CLONING, HETEROLOGOUS EXPRESSION AND CHARACTERISATION OF A RECOMBINANT CELLOBIOHYDROLASE FROM *Humicola insolens* ATCC16454 IN *Pichia pastoris*

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ABSTRACT

A cellobiohydrolase gene from the thermophilic fungus *Humicola insolens* ATCC 16454 was expressed in the methylotrophic yeast *Pichia pastoris* X-33, and the biochemical properties of the recombinant protein were characterised. The full-length cDNA of the cellobiohydrolase gene *avi2* was cloned into the *P. pastoris* expression vector pPICZαC and expressed extracellularly as a recombinant cellobiohydrolase protein with a molecular weight of approximately 52.3 kDa. The purified recombinant Avi2 enzyme displayed an optimal activity at 50°C and was found stable between temperatures of 30°C and 60°C. The optimal pH of the enzyme was pH 5.0. More than 80% of the enzyme activity was retained at pH values ranging from pH3.0 to pH9.0. Recombinant Avi2 enzyme showed its highest activity towards the substrates Avicel (0.075 U mg^{-1}) and Sigmacell-cellulose (0.018 U mg^{-1}). Very low or undetectable hydrolysis was observed with cellobiose and filter paper. Metal ions, such as Mn^{2+}, Co^{2+}, and Ba^{2+}, increased the activity of the recombinant enzyme. Manganese ions caused the highest increase in activity of approximately 1.38-fold compared to the control assay. Other ions such as Pd^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, and SDS, however, inhibited Avi2 enzyme activity. Interestingly, this recombinant enzyme showed high pH stability when it was incubated in either acidic or basic solutions.

Key words: Cellobiohydrolase, *Humicola insolens*, *Pichia pastoris*, recombinant enzyme

INTRODUCTION

Cellulose is one of the major components of lignocellulosic materials and is a complex polymer of glucose. The degradation of celluloses into glucose has been studied as an alternative source of energy for the production of bioethanol and fine chemicals. Bioethanol, a product from agricultural or forest wastes and dedicated energy crops, is considered to be the renewable energy source with the greatest potential for the replacement of petroleum-derived fossil fuels (Cardona and Sanchez, 2007).

The hydrolysis of cellulosic materials using enzymes has been studied extensively over the past few decades as a way to increase the yield of fermentable sugars using cleaner processes (Sun and Cheng, 2002). In many industrial processes, enzymatic hydrolysis is increasingly desirable because of the high specificity of the reactions, the mild processing conditions and better control of the processes. There are three components of cellulases that work synergistically to degrade celluloses, which include endoglucanase (EG, EC: 3.2.14), exoglucanase or cellobiohydrolase (CBH, EC: 3.2.1.91) and β-glucosidase (BG, EC: 3.2.1.21). Endoglucanases randomly hydrolyse the internal glycosidic linkages in the amorphous regions of the cellulose fibres. This activity opens up sites for cellobiohydrolases, which are exo-type enzymes that hydrolyse the ends of cellulose chains and release cellobiose and glucose. β-glucosidases further cleave cellobioses into glucose monomers.

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Endoglucanases, cellobiohydrolases and β-glucosidases therefore act synergistically to degrade cellulose polymers into glucose units (Vlasenko *et al*., 2010).

Thermostable cellulases have several potential advantages for the hydrolysis of lignocellulosic materials, including higher specific activity and stability (allowing for longer hydrolysis times). Carrying out the hydrolysis at higher temperatures, therefore, ultimately leads to an improved performance (Viikari *et al*., 2007). Thermostable cellulases are usually obtained from thermophilic filamentous fungi, such as *Humicola insolens*. Hayashida and Yoshioka (1980) isolated *H. insolens* YH-8 from manure and compost heaps and showed that the cellobiohydrolase that was produced by this fungus was thermostable. The optimal temperature of the enzyme was 50°C, and it was stable even after heating at 65°C for 5 min. The gene that encodes *H. insolens* cellobiohydrolase (*avi2*) was subsequently cloned and expressed in *Aspergillus oryzae* (Woldike *et al*., 1991). Moriya *et al*. (2003) also cloned and overexpressed Avi2 in *H. insolens* and observed up to 8-fold increase in expression compared to the native host. However, in both studies, detailed biochemical characterisation of the enzyme was not described. Furthermore, the expression of Avi2 in yeast has not been previously reported. Therefore, we cloned the cellobiohydrolase gene *avi2* from *H. insolens* ATCC 16454 and overexpressed the recombinant enzyme in the methylotrophic yeast *P. pastoris*. We then further characterised the biochemical properties of the purified recombinant cellobiohydrolase enzyme produced.

