

Full Length Research Paper

# Expression optimisation of recombinant $\alpha$ -L-arabinofuranosidase from *Aspergillus niger* ATCC 120120 in *Pichia pastoris* and its biochemical characterisation

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**A gene encoding  $\alpha$ -L-arabinofuranosidase (*AnabfA*) from *Aspergillus niger* ATCC 120120 was successfully cloned and expressed in *Pichia pastoris* under the control of the *AOX1* promoter. The effect of cultural conditions on recombinant *AnabfA* production was studied and the enzyme was expressed as a soluble protein. Recombinant *AnabfA* was expressed as an active enzyme at 28 °C when cultured in BMMY medium (pH 6.0) and induced with 2% methanol every 24 h. Maximum activity was observed 5 days after induction. The purified recombinant *AnabfA* before and after treatment with PNGase F migrated by SDS-PAGE had relative molecular masses of about 83 and 66 kDa, respectively, suggesting that the *AnabfA* contains N-linked oligosaccharides. Characterisation of the purified recombinant *AnabfA* showed an optimum temperature and pH of 50 °C and 4, respectively. The enzyme was stable at a pH of 3 to 6 and retained more than 80% of its activity after pre-incubation at 40 °C for 30 min. The recombinant *AnabfA* activity was stimulated by K<sup>+</sup>, Mn<sup>2+</sup>, Na<sup>2+</sup> and triton X-100 and was strongly inhibited by Cu<sup>2+</sup> and Fe<sup>2+</sup> and the enzyme activity was relatively unaffected by Ca<sup>2+</sup>, CO<sup>2+</sup>, Mg<sup>2+</sup> and EDTA. The  $K_m$  and  $V_{max}$  of the purified recombinant *AnabfA* activity towards pNPA were 0.93 mM and 17.86  $\mu$ mol/ml/min, respectively.**

**Key words:** *Aspergillus niger*,  $\alpha$ -L-arabinofuranosidase, expression, *Pichia pastoris*, characterisation.

## INTRODUCTION

$\alpha$ -L-Arabinofuranosidases ( $\alpha$ -L-arabinofuranoside arabinofuranohydrolases, EC 3.2.1.55) are accessory enzymes that cleave  $\alpha$ -L-arabinofuranosidic linkages and act synergistically with other hemicellulases and pectic enzymes for the complete hydrolysis of xylans and pectins (Spagna et al., 1998; Margolles-Clark et al., 1996). Xylans and pectins, which comprise the major

matrix polysaccharides in plant cell walls, are extensively decorated with arabinofuranose moieties.  $\alpha$ -L-arabinofuranosidases have attracted considerable research interest due to their potential industrial applications; they are used in the synthesis of oligosaccharides (Rémond et al., 2004; Rémond et al., 2002), in the pretreatment of lignocelluloses for bioethanol production (Foreman et al., 2003; Saha, 2003) and in the chlorination in pulp and paper products (Gomes et al., 2000; Mai et al., 2000).

Enzymes hydrolyzing L-arabinose linkages have been purified from several bacteria and fungi. Rombouts et al. (1988) reported that, *Aspergillus niger* produced two

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extracellular  $\alpha$ -L-arabinofuranosidases;  $\alpha$ -L-arabinofuranosidase A (*abfA*) and  $\alpha$ -L-arabinofuranosidase B (*abfB*). *A. niger* is a well-known hemicellulolytic producer and is widely known for its role as a citric acid producer (Magnuson and Lasure, 2004). As a common member of the microbial communities found in soils, *A. niger* plays a significant role in the global carbon cycle. Because this organism is a soil saprobe with a wide array of hydrolytic and oxidative enzymes, *A. niger* is involved in the breakdown of plant lignocelluloses.

In recent years,  $\alpha$ -L-arabinofuranosidases have been isolated from various microorganisms (Shi et al., 2009; Miyazaki, 2005; Tateishi et al., 2005; Birgisson et al., 2004; Kimura et al., 2000) and their genes have been cloned and expressed in heterologous expression systems (Miyazaki, 2005; Debeche et al., 2000; Margolles-Clark et al., 1996). Heterologous expression of recombinant proteins is often a convenient way to produce large amounts of protein (Kallas, 2006). *Pichia pastoris*, a methylotrophic yeast, has been shown to be a highly robust system for the production of a wide variety of recombinant proteins. It can be used to produce foreign proteins at high level, either intracellularly or extracellularly and with the proper post-translational modifications such as glycosylation, disulfide bond formation and proteolytic processing and fermentation occurs readily at a high cell density (Cereghino et al., 2002; Cereghino and Cregg, 2000). Also, because of the genetic stability of *P. pastoris*, heterologous gene expression can readily be accomplished on an industrial scale without the loss of yield (Baumgartner et al., 2002; Berrin et al., 2000).

The optimised expression of  $\alpha$ -L-arabinofuranosidase from *A. niger* ATCC 120120 in *P. pastoris* has not been reported before, so far, although, mutational analysis of  $\alpha$ -L-arabinofuranosidase 54 from *Aspergillus kawachii* expressed in *P. pastoris* has previously been performed (Koseki et al., 2006). In this paper, we present the effect of cultural conditions on the expression of  $\alpha$ -L-arabinofuranosidase (*AnabfA*) from *A. niger* ATCC 120120 in *P. pastoris* and examined some properties of the purified recombinant *AnabfA* protein.

## MATERIALS AND METHODS

### Strains, plasmids, culturing conditions, primers and materials

*A. niger* ATCC 120120, the source of the *AnabfA* gene, was obtained from the American Type Culture Collection (ATCC). *Escherichia coli* JM109 was used as the host for plasmid propagation. The *E. coli* strain was cultured in low-salt LB medium. *P. pastoris* strain X-33 (Invitrogen, USA) was used as the host for the expression of *AnabfA* and was grown in YEPD medium (1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) dextrose). The *P. pastoris* transformants were selected on YEPD medium containing 100  $\mu$ g/ml Zeocin<sup>TM</sup>. The pGEM<sup>®</sup>-T easy vector (Promega, USA) was used for cloning the PCR fragments and the

pPICZ $\alpha$  vector (Invitrogen, USA) were used for protein expression in *P. pastoris*. All synthetic oligonucleotides used in this study were obtained from the First BASE Laboratories (M) Sdn. Bhd., Malaysia. KOD hot start DNA polymerase (Novagen) was used for the amplification of the *AnabfA* gene. Restriction enzymes were obtained from Promega and Fermentas. All other chemicals were of analytical grade and were obtained from Sigma, Amresco, Fluka and Merck.

