS-PAGE gel for insoluble and soluble fractions. Lane 1: insoluble fraction; Lane 2: soluble fraction. B. 15% SDS-PAGE gel for media fraction. Lane 3: induced recombinant pSTAG with xylanase insert; Lane 4: induced empty pSTAG plasmid. C. Western blot for media fraction separated on 10% SDS-PAGE gel. Lane 5: induced recombinant pSTAG with xylanase insert; Lane 6: induced empty pSTAG plasmid.

A xylanase gene from *Bacillus* that was expressed in *E. coli* had been reported to be distributed in extracellular, intracellular and periplasmic fractions (Huang *et al.*, 2006). However, recombinant xylanases were commonly found to be in the insoluble fraction of the cytoplasm, hinting that even after optimization, the expression of the xylanase gene produces protein that is insoluble and accumulates in inclusion bodies (Yin *et al.*, 2008). In many cases the expressed protein is insoluble and accumulates in inclusion bodies, especially under conditions of high level expression. Several strategies are available to improve the solubility of the expressed protein such as change of the media composition, production of the protein at low temperature (20°C or lower), genetic modifications of *E. coli* strains, and co-production of folding modulators and fusion tags (Altenbuchner & Mattes, 2006; Garcia-Fruitòs *et al.*, 2009). However, sometimes despite of all efforts the target protein will still be expressed in the inclusion bodies. In this case, the other option available is to carry out protein denaturation followed by refolding of the denatured protein *in vitro*.

The secretion of xylanase into the medium does not only simplify purification of the xylanase, it also decreases proteolysis, reduces levels of contaminants, and cell disruption can be avoided (Jonasson *et al.*, 2002). This 29 amino-acid transport peptide could be used as a gene fusion partner, for secretion of other heterologous proteins in *E. coli* since it is recognized by both gram positive and gram negative bacteria.

The activity of the crude recombinant xylanase was 2.015 U/mL, which is more than 3 times higher compared to the activity of the crude native xylanase of *K. pneumoniae* isolated by Hussain *et al.* (2011), which was 0.642 U/mL at the maximum. Based on the latter activity value, it was expected that higher activity could be obtained once the gene was isolated and heterologously expressed as crude recombinant xylanase. This expectation was based on research by Jalal *et al.* (2009), Helianti *et al.* (2008), and Lee *et al.* (2008) where purified recombinant xylanase showed an increase in its activity compared to the activity of native xylanase.

CONCLUSION

K. pneumoniae xylanase was successfully expressed in the heterologous expression system of *E. coli* BL21 (DE3). The characterised enzyme showed an increase in activity compared to native xylanase. The *E. coli* BL21 (DE3) however showed leaky expression secretion system prior to being induced by IPTG. Analysis of the recombinant xylanase in the soluble and insoluble fractions showed that the recombinant xylanase protein is secreted out of the cell as a soluble 20 kDa mature protein. Meanwhile, in the insoluble fraction, the 20 kDa recombinant mature xylanase as well as the 25 kDa recombinant xylanase containing uncleaved transit peptide accumulated in the cell as inclusion bodies because the *E. coli* secretion system cannot cope with such high production of recombinant protein.

ACKNOWLEDGEMENTS

The authors would like to thank Ministry of Higher Education Malaysia and Universiti Malaysia Sarawak for the facilities and financial support to conduct this research through FRGS grant 961/2013(02). Special thanks to Dr Md Nuruzzaman of School of Food Science and Technology, Universiti Malaysia Terengganu for comments and suggestions during preparation of the manuscript.

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