MATERIALS AND METHODS

Fungal growth medium, condition and total RNA extraction

Humicola insolens ATCC 16454 was obtained from the American Type Culture Collection (ATCC). The fungus was cultured on potato dextrose agar (PDA) for 5 to 7 days at 40°C (Takashima *et al*., 1999). In order to obtain the fungal mycelium for RNA extraction, a fungal suspension of approximately 1 x 10^6 spores/ml was sub-cultured in 100 ml of potato dextrose yeast extract (PDYE) medium at 40°C and incubated with shaking at 180 rpm for 24 hours. The spores were then transferred into a flask that contained Mendel's medium with 5 g of filter paper (Quay *et al*., 2011). The culture was incubated at 40°C for 5 days at 150 rpm. Mycelia were then harvested and frozen in liquid nitrogen. TRIzol® reagent (Invitrogen, USA) was used for the total RNA extraction from the powdered mycelia as described by Oh *et al*. (2009).

cDNA synthesis

The Access RT-PCR System (Promega, USA) was used to synthesize *avi2* cDNA using total RNA as the template. The specific primers used were designed based on the complete *avi2* sequence of *H. insolens* FERM BP-5977, which is available in GenBank (accession no. AB048710). The sequence of the forward primer was 5' GCAGTACCTGGGA CAAG 3', and the sequence of the reverse primer was 5' GGTTTGAGGCGTTTGGCG 3'. First strand cDNA synthesis was carried out at 45°C for 45 min and 94°C for 2 min for one cycle. Second strand cDNA synthesis and PCR amplification cycles were performed as follows: 94°C for 30 sec, 64.3°C for 1 min and 68°C for 2 min for 40 cycles, with a final extension at 68°C for 15 min. The PCR product was purified from an agarose gel and cloned into the pGEM®-T Easy Vector (Promega, USA). The cDNA was then sequenced using BigDye® Terminator v3.1 Cycle Sequencing (Applied Biosystems, USA).

Construction of the expression vector

The *avi2* cDNA without the sequence that encodes its native signal peptide was cloned and sequenced. SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/; Bendtsen *et al*., 2004) was used to predict the signal peptide. Primers to amplify the cDNA fragment encoding mature Avi2 were designed to contain *Cla*I and *Xba*I restriction sites at the 5' and 3' ends, respectively. The sequence of the forward primer was 5' GCATCGATTGCTCCC GTTGTT 3', and the sequence of the reverse primer was 5' TGTTCTAGAAAGAACGGCGGA 3'. The PCR amplification cycles were as follows: 94°C for 5 min for initial denaturation, 30 cycles of 94°C for 1 min, 64.5°C for 1 min, 72°C for 1.5 min and a final extension step at 72°C for 20 min. The PCR fragment was then digested with *Cla*I and *Xba*I and ligated into the *Cla*I/*Xba*I sites of the *P. pastoris* expression vector pPICZαC (Invitrogen, USA) to produce the expression cassette avi2_pPICZαC. The cloned cDNA was then sequenced to verify the gene sequence.

Transformation of *P. pastoris* and screening for positive transformants

Transformation of *P. pastoris* was performed via electroporation. The procedure was carried out according to the protocols described by the EasySelect™ *Pichia* Expression System instruction manual (Invitrogen, USA). The expression cassette avi2_pPICZαC was linearised with *Pme*I endonuclease (NEB, USA) to allow the expression cassette to integrate into the *AOX*1 locus of *P. pastoris* strain X-33. Transformants of *P. pastoris* carrying *avi2* cDNA were selected using Yeast Extract-Peptone-Dextrose (YPD) agar plates containing 100 μg/ml Zeocin™ (Invitrogen, USA).

Zeocin-resistant colonies were replica-plated onto YPD plates that contained different concentrations of zeocin (1000 μg/ml and 2000 μg/ml) to detect multicopy transformants. PCR amplification was subsequently carried out using the 5'*AOX*1 primer (5'-GACTGGTTCCAATTGACAAGC-3') and the 3'*AOX*1 primer (5'-GCAAATGGCATTCTGACA TCC-3') to verify the integration of the targeted gene at the *AOX*1 locus in the *P. pastoris* genome.

