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CHARACTERISATION OF RECOMBINANT *Trichoderma reesei* CELLOBIOHYDROLASE AND THE POTENTIAL OF CELLULASE MIXTURE IN HYDROLYZING OIL PALM EMPTY FRUIT BUNCHES

DORIS QUAY HUAI XIA[1], YONG HUI YEE[1], ROSLI MD ILLIAS[2], NOR MUHAMMAD MAHADI[3], FARAH DIBA ABU BAKAR[1] and ABDUL MUNIR ABDUL MURAD[1*]

[1]*School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia*
[2]*Department of Bioprocess Engineering, Faculty of Chemical Engineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia*
[3]*Malaysia Genome Institute, Jalan Bangi, 43000 Kajang, Selangor, Malaysia*
[*]*Email: munir@ukm.edu.my/ munir8488@gmail.com*

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ABSTRACT

The potential for recombinant cellulases from fungi to hydrolyze oil palm empty fruit bunches (OPEFB) into simple sugars was investigated. A mixture of two recombinant enzymes consisting of a cellobiohydrolase from *Trichoderma reesei* and an endoglucanase from *Aspergillus niger,* was evaluated for OPEFB hydrolysis. The development of *A. niger* endoglucanase has been described previously. In this work, the development of a recombinant cellobiohydrolase and the activity of this enzyme mixture towards OPEFB hydrolysis were described. To obtain recombinant cellobiohydrolase, the cDNA encoding for cellobiohydrolase, *cbhII*, was isolated from the *T. reesei* strain M5, and the gene was expressed in the methylotrophic yeast, *Pichia pastoris*. Partially purified CbhII demonstrated optimum activity at 50°C and pH 5.0. This enzyme was shown to hydrolyze Avicel at a concentration of 0.486 U/mg under optimum conditions. Hydrolysis of pretreated OPEFB using CbhII and endoglucanase EglA showed that the total reducing sugar produced was higher in the enzyme mixture reaction than were produced in an individual enzyme reaction. Addition of β-glucosidase and an enzyme cofactor significantly increased enzyme activity and OPEFB cellulose hydrolysis. This study demonstrated the capability of an enzyme mixture to produce sugars from oil palm lignocellulosic waste.

Key words: Cellobiohydrolase, *Trichoderma reesei*, enzyme mixture, oil palm empty fruit bunch

INTRODUCTION

Cellulose, the most abundant component of plant biomass, is found in most agricultural waste products. This biopolymer is made up of glucose monomers linked together by β-1,4-glycosidic bonds. The cellulose fibers are usually embedded in a matrix of other structural biopolymers, such as hemicellulose and lignin. These matrix interactions are the dominant structural feature that limits the rate and extent of cellulose utilization (Lynd *et al*., 2002). Oil palm empty fruit bunch (OPEFB) is an underutilized plant biomass byproduct from the oil palm industry that is typically discarded. OPEFB consists of approximately 43.8% cellulose, 35.0% hemicellulose and 16.4% lignin (Hamzah *et al*., 2011) and can be obtained easily and inexpensively in Malaysia, the second largest palm oil producer in the world. Hence, it has potential as a cellulosic raw material for degradation into simple sugars. The simple sugars produced, in turn, could be converted into various value-added products, such as bioethanol, fine chemicals and animal feed (Rahman *et al*., 2007).

Cellulose degradation requires three types of cellulases: 1,4-β-D-endoglucanase (EC 3.1.2.4), 1,4-β-D-cellobiohydrolase (Cbh; EC 3.1.2.91) and β-glucosidase (EC 3.2.1.21) [1]. Cellobiohydrolases hydrolyze crystalline cellulose by starting at the ends of the cellulose chains and producing primarily

* To whom correspondence should be addressed.

cellobiose. Endoglucanases catalyze hydrolysis internally in the cellulose chain and attack the amorphous regions in cellulose, providing new chain ends for cellobiohydrolases. β-glucosidases hydrolyze cellobiose to glucose. During degradation of cellulose, these different types of enzymes act synergistically to hydrolyze cellulose polymer into glucose (Lynd *et al*., 2002). The ability of these enzymes to degrade cellulose and modify wood fibers have allowed cellulases to be used in various industries, including the animal and human feed industry, the textile and detergent industry, the paper and pulp industry and for bioethanol production (Kuhad *et al*., 2011).