### Construction of expression plasmids

To achieve secretive expression of *AnabfA*, an *E. coli* *P. pastoris* shuttle vector, pPICZ $\alpha$ , was used. The pPICZ $\alpha$  vector contains the tightly regulated *AOX1* promoter and the *Saccharomyces cerevisiae*  $\alpha$ -mating factor secretion signal located immediately upstream of the multiple cloning site (Higgins et al., 1998). The mature *AnabfA* gene was amplified using gene-specific primers. The mature *AnabfA* DNA to be cloned into the pPICZ $\alpha$  vector was amplified using the forward primer *FabfA* (5'-AATCCGCGGATCTCCTTGAAGGTCTCCACC-3'), which contained a *SacI* site (underlined) and the reverse primer *RabfA* (5'-TTTTCTAGAGAGTTCGCCGCCAGGACAGCCAC-3'), which contained an *XbaI* site (underlined). The newly amplified gene contained two new flanking restriction sites, *SacI* and *XbaI*, which allowed the *AnabfA* gene to be ligated in-frame with the start codon of the  $\alpha$ -factor secretion signal in pPICZ $\alpha$ . The amplification was carried out under the following conditions: 3 min initial denaturation at 94°C, followed by 30 cycles for 2 min at 94°C, 1 min at 58°C and 3 min at 72°C, followed by a final cycle for 10 min at 72°C for final extension. The PCR product obtained was gel-purified and then, digested with *SacI* and *XbaI* (Promega, USA) before cloning into pPICZ $\alpha$  and transformation into *E. coli* JM109 competent cells. The constructed vector designated as pPICZ $\alpha$ -*abfA* was screened on low-salt LB agar plate containing 25  $\mu$ g/ml of Zeocin<sup>TM</sup> to detect the presence of the recombinant plasmid. The insertion was checked by restriction analysis and sequencing.

### Construction of yeast transformants

For *P. pastoris* integration, about 10 to 20  $\mu$ g of the recombinant plasmid was linearized using *PmeI* (Fermentas) and was transformed into *P. pastoris* X-33 by electroporation as described by the manufacturer. The parent construct pPICZ $\alpha$ , which lacked an insert, was linearized with *PmeI* and was transformed as the negative control. Transformants were selected at 28°C on YEPD agar plates containing 100  $\mu$ g/ml of Zeocin<sup>TM</sup>. The integration of the *AnabfA* gene into the genome of *P. pastoris* was confirmed by colony PCR using 5' *AOX1* and 3' *AOX1* primers. The methanol utilisation phenotype (Mut) was checked by plating the transformants onto MM (1.34% YNB, 4X10<sup>-5</sup>% biotin and 0.5% methanol) and MD (1.34% YNB, 4X10<sup>-5</sup>% biotin and 2% dextrose) plates as described by the manufacturer. GS115/Mut<sup>S</sup> Albumin and GS115/pPICZ//*lacZ* Mut<sup>+</sup> strains were plated as controls for the Mut<sup>S</sup> and Mut<sup>+</sup> phenotypes, respectively.

### Expression of recombinant *AnabfA* in *P. pastoris*

*P. pastoris* transformants were grown in 100 ml fresh BMGY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate (pH 6.0), 1.34% YNB, 4X10<sup>-5</sup>% biotin and 1% (v/v) glycerol) in a 250 ml baffled flask at 28°C and 250 rpm until the culture reached an A<sub>600</sub> of 2 to 6 (approximately 18 to 20 h). To induce *AnabfA* production in *P. pastoris*, the cell pellet was then,

harvested and resuspended in BMMY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate (pH 6.0), 1.34% YNB,  $4 \times 10^{-5}$ % biotin and 0.5% (v/v) methanol) using 1/5 of the original culture volume (20 ml). Absolute methanol was added every 24 h to a final concentration of 0.5% (v/v) to maintain induction. In order to analyse the expression levels and the optimal time post-induction to harvest, the culture supernatant was collected at 0, 1, 2, 3, 4, 5, 6 and 7 days. Expression of secreted proteins was analysed by SDS-PAGE.

#### Effect of cultural conditions on recombinant *AnabfA* expression

The production of recombinant enzymes by microbial cells is governed by genetic and biochemical controls including induction (Kumar et al., 2008). In this study, several efforts were made to increase the production of *AnabfA*. Various parameters for *AnabfA* production were optimised by maintaining all the factors at a constant level except for the one being studied. The transformants were grown in different culture media (BMGY-BMMY, BMG-BMM and MGY-MM); in medium pH levels ranging from 4.0 to 8.0 and at cultivation temperatures ranging from 25 to 37°C. Absolute methanol was added every 24 h to achieve concentrations ranging from 0.5 to 4% (v/v) to sustain the expression conditions and the induction.

#### Enzyme assay and protein determination

The following procedure was modified from Kengo et al. (2004) for the enzymatic assay of  $\alpha$ -L-arabinofuranosidase. *AnabfA* enzymatic activity was measured using *p*-nitrophenyl- $\alpha$ -L-arabinofuranoside (pNPA, Sigma N3641) as the substrate. *AnabfA* activity was assayed in a reaction mixture (1 ml) containing 2 mM pNPA in 50 mM acetate buffer (pH 5.0) and the appropriately diluted enzyme solution. The reaction mixture was incubated for 15 min at 50°C, the reaction was terminated with the addition of ice-cold 0.5 M  $\text{Na}_2\text{CO}_3$  (1 ml) and the amount of *p*-nitrophenol (pNP) liberated was determined at 415 nm. One unit (U) of *AnabfA* activity was defined as the amount of enzyme that produced 1  $\mu\text{mol}$  of pNP per minute using the earlier mentioned assay conditions. The protein content was measured according to the method of Lowry et al. (1951) using bovine serum albumin (BSA) as the protein standard. The reaction was measured at 750 nm.

#### Purification of the recombinant *AnabfA* enzyme

All the purification steps were performed at 4°C. A 5 day old culture was centrifuged at 4600 x *g* for 10 min to remove the cells. The supernatant was then subjected to ammonium sulphate fractionation (80% saturation). The pellet obtained at 80% ammonium sulphate saturation was resuspended in 50 mM sodium acetate buffer (pH 5.0) and dialysed against 10 mM sodium acetate buffer (pH 5.0) overnight at 4°C. The purified proteins were collected and characterised further. About 2  $\mu\text{g}$  of the purified proteins were analysed by SDS-PAGE on a 10% gradient gel.