Expression of recombinant cellobiohydrolase enzyme Avi2 in *P. pastoris*

Expression in *P. pastoris* was carried out by culturing the yeast in 50 ml of Buffered Glycerol Complex Medium (BMGY, containing 1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base and 1% glycerol) at 30°C and 250 rpm for 24 hr or until the cell density reached an OD_{600} between 2 to 6. Yeast cells from the BMGY were then harvested by centrifugation and resuspended in 50 ml of Buffered Minimal Methanol Medium (BMMY). BMMY medium had the similar components as BMGY medium except that the 1% glycerol was replaced with 1% methanol. Cultures were grown under the same conditions for 3 days, and 3% of absolute methanol was added every 24 hr into the culture to maintain induction. The culture supernatants were centrifuged at 4°C for 5 min at 4000 rpm and concentrated using Vivaspin Centrifugal Concentrators with a 10-kDa cut-off (Sartorius-stedim, USA).

Purification of the recombinant enzyme

To purify the enzyme, the pre-concentrated supernatant was applied to a HisTrapTM column (GE Healthcare, USA). The HisTrapTM column was pre-equilibrated with binding buffer (pH 7.4) containing 20 mM $NaHPO_4$, 1 M NaCl and 20 mM imidazole using an automated AKTA prime system (GE Healthcare, USA). The bound enzyme was then eluted with a linear gradient of elution buffer (pH 7.4) containing 20 mM NaH_2PO_4, 1 M NaCl and 300 mM imidazole. Fractions that contained the bound protein were collected, concentrated and buffer-exchanged with 50 mM sodium acetate buffer pH 5.0 using a Vivaspin device with a molecular weight cut-off of 10 kDa (Sartorius-stedim, USA). The recombinant Avi2 enzyme was analysed by SDS-PAGE and Coomassie blue staining or western blotting.

Enzyme activity and substrate specificity assays

The amount of the expressed protein was determined using a Bradford assay. For standard cellobiohydrolase assays, the reaction mixtures contained 0.2 ml of enzyme solution, 0.3 ml of 3% Avicel and 0.5 ml of 100 mM sodium acetate buffer (pH 5.0). The reaction components were mixed and incubated for 1 hr at 60°C and 1000 rpm using an Eppendorf thermomixer (Eppendorf, Germany). The optimal temperature for the purified Avi2 was determined by incubating 0.2 ml of the purified enzyme, 0.3 ml of 3% Avicel and 0.5 ml of 100 mM sodium acetate buffer (pH 5.0) at the following temperatures for 1 hr: 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, and 90°C. Enzyme temperature stability was determined by measuring the residual activity under the optimal assay condition after pre-incubation of the enzyme for 30 min in the absence of substrate at various temperatures between 30°C and 90°C. The optimal pH for the activity of the purified Avi2 was determined by incubating 0.2 ml of the purified enzyme and 1% Avicel in a 50 mM buffer solution with different pH values that ranged from 3.0 to 9.0 at the pre-determined optimal temperature. Recombinant Avi2 enzyme pH stability was studied by pre-incubating the enzyme for 30 min at 30°C in the absence of substrate at pH values that ranged from 3.0 to 9.0. The remaining enzyme activity was measured using the standard cellobiohydrolase assay. The substrate specificity test of the purified recombinant enzyme was performed with several cellulose-derived substrates, including avicel, Sigmacell-cellulose, carboxy-methylcellulose (CMC) and cellobiose. The reaction mixture contained 0.2 ml of pure enzyme in 0.8 ml of 50 mM sodium acetate buffer (pH 5.0), and the different substrates were incubated at 60°C for 1 hr. The amount of produced reducing sugar was measured using the DNS (Dinitrosalycyclic Acid) method (Miller 1959). One unit activity was defined as the amount of enzyme that produced a reducing sugar equivalent to 1 μmol of D-glucose per minute under the assay conditions.