Cellulases are known from a wide range of organisms, including fungi, bacteria and plants. Among these organisms, fungal cellulases have been the most widely studied and used in industrial applications, mainly because fungi are good producers of cellulases compared to other microorganisms (Lynd *et al*., 2002). Fungi that produce cellulases are from the genus of *Trichoderma*, *Aspergillus* and *Chrysosporium*. There are various studies that clone different classes of cellulase genes from these organisms and produce recombinant enzymes in heterologous hosts (Okada *et al*., 1998; Takashima *et al*., 1998; Quay *et al*., 2011; Abdul Fattah *et al*., 2015). These approaches could produce recombinant enzymes in large quantities and allow the study of individual cellulase activities during amorphous and crystalline cellulose hydrolysis. In addition, this approach would also allow researchers to design specialized enzyme cocktails that are highly active towards specific lignocellulosic biomasses.

In this work, the potential of an enzyme mixture containing *T. reesei* CbhII (cellobiohydrolase) and *A. niger* EglA (endoglucanase) to hydrolyze OPEFB was investigated. Both *T. reesei* and *A. niger* are cellulolytic fungi that have been widely studied, and their cellulase enzymes are used commercially in industry (Bhat and Bhat, 1997). Previously, we produced and characterized recombinant EglA (Quay *et al*., 2011). This enzyme displayed optimum activity at 50°C and pH 4.0. It is stable between 30 and 55°C and between pH 2.0 and 7.0 and shows good activity towards amorphous cellulose. To develop an enzyme mixture, we have produced recombinant *T. reesei* CbhII in *P. pastoris* and characterized its biochemical properties. This enzyme was selected because it is reportedly capable of binding and degrading insoluble crystalline cellulose, and its avicelase activity is two-fold higher than the second cellobiohydrolase produced by *T. reesei*, CbhI (Teeri *et al*., 1987). Subsequently, the ability of the CbhII and EglA enzyme mixture to produce reducing sugars from OPEFB hydrolysis was tested.

MATERIALS AND METHODS

Strains and plasmids

T. reesei strain M5 (ATCC 26921) was obtained from the American Type Culture Collection (ATCC), USA. *Pichia pastoris* strain X-33 was purchased from Invitrogen, USA, and *Escherichia coli* strain DH5α was obtained from the Molecular Mycology Laboratory, Universiti Kebangsaan Malaysia, Malaysia. *P. pastoris* overexpressing the *A. niger* endoglucanase, EglA, was developed in our previous study (Quay *et al*., 2011). The pGEM-T® Easy Vector used for DNA cloning procedures was purchased from Promega, USA, and the pPICZαA vector, which was used for heterologous expression of recombinant cellobiohydrolase, was obtained from Invitrogen, USA. Celluclast™ and β-glucosidase was purchased from Novozyme, USA.

Total RNA extraction and *cbhII* cDNA synthesis

Fungal spores were cultured on Potato Dextrose Agar (PDA) at 30°C for 4 days for mycelial growth and spore formation. Approximately $1x10^6$ spores/ml were cultured in 100 ml of Mandel's medium (Juhasz *et al*., 2005) containing filter paper (Whatman No.1) at pH 5 with shaking at 180 rpm and incubated at 30°C for 5 days. The mycelia were harvested by centrifugation at 5,000 rpm for 10 min at 4°C. Total RNA was isolated using TRIzol reagent (Invitrogen, USA) as described by Oh *et al.* (2009). Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed using the Access RT-PCR Kit (Promega, USA), the forward primer (cbhII_RT_F) 5'-GCTCACCTGAAGAGGCTT GTAAGAT-3' and the reverse primer (cbhII_RT_R) 5'-GGTCACGAAAGCCTTACAGGAACGA-3'.