#### Glycosylation analysis of the recombinant *AnabfA* expressed in *P. pastoris*

The purified recombinant *AnabfA* was digested with PNGase F (New England BioLabs) and *O*-glycosidase (Sigma) to determine whether the recombinant endoglucanase was *N*-linked or *O*-linked glycosylated, respectively. The reaction was set up according to the

manufacturer's instructions. The digested protein was analysed using SDS-PAGE.

#### Optimum temperature and thermostability

The optimum temperature for the purified *AnabfA* activity was measured by incubating the purified enzyme for 15 min at temperatures ranging from 10 to 100°C. The thermostability of *AnabfA* was investigated at temperatures 10 to 100°C after incubation of the enzyme solutions in the absence of the substrate for 30 min. A temperature profile was produced with the enzyme activity at the optimum temperature set at 100%.

#### pH optimum and stability

The optimum pH for the purified *AnabfA* was evaluated at 50°C over a pH range of 3.0 to 8.0, using appropriate buffers (50 mM): acetate buffer (pH 3.0 to 5.0) and phosphate buffer (pH 6.0 to 8.0) under *AnabfA* assay procedures. The pH stability of the enzyme was investigated further at 50°C by pre-incubation of the enzyme solutions in the described buffer systems in the absence of substrate at 40°C for 30 min. Then, they were subjected to *AnabfA* assay and a pH profile was produced with the enzyme activity at the optimum pH set at 100%.

#### Effects of metal ions on *AnabfA* activity

Metal ions are generally considered to be important factors affecting microbial enzyme activity. The reaction mixture consisted of purified enzyme with 50 mM acetate buffer (pH 5.0) containing 1 mM metal ions ( $\text{Ca}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{CO}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Na}^{2+}$  and  $\text{Zn}^{2+}$ ) and different reagents (such as 1 mM EDTA, 5% DMSO, 1% Triton X-100 and 1 M urea). The effects of these metals were investigated after 30 min of incubation at 40°C.

#### Kinetic parameters

The kinetic parameters ( $K_m$  and  $V_{max}$ ) of the purified enzyme were studied. Different substrate (pNPA) concentrations were used, ranging from 0.5 to 5.0 mM. The reaction rate versus substrate concentration was plotted to determine whether the enzyme obeys Michaelis-Menten kinetics. The Michaelis-Menten constant ( $K_m$ ) and maximum velocity of the substrate hydrolysis ( $V_{max}$ ) were determined from the Lineweaver-Burk plots.

## RESULTS AND DISCUSSION

### Amplification and over expression of the recombinant *AnabfA* in *P. pastoris*

A 2239 bp gene encoding *AnabfA* was amplified via PCR from the genomic DNA of *A. niger* ATCC 120120. The complete sequence of the *AnabfA* gene reported in this work (Figure 1) has been deposited into the GenBank database (Accession No. HM004501). Sequence analysis revealed that the *AnabfA* gene contained eight exons and seven introns.

The gene had an 1887 bp open reading frame

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1      ATGGTGGCCTTTTCAGCTCTTTCGGGCGTCAGCGCCGTTTCTTTACTACTATCCCTCGTT
1      m v a f s a l s g v s a v s l l l s l v
61     CAAAATGCACACGGAATTCCTTGAAGGTCTCCACCCAGGGTGGCAACTCATCCAGCCCC
21     q n a h g I S L K V S T Q G G N S S S P
121    ATCCTATATGGCTTCATGTTTGAGGTAGGCCGAGACTAAGCAGTAAAAACAATTGGTT
41     I L Y G F M F E                               INTRON 1
181    CTCTGACGGTGATTAGGATATCAATCACTCAGGAGATGGAGGAATTTATGGCAAATGCT
49     D I N H S G D G G I Y G Q M L
241    GCAGAACCTGGCCTTCAGGGAACGGCGCCTAATCTGACTGCCTGGGCGGCTGTCGGTGA
64     Q N P G L Q G T A P N L T A W A A V G D
301    CGCTACCATCGCCATTGATGGCGACAGTCCATTGACCTCTGCCATCCCCAGCACCATCAA
84     A T I A I D G D S P L T S A I P S T I K
361    GCTTAATATTGCGGACGATGCTACCGGTGCGGTGGGTCTCACCAATGAGGGATATTGGGG
104    L N I A D D A T G A V G L T N E G Y W G
421    CATCCAGTCGATGGCAGCGAATTCATAGTTCCTTCTGGATAAAGGGAGATTACTCCGG
124    I P V D G S E F H S S F W I K G D Y S G
481    CGACATCACCGTCCGACTGGTTGAAACTACACCGGCACGGAGTACGGCTCTACCACTAT
144    D I T V R L V G N Y T G T E Y G S T T I
541    CACCCATACGTCCACAGCAGACAACCTTACCCCAAGCCTCCGTCAAGTTCCCCACCACCAA
164    T H T S T A D N F T Q A S V K F P T T K
601    GGCTCCAGATGGCAACGTTTTGTACGAGCTCACAGTTGATGGAAGCGTGGCTGCTGGCTC
184    A P D G N V L Y E L T V D G S V A A G S
661    GTCTTTGAACTTCGGCTACTTGACGCTTTTTGGCGAAACCTATAAGTCAAGGTTTGCTTC
204    S L N F G Y L T L F G E T Y K S R
721    CCTATACTCCGAAGAGTGAATTAGGGGCTAATGTGTAGGGAAAAATGGCCTGAAGCCCCA
221    INTRON 2                               E N G L K P Q
781    GCTTGCCAATGIGTTGGATGATATGAAAGGATCCTTCTGAGATTTCCCGGCGGTAACAA
228    L A N V L D D M K G S F L R F P G G N N
841    CCTGTAAGTCTCAGCTCGCCCGTAAGTGTATAGAAGCTCATCAGAGGTAGTGAGGGAAA
248    L                               INTRON 3                               E G N
901    CAGCGCAGAAAACCGCTGGAAGTGAACGAGACAATCGGCGATCTTTGTGATCGTCCCGG
251    S A E N R W K W N E T I G D L C D R P G
961    ACGTGAAGGTATGTCTCATTATTAGGATTGAAGCATTTCATCCCTGACGGTATATAGGCAC
271    R E G                               INTRON 4                               T
1021   TTGACTTACTATAACACCGACGGACTGGGTAAGTAAAGGGCTATATACAAGTACCTAGA
275    W T Y Y N T D G L G                               INTRON 5
1081   TACTGTACTAACGCTTGTAGGCCCTTACGAATACTTTTACTGGTGTGAGGATTTGGGGCT
285    L H E Y F Y W C E D L G L

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**Figure 1.** The *A. niger* ATCC 120120 *AnabfA* gene. The deduced amino acid sequence is shown in one-letter code under the DNA sequence. The amino acid signal peptides are shown in small letters. The start of the mature protein, which is preceded by residue I, appears in italics and is underlined. Introns are shown in italics. The ten predicted *N*-glycosylated residues are in boxes. (\*) denotes the end of the sequence.