Enzyme kinetic analysis

Enzyme kinetic analyses were carried out to determine the Michaelis-Menten constant (K_m) and the maximum velocity of substrate hydrolysis (V_{max}). By plotting a Lineweaver-Burk plot, the K_m and V_{max} of Avi2 towards Avicel and Sigmacell-cellulose were determined. The reactions were performed by incubating 0.2 ml of the enzyme solution with 0.8 ml of substrate at different concentrations in 50 mM sodium acetate buffer (pH 5.0) for 1 hr at 60°C and 1000 rpm using a thermomixer. The concentrations of the substrates that were used include a range of 2 to 10 mg/ml of Avicel and 0.5 to 3.0 mg/ml of Sigmacell-cellulose.

Effect of metal ions and reagents on purified Avi2

The effects of metal ions and chemical reagents were studied by incubating 0.2 ml of the puried recombinant enzyme with 0.4 ml of 50 mM sodium acetate buffer at pH 5.0, 0.3 ml of 3% avicel and

0.1 ml of a solution containing 10 mM of different metal ions (Ca^{2+}, K^+, Na^+, Zn^{2+}, Co^{2+}, Ba^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Pd^{2+}) or different reagents, such as SDS (1%), EDTA (1 mM) and urea (1 M). The reaction mixtures were incubated at 60°C for 1 hr, and the enzyme activity was then determined. To examine the activation or inhibition activities of the chemical reagents on recombinant Avi2 enzyme activity, EDTA was mixed in the Avi2 enzyme assay together with the activator or the inhibitor.

RESULTS

Isolation and cloning of the full-length *avi2* gene cDNA from *H. insolens* ATCC 16454

A full-length cDNA of *H. insolens* ATCC 16454 *avi2*, with the size of 1431 bp, was amplified by reverse-transcription PCR (RT-PCR) using total RNA as the template. The *avi2* cDNA sequence that was obtained showed three nucleotide differences compared to the *H. insolens* FERM BP-5977 *avi2* gene sequence that is available in GenBank (accession no. AB048710). These nucleotide changes resulted in amino acid changes at positions 28 (proline to serine), 88 (arginine to serine) and 131 (tyrosine to histidine) (Figure 1). These differences may be due to strain differences between *H. insolens* ATCC 16454 and *H. insolens* FERM BP-5977.

Expression and purification of Avi2 in *P. pastoris*

Humicola insolens ATCC 16454 *avi* gene has been successfully cloned and the protein was expressed in *P. pastoris* X-33. Based on the SDS-PAGE profile and western blot analyses (Figure 2), protein expression using BMGY medium as the biomass-generating medium and BMMY medium containing 3% absolute methanol as the protein-induction medium for 3 days resulted in the overexpression of a recombinant protein with a molecular weight of ~53.2 kDa. Approximately 125 mg/l of crude recombinant Avi2 was produced by *P. pastoris*. This result was in close proximity to the expression level of *Volvariella volvacea* V14 endoglucanase in *P. pastoris*, which was reported at 65-100 mg/l (Ding *et al.*, 2002).

A polyhistidine affinity tag was used to simplify the subsequent purification processes. The tag was fused with recombinant Avi2, and the recombinant enzyme was purified to homogeneity using an immobilised metal affinity chromatography (IMAC) column. The purified enzyme showed a single band with a molecular weight of ~53.2 kDa on SDS-PAGE and verified by western blotting analyses (Figure 2). Purified Avi2 was obtained after two steps of protein purification, including ultrafiltration with a MWCO of 10 kDa and IMAC (Figure 2).

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ATCC16454      1  MAKFFLTAAFAAAALAAPVVEERQNCAS TWGQCGGIGFNGPTCCQSGSTCVKQNDWYSQC
FERM BP5977    1  MAKFFLTAAFAAAALAAPVVEERQNCAP TWGQCGGIGFNGPTCCQSGSTCVKQNDWYSQC

ATCC16454     61  LPGSQVTTTSTTSTSSSSTTSRATSTT S TGGVTSITTAPTRTVTIPGGATTTASYNGNPF
FERM BP5977   61  LPGSQVTTTSTTSTSSSSTTSRATSTT R TGGVTSITTAPTRTVTIPGGATTTASYNGNPF

ATCC16454    121  EGVQLWANNY H RSEVHTLAIPQITDPALRAAASAVAEVPSFQWLDRNVTVDTLLVETLSE
FERM BP5977  121  EGVQLWANNY Y RSEVHTLAIPQITDPALRAAASAVAEVPSFQWLDRNVTVDTLLVETLSE