Cloning of *cbhII* cDNA and construction of the CbhII expression cassette

The *cbhII* cDNA was cloned into the pGEM®-T Easy vector and transformed into the *E. coli* strain DH5α. A sequencing reaction was carried out to verify the *cbhII* gene sequence using the universal T7, SP6, internal forward (cbhII_Int_F) 5'-GCAGCCTCGCTATTCCT-3' and internal reverse (cbhII_Int_R) 5'-CAGCGTCCAAATACATCG-3' primers. The *cbhII* nucleotide sequence was translated into amino acid sequence using the program 'Translate' available at the Expasy server (www.expasy.ch/tools/dna.html). The full length cDNA encoding CbhII without its native signal sequence was amplified using *Taq* polymerase

(Invitrogen, USA) and the specific primers CbhII_ClaI (5'-ACACTCGCATCGATAGTGCC TCTAG-3') and CbhII_KpnI (5'-GGTCACGAGGTA CCAACAGGAACGA-3'). These primers added the restriction enzyme sites *Cla*1 and *Kpn*1, which were then used for cloning into pPICZαC. Sequencing was performed by a commercial service (1st Base, Malaysia).

Transformation of *P. pastoris* and screening of recombinant colonies

Competent cells of the *P. pastoris* strain X-33 were prepared according to the instruction manual for the EasySelect™ *Pichia* expression system (Invitrogen, USA). The CbhII cassette was linearized by *Pme*I at the 5' end of the *AOX1* promoter and purified for electroporation. *P. pastoris* was transformed by electroporation (Eppendorf, USA) using 0.2 cm cuvettes (1.5 kv for 5.9 ms) with 10 µg of the CbhII cassette DNA fragment. After electroporation, 1 ml of ice-cold 1 M sorbitol solution was added to the cuvette, and the contents of the cuvette were transferred to 1.5-ml tube. After incubation at room temperature for 1 h without shaking, YPD medium was added, and the sample was incubated at 30°C with mixing at 500 rpm for 1 h. Subsequently, the sample was centrifuged at 13,200 rpm for 1 min to obtain the cell pellet. Cells were spread on two YPD agar plates and incubated at 30°C for 3 days. The same cassette without an insert was also transformed into *P. pastoris* as a control. Positive transformants were verified by PCR using the primers 5'AOX1 (5'-GACTGGTTCCAATTGACAAGC-3') and 3'AOX1 (5'-GCAAATGGCATTCTGACATCC-3') and subsequently screened for the multicopy clone by subculturing the positive transformants on agar plates containing 500, 1,000 and 2,000 µg/ml Zeocin.

Expression and partial purification of CbhII

Expression of CbhII was carried out using BMGY (Buffered complex medium containing glycerol) medium to produce cell mass and then BMMY (Buffered complex medium containing methanol) medium for induction. Protein expression was carried out for 5 days with 0.5% methanol added for induction every 24 h as described by Al-Rashed *et al.* (2011). Culture supernatant was collected after day 5 and concentrated using an Ultra Centrifugal Filter Device with a 10 kDa MWCO (Millipore, USA). Potassium phosphate buffer (50 mM, pH 7.0) was used for buffer exchange during ultrafiltration to remove the culture medium. A Bradford assay was carried out to determine the protein concentration. Secreted proteins were analyzed by SDS-PAGE (12% gel). Western blot analysis was performed using an anti-His antibody (Novagen, Germany) and an anti-mouse IgG HRP conjugate (Promega, USA) to detect the recombinant his-tagged CbhII protein.

Enzyme activity assays

To quantitate the activity of the CbhII, Avicel was used as the substrate in an enzyme assay, and the amount of reducing sugar released was detected using the DNS assay. A reaction mixture for the CbhII assay, containing 0.2 ml protein sample (10 µg) and Avicel (1%) in 50 mM sodium acetate buffer, pH 5.0, was performed at 50°C for 1 h. A DNS assay was carried out as described by Quay *et al.* (2011). One unit of enzyme was defined as the amount of enzyme that produced 1 µmol of reducing sugar per minute under the standard assay conditions.