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1141 CGTACCGGTGCTCGGTGTCTGGGATGGGTTTCGCTCTGGAGTCGGGTGGCAACACCCCCCT
298   V P V L G V W D G F A L E S G G N T P L

1201 CACGGGCGACGCACTGACCCCTTATATCGACGATGTCTTGAACGAGCTCGAGGTATGTTG
318   T G D A L T P Y I D D V L N E L E

1261 AGCGGCATATCAAATTGATAGCTGAAGCTAACCCATTGGCAGTACATCTTGGGTGATACG
335   INTRON 6                               Y I L G D T

1321 AGCACGACCTATGGAGCGTGGCGCGCGGCAAACGGACAGGAGGAGCCGTGGAACCTTACC
341   S T T Y G A W R A A N G Q E E P W N L T

1381 ATGGTCGAGATTGGCAATGAGGACATGCTGGGAGGCGGATGCGAGTCTTACGCGGAACGT
361   M V E I G N E D M L G G G C E S Y A E R

1441 TTCACTGCCTTCTATGATGCGATTTCATGCTGCTTATCCGGACCTTATCCTGATTGCCAGC
381   F T A F Y D A I H A A Y P D L I L I A S

1501 ACCAGCGAGGCGGATTGCTTGCCCGAGTCAATGCCCGAGGTTAGCTGGGTGACTACCAC
401   T S E A D C L P E S M P E G S W V D Y H

1561 GACTACAGCACGCCTGATGGACTGGTGGGCCAGTTCAACTACTTCGACAATTTAAACCGC
421   D Y S T P D G L V G Q F N Y F D N L N R

1621 TCGGTACCATACTTCATCGGCGAGTACTCGCGCTGGGAGATTGACTGGCCCAACATGAAG
441   S V P Y F I G E Y S R W E I D W P N M K

1681 GGATCGGTCGCTGAAGCCGCTTTCATGATCGGGTTCGAGAGGAACAGCGACGTGGTGAAG
461   G S V A E A V F M I G F E R N S D V V K

1741 ATGGCGCGGTATGCGCCTTTGCTCCAGCTAATCAACTCGACTCAGTGGACGGTAAGTCAC
481   M A A Y A P L L Q L V N S T Q W T

1801 GACTGAGCAGCGGGGTTTCAGGTATGAATGAGCTAACGAGTGGTAGCCGGACCTGATCGG
498   INTRON 7                               P D L I G

1861 ATACACGCAGTCACCCGGTGACATTTTCTGTGCGACCAGCTACTACGTGCAGGAGATGTT
503   Y T Q S P G D I F L S T S Y Y V Q E M F

1921 CTCGCGCAACCGGGGTGATACAATTAAGGAGGTGACGTGGACAGCGACTTCGGACCGTT
523   S R N R G D T I K E V T S D G D F G P L

1981 GTACTGGGTTGCGTCGAGCGCCGGGACTCGTACTACGTGAAGCTGGCCAACTATGGCTC
543   Y W V A S S A G D S Y Y V K L A N Y G S

2041 CGAGACGCAAGACCTCACGGTGAGCATCCAGGAACGAGCACAGGCAAGTTGACGGTGCT
563   E T Q D L T V S I P G T S T G K L T V L

2101 GGCGACAGTGATCCGGATGCGTATAACTCGGACACCCAGACGCTGGTCACGCCGAGTGA
583   A D S D P D A Y N S D T Q T L V T P S E

2161 ATCGACGGTGCAGGCGAGCAATGGCACTTTTACCTTTAGTTTGCCGGCATGGGCGGTGGC
603   S T V Q A S N G T F T F S L P A W A V A

2221 TGTCTGGCGGCGAACTAG
623   V L A A N *

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**Figure 1.** Continued.

encoding 628 amino acid (aa) residues. Based on the computer analysis of the deduced amino acid sequence by signal 3L server, the mature *AnabfA* was predicted to be preceded by residue I (Isoleucine) at position 26. Thus, the mature protein was predicted to be 603 aa

long.

Comparison of the amplified 1887 bp *AnabfA* fragment with other  $\alpha$ -L-arabinofuranosidases was performed using the BLAST program to search the NCBI database. The *AnabfA* nucleotide sequence showed very high identity

(100%) to *A. niger* CBS 120.49 *abfA* (L29005), 99% identity to *A. niger* CBS 513.88 *abfA* (XM001388445) and 96% identity to both *Aspergillus awamori* *AwabfA* (AB046702) and *A. kawachii* *AkabfA* (AB085903). The accession numbers of the nucleotide sequence data are shown in parentheses. On the basis of its similarity to other  $\alpha$ -L-arabinofuranosidases, *A. niger* ATCC 120120 *AnabfA* was found to belong to the GH51 family.

The gene coding the mature *AnabfA* was fused in-frame to the  $\alpha$ -mating factor secretion signal and this construction was ligated with the *P. pastoris* expression vector pPICZ $\alpha$ A to produce the recombinant expression plasmid pPICZ $\alpha$ A-*abfA*. pPICZ $\alpha$ A-*abfA* was then transformed and integrated into the *P. pastoris* X-33 genome. Integration of multiple gene copies of the gene increases the gene dosage, which affect the expression level of *AnabfA*. Selection of recombinant *P. pastoris* containing a high number of copies of the *AnabfA* gene was done using antibiotic screening.

The transformants were selected on YEPD plates supplemented with Zeocin™ and the integration of the *AnabfA* gene into the *AOX1* location in the *Pichia* genome was further confirmed by colony PCR (data not shown). The  $\alpha$ -factor signal peptide from *S. cerevisiae* aided in the secretion of *AnabfA* into the culture medium. The transformants were found to have a methanol utilisation plus (*Mut*<sup>+</sup>) phenotype; they grew normally on MM and MD plates after two days of incubation at 28°C.

Colonies found to be positive using PCR were selected for small-scale induction. The *AnabfA* activity was monitored after 1, 2, 3, 4, 5, 6 and 7 days of the methanol induction. No *AnabfA* activity was detected in the supernatant of the *P. pastoris* transformant containing only pPICZ $\alpha$ A (negative control). The highest level of the recombinant enzyme was produced 5 days after induction.