ATCC16454    181  IRAANQAGANPPYAAQIVVYDLPDRDCAAAASNGEWAIANNGANNYKGYINRIREILISF
FERM BP5977  181  IRAANQAGANPPYAAQIVVYDLPDRDCAAAASNGEWAIANNGANNYKGYINRIREILISF

ATCC16454    241  SDVRTILVIEPDSLANMVTNMNVAKCSGAASTYRELTIYALKQLDLPHVAMYMDAGHAGW
FERM BP5977  241  SDVRTILVIEPDSLANMVTNMNVAKCSGAASTYRELTIYALKQLDLPHVAMYMDAGHAGW

ATCC16454    301  LGWPANIQPAAELFAKIYEDAGKPRAVRGLATNVANYNAWSISSPPPYTSPNPNYDEKHY
FERM BP5977  301  LGWPANIQPAAELFAKIYEDAGKPRAVRGLATNVANYNAWSISSPPPYTSPNPNYDEKHY

ATCC16454    361  IEAFRPLLEARGFPAQFIVDQGRSGKQPTGQKEWGHWCNAIGTGFGMRPTANTGHQYVDA
FERM BP5977  361  IEAFRPLLEARGFPAQFIVDQGRSGKQPTGQKEWGHWCNAIGTGFGMRPTANTGHQYVDA

ATCC16454    421  FVWVKPGGECDGTSDTTAARYDYHCGLEDALKPAPEAGQWFQAYFEQLLRNANPPF
FERM BP5977  421  FVWVKPGGECDGTSDTTAARYDYHCGLEDALKPAPEAGQWFQAYFEQLLRNANPPF
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Fig. 1. Amino acid sequence alignment of Avi2 from *H. insolens* ATCC 16454 with Avi2 from *H. insolens* FERM BP-5977. Identical residues are marked with the black background, and different residues are marked with the white background.
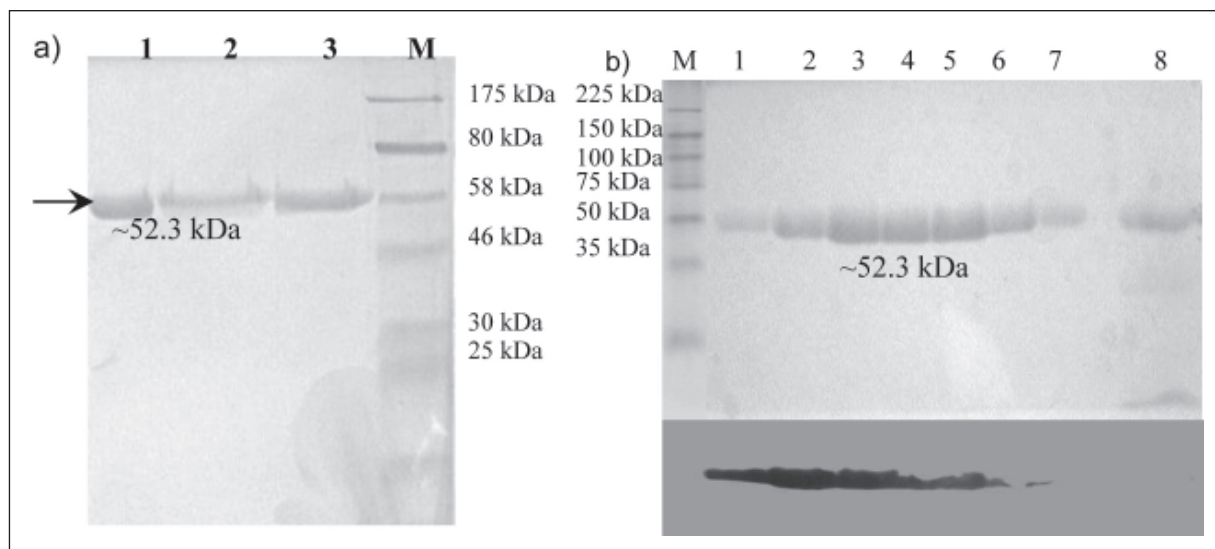
Fig. 2. SDS-PAGE and western blot analysis of Avi2 expression in *P. pastoris*, X-33. (a) SDS-PAGE analyses of Avi2 from *P. pastoris* strain X-33. Lanes 1, 2 and 3: culture supernatants of *P. pastoris* strain X-33 induced with 0.5% methanol; M: protein marker. (b) SDS-PAGE analyses of Avi2 from IMAC purification fractions. Lane M: Protein marker (New England Biolabs); Lanes 1 to 7: Avi2-targeted protein elution fractions; Lane 8: Avi2 crude extract. The bottom figure is a western blot result showing the detection of purified Avi2 using an anti-His antibody.