### Effect of cultural conditions on recombinant *AnabfA* expression

In order to improve recombinant *AnabfA* production, several different cultural conditions were manipulated. Tanfous et al. (2006) reported that, cultural conditions are considered to be critical parameters that significantly influence the yield of recombinant product. Temperature and pH are among the factors that dramatically influence protein expression. Because of this, a study on the effects of medium, pH, cultivation temperature and methanol concentration on the expression of *AnabfA* by *P. pastoris* was performed.

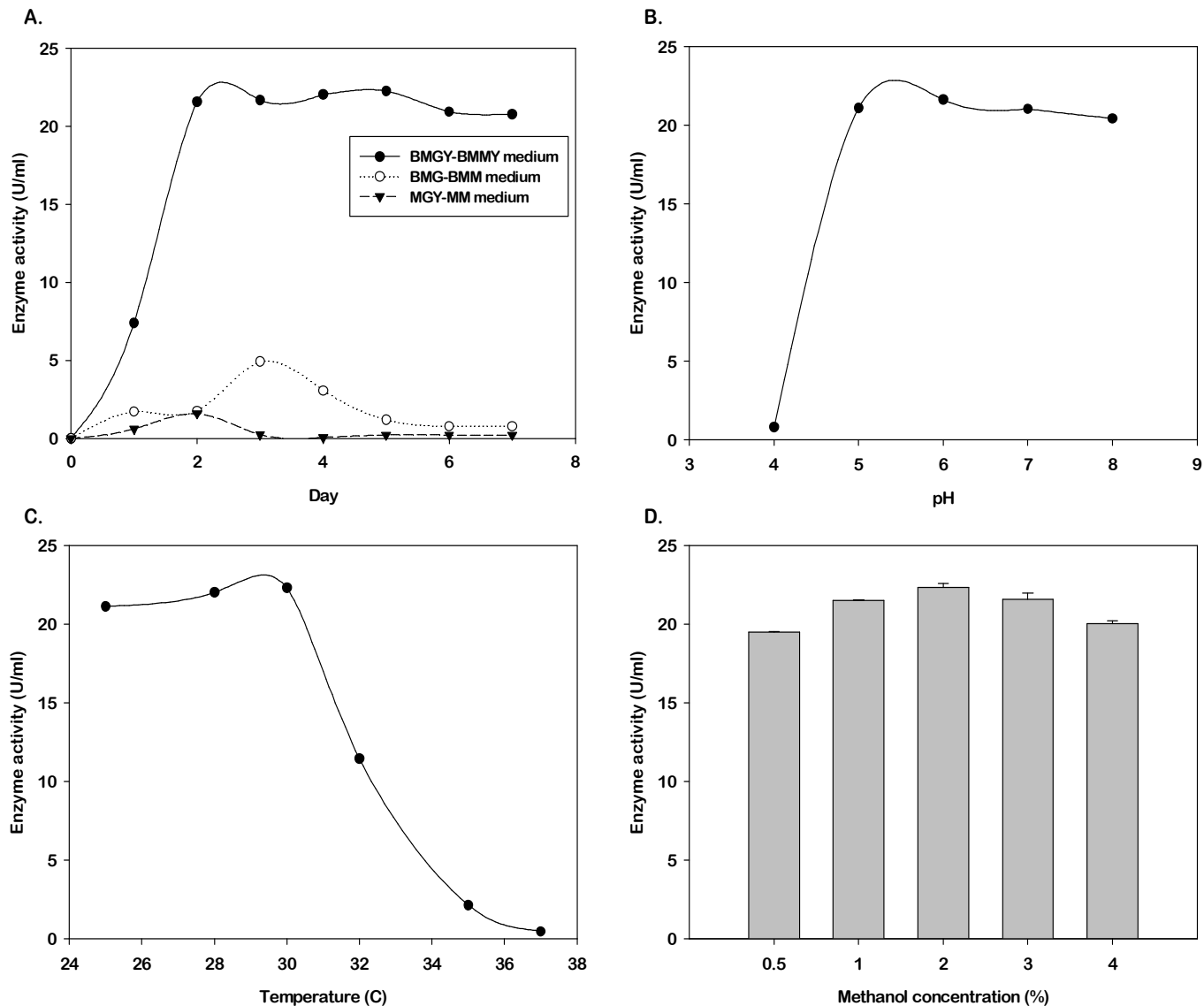
*P. pastoris* X-33 (along with the negative control) was inoculated in three different media (BMGY, BMG and MGY media) in 250 ml shake flasks. BMGY, BMG and MGY media were used to repress expression before the inoculums were transferred into BMMY, BMM and MM media to induce expression. The *AnabfA* activity of the

supernatant was monitored for 7 days. Figure 2a shows that, expression of recombinant *AnabfA* was found to be significantly higher in the supernatant of BMMY medium than in BMM and MM media. The addition of complex components (yeast extract and peptone) to the BMMY medium was shown to enhance protein expression levels. A previous study showed that, the heterologous expression of the xylanase gene from *A. niger* BCC14405 in *P. pastoris* strain KM71 was expressed at high levels when cultured in BMMY induction medium (Ruanglek et al., 2007). When the growth kinetics of the recombinant *P. pastoris* producing *A. niger* BCC14405 xylanase was studied in BMMY and BSM media using the batch cultivation process (Ruanglek et al., 2007), the volumetric xylanase production (U/ml) was found to be 2-fold higher in BMMY medium than in the BSM medium. Because the complex media (yeast extract and peptone) and mineral media for *P. pastoris* (containing glycerol or methanol, biotin, salts and trace elements) are economical and well-defined, this yeast is nearly ideal for use in large-scale production of heterologous proteins in fermenters (Cereghino et al., 2002).

One of the important parameters that affects the recombinant *A. niger* BCC14405 xylanase production in *P. pastoris* KM71 (Ruanglek et al., 2007) is the pH of the medium. In this study, the effect of pH on *AnabfA* production was assessed by cultivating the yeast in BMMY media at various pH levels (pH 4.0 to 8.0). Maximum *AnabfA* activity was reported when the cultivation was carried out in BMMY medium at pH 6.0. As shown in Figure 2b, the production of recombinant *AnabfA* was still detected at higher pH levels. The recombinant *P. pastoris* totally lost its ability to produce *AnabfA* when cultivated in BMMY medium at pH 4.0.

Temperature is a critical parameter in *P. pastoris* fermentation. It may also affect recombinant protein production at ultra-high cell densities. In this study, the effect of cultivation temperature on *AnabfA* production was assessed by cultivating the yeast in BMMY medium (pH 6.0) at various temperatures including 25, 28, 30, 32, 35 and 37°C. Figure 2c shows that, the expression of the recombinant *AnabfA* was best observed at 28°C. When the cultivation temperature was reduced to 25°C, the expression was slightly decreased. A decrease in *AnabfA* expression was observed when the cultivation temperature was increased to 32°C. At higher temperatures (35 to 37°C), *AnabfA* activity was barely detectable. Hong et al. (2002) reported that, high cultivation temperatures can induce the release of protease from dead cells and may also affect the protein folding process.

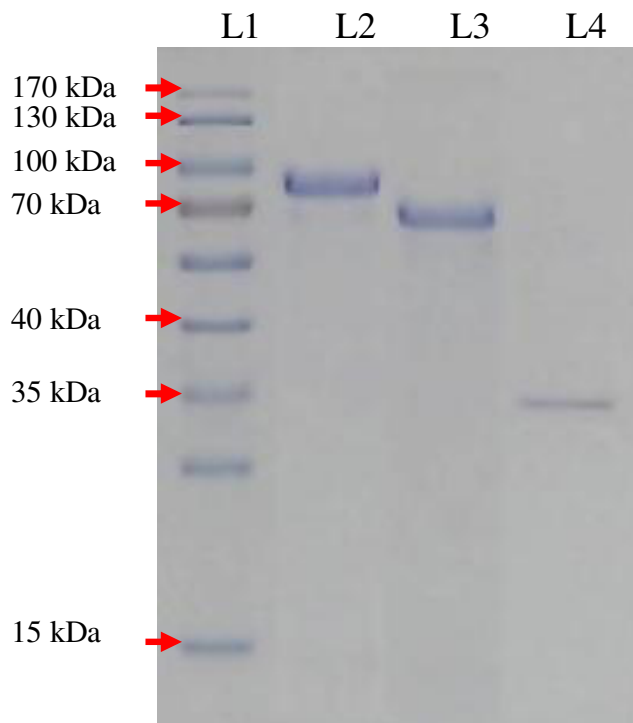
The *P. pastoris* expression system is popular for many reasons. One reason is that, the *AOX1* gene contains an unusually efficient and tightly regulated promoter that can be used to drive the expression of foreign genes (Cregg and Madden, 1988). The *AOX1* promoter is strongly repressed in cells grown on glucose and most other carbon sources, but it is induced over 1000-fold when



**Figure 2.** A, Effect of medium on recombinant *AnabfA* expression. Based on the enzymatic assay analysis, the production of *AnabfA* was found to be significantly higher in the supernatant of BMGY-BMMY. The addition of yeast extract and peptone in BMGY-BMMY medium was shown to enhance protein expression level; B, effect of pH on recombinant *AnabfA* expression. When the cultivation was carried out in a BMGY-BMMY medium at pH 6, the maximum *AnabfA* activity was reported; C, effect of cultivation temperature on recombinant *AnabfA* expression. Expression of the recombinant *AnabfA* in *P. pastoris* X-33 was best observed when the cultivation temperature was maintained at 28°C; D, effect of methanol concentration on recombinant *AnabfA* expression. The highest level of expression of recombinant *AnabfA* appeared after 2% (v/v) methanol induction.

cells are shifted to a medium containing methanol as the sole carbon source (Cereghino et al., 2002). In this study, *P. pastoris* X-33 was induced every 24 h using various concentrations of methanol (0.5, 1, 2, 3 and 4%), when cultured in BMMY medium (pH 6.0) at 28°C at 250 rpm. The highest level of *AnabfA* production occurred with 2% methanol (Figure 2d). However, induction using methanol at a level either lower or higher than 2% reduced the expression level of the recombinant *AnabfA* in *P. pastoris* X-33. On the other hand, the highest level of expression

for recombinant xylanase isolated from *Aspergillus usarii* E001 expressed in *P. pastoris* GS115 occurred only after induction with 1.5% methanol and the expression decreased when induced with 2% methanol (Zhou et al., 2008). Induction with 3% methanol was reported for expression of the xylanase from *A. niger* BCC14405 in *P. pastoris* KM71 (Ruanglek et al., 2007). They also reported that, a higher concentration of methanol (3%) was not toxic, yet it could be a major factor in for higher xylanase production.



**Figure 3.** Purification and glycoprotein analysis of the recombinant *AnabfA* expressed in *P. pastoris* X-33. L1, PageRuler™ Prestained Protein Ladder (Fermentas); L2, 83 kDa purified recombinant *AnabfA* (sample after dialysis); L3, 66 kDa purified recombinant *AnabfA* digested using the PNGase F enzyme; L4, 36 kDa PNGase F enzyme (control).

### Purification and glycoprotein analysis of recombinant *AnabfA*

To examine the catalytic properties of *AnabfA* produced by *P. pastoris*, the recombinant enzyme was purified using a two-step procedure involving ammonium sulphate precipitation and dialysis. The purified enzyme was resolved as a single band by SDS-PAGE when visualised by Coomassie brilliant blue R-250 staining. The molecular weight of the purified *AnabfA* protein was estimated to be 83 kDa (Figure 3).

Previous studies reported that, the  $\alpha$ -L-arabinofuranosidases from various *Aspergillus* species had a molecular weight of about 30 to 118 kDa. Purified recombinant  $\alpha$ -L-arabinofuranosidase from *Aspergillus oryzae* (*AbfA*) had a molecular mass of 55 kDa (Kengo et al., 2004) and the enzyme purified from *Aspergillus sojae* (*AFdase*) was found to have a molecular mass of 52.3 kDa (Kimura et al., 2000). Moreover, *A. awamori* IFO 4033 produced two extracellular  $\alpha$ -L-arabinofuranosidases,  $\alpha$ -L-AFases I and II. The molecular weights of the purified  $\alpha$ -L-AFases I and II were estimated to be 81 kDa and 62 kDa, respectively (Kaneko et al., 1998).