Enzymatic properties of purified Avi2

To characterise the recombinant Avi2 protein, the effect of temperature (30-90°C) and pH (pH 3.0-9.0) on enzyme activity was evaluated. The purified enzyme exhibited an optimal activity at 50°C, and retained over 40% of its activity when assayed at temperatures up to 80°C (Figure 3a). The Avi2 recombinant enzyme was stable when exposed to temperatures between 30°C to 90°C for 30 min in the absence of substrate. The enzyme gradually lost its activity with the increase in temperature, as such that only 40% of the activity remained after heat treatment at 90°C for 30 min (Figure 3a).

To determine the effect of pH on the recombinant Avi2 enzyme activity and stability, several buffers with different pH values were used, including sodium citrate buffer (pH 3.0-6.0) and potassium phosphate buffer (pH 7.0-9.0). The optimal pH for Avi2 enzyme activity was pH 5.0 to pH 6.0 (Figure 3b), and the enzyme was stable from pH 3.0 to pH 9.0 with more than 80% of residual activity (Figure 3b). This result suggested that this purified recombinant cellobiohydrolase Avi2 was stable in solutions of varying pH values.

The specific activity of this enzyme was 0.075 U mg^{-1} when Avicel was used as a substrate and 0.018 U mg^{-1} when Sigmacell-cellulose was used as a substrate. There was no detectable activity when either CMC or cellobiose was used as the substrate. Enzyme analyses were subsequently carried out by measuring the initial reaction rates of the purified Avi2 protein at various concentrations of Avicel and Sigmacell-cellulose. The initial reaction rates were analysed by using a Lineweaver-Burke plot. The Michaelis-Menten constants (K_m) for Avicel and Sigmacell-cellulose as substrates were 8.104 mg/ml and 10.477 mg/ml, respectively. These results indicate that the purified Avi2 protein showed a higher affinity towards hydrolysing Avicel than Sigmacell-cellulose.
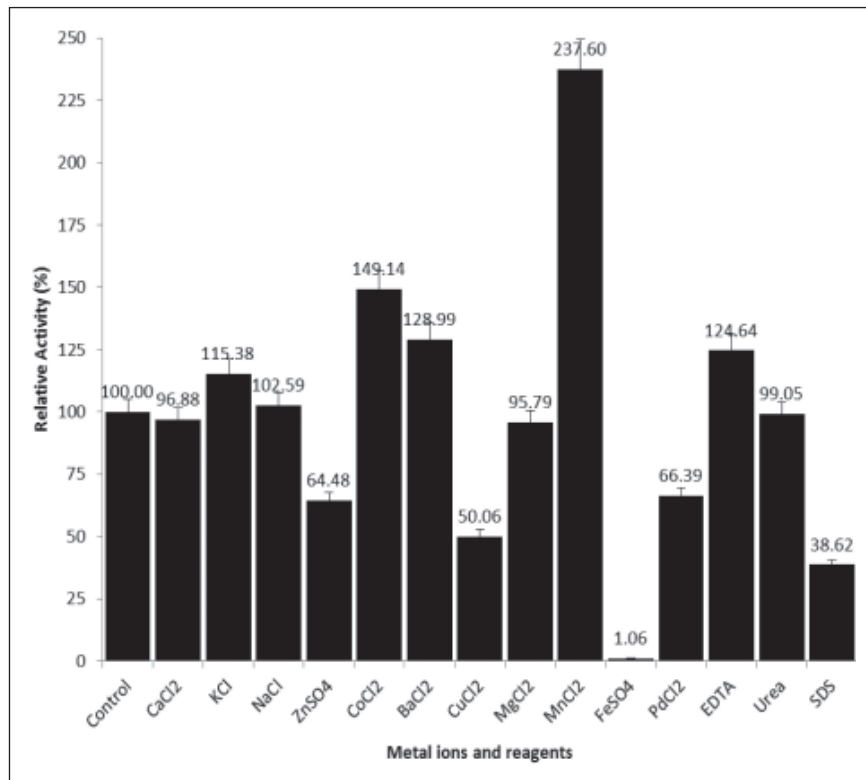
Effect of metal ions and other reagents