In this study, potential *N*- and *O*-glycosylation sites were identified in the mature *AnabfA* using the NetNGlyc

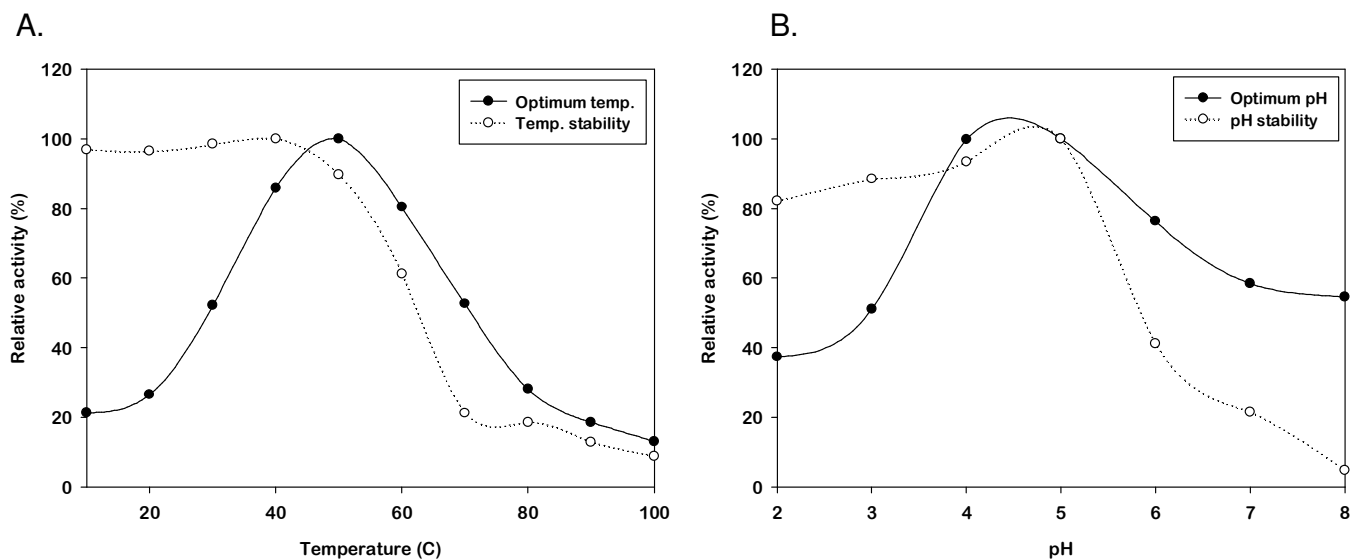
3.1 and NetOGlyc 3.1 server. Ten potential *N*-glycosylation sites and no potential *O*-glycosylation sites were found. Protein glycosylation in the eukaryotic cells is thought to be important for proper protein folding, transport and protein stability (Koseki et al., 2006). Thus, the purified *AnabfA* was treated with PNGase F (New England BioLabs) in order to confirm that the enzyme was *N*-linked glycosylated (PNGase F cleaves *N*-linked glycoproteins). As shown in Figure 5, the purified *AnabfA* from *A. niger* ATCC 120120 before and after treatment with PNGase F migrated by SDS-PAGE with a relative molecular mass of 83 and 66 kDa, suggesting that the native enzyme contained *N*-linked oligosaccharides. The 66 kDa band is in agreement with the molecular weight of the deduced amino acid sequence of mature *AnabfA* from *A. niger* ATCC 120120, which was calculated to be 65.334 kDa using CLC sequence viewer (Version 6.0.1) software. The *myc* epitope and the polyhistidine tag at the C-terminal of the protein corresponded to 2.5 kDa.

### Enzymatic properties of the purified recombinant *AnabfA*

The biochemical properties of the purified recombinant *AnabfA* were examined. As shown in Figure 4a, the purified enzyme displayed optimum activity at 50°C in 50 mM acetate buffer (pH 5.0) using pNPA as a substrate. In the absence of the substrate, *AnabfA* exhibited stability at temperatures up to 50°C; retaining 90% of its activity after pre-incubation at 40°C for 30 min. Higher temperatures resulted in a complete decrease in the enzyme activity. Most *Aspergillus*  $\alpha$ -L-arabinofuranosidases reported in literature exhibited optimum activity at temperatures range from 50 to 65°C (Saha, 2000). Extracellular  $\alpha$ -L-arabinofuranosidases from *A. kawachii*, *AkabfA* and *AkabfB* were most active at 55°C and were stable at temperatures up to 60 and 55°C, respectively (Koseki et al., 2003). On the other hand,  $\alpha$ -L-arabinofuranosidase B (*abfB*), from the fungal plant pathogen *F. oxysporum* f. sp. *dianthi*, was most active at 50°C (Carlos et al., 2004).

The effect of pH on the activity and stability of the purified recombinant *AnabfA* was also determined. The enzyme exhibited optimum activity at pH 4.0 and was stable from pH 3.0 to 6.0 with a gradual loss of activity at higher and lower pH values as shown in Figure 4b. The pH stability assay showed that more than 80% of the initial activity was retained after pre-incubation at 40°C in the absence of the substrate. Recombinant *AnabfA* was slowly inactivated at pH levels above 7.0. Such inactivation also occurred in arabinofuranosidases ( $\alpha$ -L-AFases) I and II from *A. awamori* IFO 4033 (Kaneko et al., 1998). In addition,  $\alpha$ -L-arabinofuranosidases isolated from *A. kawachii* (*AkabfA* and *AkabfB*) and *A. awamori* (*AwabfA* and *AwabfB*) were found to be acidophilic and acid-stable enzymes with an optimum pH of 4.0 and stability at pH 3.0 to 7.0. These data suggest that these





**Figure 4.** A, Effect of temperature on purified recombinant AnabfA activity and stability. The purified recombinant *A. niger* ATCC 120120 AnabfA had optimum activity at 50°C in 50 mM acetate buffer (pH 5) and exhibited stability at temperatures up to 50°C; B, effect of pH on purified recombinant AnabfA activity and stability. The purified recombinant *A. niger* ATCC 120120 AnabfA exhibited an optimum activity at pH 4 and was stable from pH 2 to pH 6 with a gradual loss of activity at higher and lower pH values.

enzymes, like the enzyme described in this paper, can function in a low-pH environment (Koseki et al., 2003). The recombinant AnabfA secreted by *P. pastoris* X-33 might be suitable for use in the animal feed industry because it is active over pH levels ranging from 3.0 to 6.0 and is stable below 50°C; these conditions closely resemble the environment found in the digestive systems of animals (Zhou et al., 2008).

#### Effect of cations and reagents on AnabfA activity

Metal ions are generally considered to be important factors affecting microbial enzyme activity (Zhou et al., 2008). In this study, each metal ion was added to the diluted enzyme and the effects of these metal ions were investigated after 30 min of incubation. The effects of the metal ions on the purified recombinant AnabfA activity are summarised in Table 1. It was found that several ions, such as  $K^+$ ,  $Mn^{2+}$ ,  $Na^{2+}$  as well as triton X-100, could enhance and activate the enzyme, while other ions ( $Cu^{2+}$  and  $Fe^{2+}$ ) inhibited AnabfA activity significantly. The enzyme was relatively unaffected by  $Ca^{2+}$ ,  $CO^{2+}$ ,  $Mg^{2+}$  and EDTA.