The effects of metal ions and some reagents on enzyme activity were summarised in Figure 4. The addition of the metal ions, Mn^{2+}, Co^{2+}, and Ba^{2+} resulted in the increase in the activity of the purified Avi2 recombinant enzyme. Among others, Mn^{2+} ions caused the highest increase in activity of approximately 1.38-fold compared to the control assay. At the same concentrations, Co^{2+} and Ba^{2+} increases the enzyme activity to approximately 0.5-fold and 0.3-fold, respectively, compared to the activity of the enzyme in the absence of metal ion. In contrast, a significant reduction of reducing sugars was observed with the addition of Fe^{2+}, Cu^{2+}, Pd^{2+} or Zn^{2+}. Furthermore, Fe^{2+} ions at 10 mM was found to lower the reducing sugars level to less than 5%, which was almost a complete inhibition of the recombianant enzyme activity.

DISCUSSION

In this work, Avi2 was produced as a recombinant cellobiohydrolase enzyme and its biochemical properties were characterised. The *avi2* cDNA from
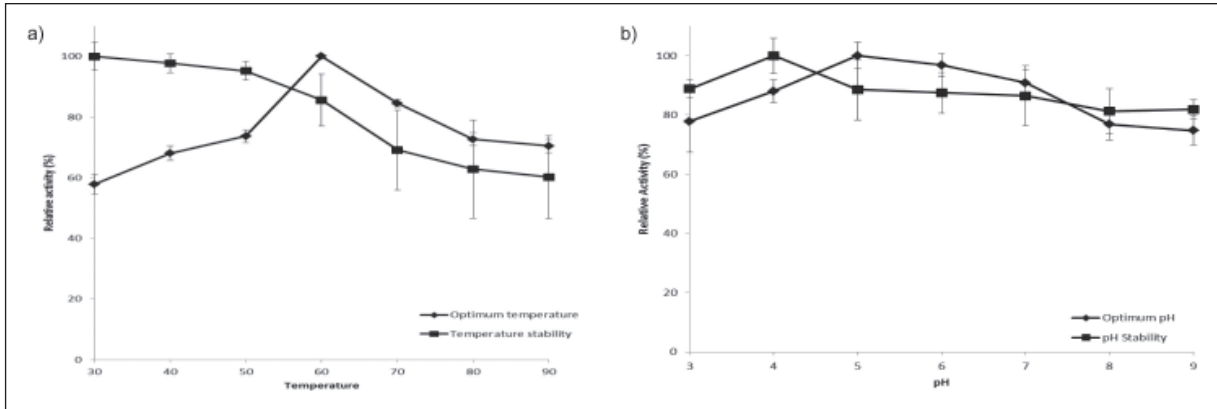
Fig. 3. Biochemical properties of the purified recombinant Avi2. (a) Effects of temperature and temperature stability of Avi2; (b) Effects of pH on Avi2 activity and the pH stability of Avi2.

Fig. 4. Effects of metal ions and chemical reagents on purified Avi2 compared to the activity of Avi2 without metal ions and chemical reagents.

H. insolens ATCC 16454 was successfully cloned and expressed in the methylotrophic yeast *P. pastoris* X-33 as an active protein. Based on the deduced amino acid sequence, three domains were predicted to be present in Avi2, including the catalytic domain, the linker domain, and the cellulose-binding domain. The Avi2 amino acid sequence from *H. insolens* ATCC 16454 is almost identical to the *H. insolens* FERM BP-5977 Avi2 sequence that is available in GenBank. However, the specific activity of the recombinant Avi2 that

was produced in *P. pastoris* towards Avicel was lower compared to the reported values of Avi2 that were produced by *H. insolens* FERM BP-77 (0.18 U mg^{-1}) (Moriya *et al*., 2003). This result may be due to differences between the hosts that were used in the expression of the proteins. Furthermore, the presence of the His-tag and Myc-epitope amino acid sequence at the C-terminal region of the recombinant Avi2 that was produced in *P. pastoris* may also affected the activity of the recombinant enzymes (Quay *et al*., 2011). In addition, the slight

differences in the gene sequence of the different strains have resulted in the differences between the two enzymes.

The amount of the recombinant Avi2 produced in *P. pastoris* is considered to be high, and it is in agreement with the previous work described by Ding *et al*. (2002). They reported 65-100 mg/l of *V. volvacea* V14 endoglucanase was produced in *P. pastoris*. In this study, *P. pastoris* was selected to produce the recombinant protein because it is widely known for its potential to produce soluble, correctly folded recombinant proteins that need to undergo post-translational modifications that are required for functionality (Daly and Hearn, 2005). However, to obtain high amounts of recombinant protein, the expression has to be carried out in a fermenter. The yield of protein that can be obtained from shaker-flasks is generally 10-fold lower than that potentially achievable with fermenters because of the lower cell density that limits the extent of aeration (Romanos, 1995). Jang and Chang (2005) reported that the production of avicelase from *Streptomyces* sp. T3-1 is 15% higher in a 50-l fermenter than in shaker-flask cultures.

The biochemical characterisation of the purified Avi2 categorised this enzyme as a thermostable cellobiohydrolase. The cellobiohydrolase activity of *H. insolens* YH-8 that was characterised by Hayashida and Yoshioka (1979) had an optimal temperature at 60°C, which is similar to the recombinant Avi2 expressed in this study. In their work, Hayashida and Yoshioka (1979) also showed that after a 5 min exposure at 70°C, only 20% of the enzyme activity remained. However, in this study, a higher enzyme activity was observed after the exposure at the same temperature. Moriya *et al*. (2003) and Hayashida and Yoshioka (1979) assayed the enzyme at pH 5.5. In this work, the recombinant Avi2 demonstrated similar pH optimum. This enzyme was able to maintain more than 80% of its initial activity throughout a range of acidic and basic conditions (ranging from pH 3.0 to pH 9.0). The thermostability and pH stability of Avi2 may contribute to the structural stability of this enzyme because of the presence of glycosylation sites. A structural study carried out on CBHII of *H. insolens* FERM 1577, which shares 97.2% similarity to Avi2 at its core domain, revealed that the crystal structure of CBHII has one N-glycosylation site and two O-glycosylation sites (Varrot *et al*., 1999). The addition of polysaccharides to the protein structure often contributes to a high level of solubility of the protein and increases its stability against proteolysis. Moreover, the covalent binding of glycans to the protein surface may enhance the thermal and kinetic stability of proteins (Shental-Bechor and Levy, 2008).

The recombinant Avi2 showed activity towards microcrystalline-cellulose (Avicel and Sigmacell-cellulose) but none towards amorphous cellulose (CMC) or shorter cellulose chains (cellobiose). Both avicel and Sigmacell-cell are cellulose with linear cellulose chains that are bundled together as microfibrils. Furthermore, each microfibril exhibits a high degree of three-dimensional internal bonding, which results in a crystalline structure that is insoluble in water and resistant to reagents. Avi2 is therefore similar to other cellobiohydrolases, which are usually most efficient on highly ordered crystalline cellulose and cleave mainly cellobiose from the opposite ends of the glucose chain (Lahjouji *et al*., 2007).

Metal ions may influence the maximal enzyme catalytic activity by acting as enzyme cofactors or inhibitors (Jaabar *et al*., 2008). The increase in enzyme activity with the addition of manganese ions was observed for an endoglucanase from *Penicilium chrysogenum* in which a more than 3-fold stimulatory effect in enzyme hydrolytic activity occurred (Chinedu *et al*., 2008). A total inhibition of the recombinant Avi2 activity by ferum ions was observed in this work. Based on Khademi *et al*. (2002), metal ions directly bind to and block the active sites of enzymes (endoglucanase), thus inactivating the enzyme. The same mechanism may occur and cause a decrease in Avi2 activity. The reduction of the recombinant Avi2 activity in the presence of SDS, indicating that the enzyme is sensitive to anionic detergents.

CONCLUSION

In this work, *H. insolens* ATCC 16454 Avi2 was successfully cloned, expressed and characterised using the *P. pastoris* expression system. This enzyme displayed an optimal activity at 50°C and pH 5.0. It is stable over a broad range of pH values and has a higher preference towards microcrystalline-cellulose. Due to its unique properties, this recombinant enzyme has the potential to be exploited for the enzymatic hydrolysis of locally produced agricultural biomass.

ACKNOWLEDGEMENTS

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