$K^+$ ,  $Mn^{2+}$  and  $Na^{2+}$  ions enhanced the activity of the purified AnabfA by about 1.14- (13.85%), 1.36- (36.48%) and 1.12-fold (11.77%), respectively. The addition of the chemical reagent triton X-100 strongly stimulated the activity by about 1.50-fold (49.58%). However, the non-ionic detergent triton X-100 had a mildly stimulatory effect on  $\alpha$ -L-arabinofuranosidase from *Thermobacillus xylanilyticus* (Debeche et al., 2000). A study to evaluate

the effects of metal ions on the activity of  $\alpha$ -L-arabinofuranosidase from Japanese pear fruits showed that 1 mM  $Mn^{2+}$  enhanced the activity of the enzyme by about 1.17-fold (17%). Nevertheless, the addition of 0.3 mM  $Mn^{2+}$  had little or no effect on  $\alpha$ -L-arabinofuranosidase activity (Tateishi et al., 1996), which seems to contradict our results.

The activity of the purified AnabfA was strongly inhibited by  $Cu^{2+}$  and  $Fe^{2+}$  at a level of about 65.63% for both ions. Thus, these heavy metal ions ( $Cu^{2+}$  and  $Fe^{2+}$ ) had a significantly inhibitory effect on the purified recombinant AnabfA activity. In the presence of  $Zn^{2+}$ , DMSO and urea, the activity was absolutely inhibited, but the enzyme activity was reduced to less than 50%. Strong inhibition by  $Cu^{2+}$  and  $Fe^{2+}$  has been reported for  $\alpha$ -L-arabinofuranosidase enzyme purified from *Bifidobacteriu breve* K-110 (Shin et al., 2003).  $Cu^{2+}$  had an inhibitory effect; decreased the activity of  $\alpha$ -L-arabinofuranosidase from *Thermotoga maritima* MSB8 (Miyazaki, 2005) to about 50%. Also, Shi et al. (2009) reported that the addition of  $Cu^{2+}$  inhibited the activity of *Streptomyces* sp. S9  $\alpha$ -L-arabinofuranosidase (Abf51S9) significantly, suggesting that Abf51S9 is a thiol-sensitive enzyme because these heavy metal ions bind free mercapto groups (-SH) in Cysteine residues. Based on DNAMAN software version 5.2.10, there were four Cysteine residues in *A. niger* ATCC 120120 AnabfA: Cys267, Cys293, Cys375 and Cys407, which could behave like the Cysteine in Abf51S9.

The addition of other metal ions and chemical reagents ( $Ca^{2+}$ ,  $CO^{2+}$ ,  $Mg^{2+}$  and EDTA) had little or no effect on the

Table 1. Effects of various cations and reagents on AnabfA activity.

Metal ion	Relative activity (100%)
Ca <sup>2+</sup>	104.96
Cu <sup>2+</sup>	34.37
CO <sup>2+</sup>	99.51
Fe <sup>2+</sup>	34.37
K <sup>+</sup>	113.85
Mg <sup>2+</sup>	106.42
Mn <sup>2+</sup>	136.48
Na <sup>2+</sup>	111.77
Zn <sup>2+</sup>	89.33
<b>Reagent</b>	
EDTA	103.47
DMSO	87.55
Triton X-100	149.58
Urea	84.86

\*Control (100% relative activity), no metals added to the enzyme solutions.

purified recombinant *AnabfA* activity. Enzyme activity was increased to less than 10% by the addition of Ca<sup>2+</sup> (4.96%), Mg<sup>2+</sup> (6.42%) and EDTA (3.47%). However, a study conducted by Miyazaki (2005) reported that, when EDTA, Mg<sup>2+</sup> and Ca<sup>2+</sup> were added to diluted *T. maritima* MSB8  $\alpha$ -L-arabinofuranosidase, the enzyme's activity did not change. Le Clinche et al. (1997) found that CO<sup>2+</sup>, Ca<sup>2+</sup> and EDTA did not affect the *A. terreus*  $\alpha$ -L-arabinofuranosidase activity. These findings suggest that Ca<sup>2+</sup>, CO<sup>2+</sup>, Mg<sup>2+</sup> and EDTA play minor roles in enzyme activity.

### Kinetic study

The study of the initial reaction rates versus substrate concentration showed that, the enzyme obeyed Michaelis-Menten kinetics. The data revealed that, the Michaelis-Menten constant ( $K_m$ ) and maximum forward velocity of the reaction ( $V_{max}$ ) for the purified recombinant *A. niger* ATCC 120120 *AnabfA* activity towards pNPA were 0.93 mM and 17.86  $\mu$ mol/ml/min, respectively. The  $K_m$  value represents the substrate concentration at which half of the enzyme sites in the sample are filled. Lower  $K_m$  value indicates a higher affinity of the substrate for the enzyme.

The recombinant *A. niger* ATCC 120120 *AnabfA* expressed in *P. pastoris* X-33 showed a higher affinity towards the substrate (pNPA) compared with the wild-type  $\alpha$ -L-arabinofuranosidase 54 from *A. kawachii* (Koseki et al., 2006). Koseki et al. (2006) reported that,  $K_m$  and  $V_{max}$  values of wild-type  $\alpha$ -L-arabinofuranosidase 54 from *A. kawachii* expressed in *P. pastoris* GS115 were

1.04 mM and 0.92  $\mu$ mol/min/mg, respectively. Prior to this study, there were no reported  $K_m$  and  $V_{max}$  values for the recombinant  $\alpha$ -L-arabinofuranosidase isolated from *A. niger* ATCC 120120 and expressed in *P. pastoris*.

### Conclusions

Heterologous expression is currently the most important tool for the production of industrial enzymes. The *P. pastoris* expression system has been successfully employed for the production of various recombinant heterologous proteins. A number of properties make *Pichia* well-suited for this task. In this study, the recombinant *A. niger* ATCC 120120  $\alpha$ -L-arabinofuranosidase (*AnabfA*) was expressed and efficiently secreted as a glycosylated protein by *P. pastoris* X-33. The effects of culture conditions on enzyme expression were studied in order to improve *AnabfA* production. The recombinant *AnabfA* was characterised and the kinetic parameters were determined. To our knowledge, this is the first report describing the expression of the  $\alpha$ -L-arabinofuranosidase gene from *A. niger* using the *P. pastoris* system.

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