Effect of temperature and pH on enzyme activity

The optimum pH for enzyme activity was determined over the pH range of 2.0 to 8.0 using glycine-HCl buffer for pH 2.0-3.0, sodium citrate buffer for pH 3.0-6.0 and potassium phosphate buffer for pH 6.0-8.0, all at a concentration of 50 mM. To determine pH stability, CbhII was incubated in buffer without substrate at a pH range of 2.0 to 8.0 for 30 min at 50°C using buffers of various pHs. Next, the substrate was added and the enzymatic reaction was incubated for 30 min at 50°C to determine cellobiohydrolase activity. The optimum temperature for the enzyme was determined in a temperature range between 30 and 60°C. To determine its temperature stability, the enzyme was incubated in 50 mM sodium citrate buffer, pH 5.0 without substrate in a temperature range between 30 and 60°C for 30 min. Next, the substrate was added to the reaction mixture and incubated for 30 min at 50°C to determine enzyme activity. The effect of temperature on CbhII hydrolysis was determined out by incubating an enzyme sample in buffer without substrate at 50°C for 0, 2, 5, 24 and 30 h. Substrate was then added, and an enzyme assay reaction was performed for 30 min to determine the residual activity of CbhII.

OPEFB pretreatment

OPEFB fibers 2 mm in size were ground using a MF10 grinding machine (IKA, USA) and filtered with a 0.5-mm sieve. The finely ground OPEFB were then soaked in a solution containing 70% glycerol and 1% H_2SO_4 at a ratio of 1:30 for 3 h at 120°C. The OPEFB were then filtered through muslin cloth and washed under distilled water until the pH reached 7.0. The OPEFB was then dried at 50°C for 2 days.

OPEFB cellulose hydrolysis

The expression of EglA from *P. pastoris* was carried out using BMGY medium to produce cell mass and BMMY medium for induction as described by Quay *et al.* (2011). The enzyme hydrolysis reaction was carried out with pretreated OPEFB mixed with partially purified enzymes in sodium citrate buffer (50 mM, pH 5.0). The partially purified CbhII and EglA enzymes used were 36 U/ml each, and the hydrolysis was carried out for 24 h with mixing at 150 rpm. An optimum ratio of enzyme was determined so that the enzyme components could react synergistically. To determine the best enzyme ratio for reducing sugar production, endoglucanase and cellobiohydrolase enzyme were mixed in 0:1, 3:1, 1:1 and 1:3 ratios, with a total enzyme concentration of 72 U/ml. The hydrolysis reaction was terminated by boiling the sample for 5 min. Then, the sample was centrifuged at 13,200 rpm for 5 min, and the reducing sugar produced in the supernatant was measured using the DNS assay. Every hydrolysis experiment was performed in triplicate. The structural morphology of the hydrolyzed OPEFB fibers was analyzed using scanning electron microscopy (SEM) (Microscopy Unit, Universiti Kebangsaan Malaysia).

To prevent end product inhibition, the hydrolysis reaction was supplemented with β-glucosidase (Novozyme 188) at a ratio of 3:1 for the CbhII/EglA mixture to β-glucosidase (Xiao *et al*., 2004; Sun and Chen, 2007), with a total enzyme concentration of 72 U/ml. Additionally, $MnCl_2$ (10 mM), an enzyme cofactor for EglA, was added to the enzyme mixture to improve enzyme efficiency. All enzymatic hydrolysis was performed at 50°C in sodium citrate buffer (50 mM, pH 5.0) with 1% OPEFB as the substrate. OPEFB hydrolysis with Celluclast^{TM} (Novozyme, USA) using the same enzyme unit under the standard condition was performed for the comparison of reducing sugar production.

RESULTS

Isolation and sequence analysis of *T. reesei cbhII* cDNA

T. reesei strain M5 (ATCC 26921) *cbhII* cDNA was amplified, cloned and sequenced. The amplified cDNA has a length of 1,416 bp and encodes a 472 amino acid protein with a putative molecular mass of 50 kDa. This sequence is available in GenBank with the accession number GU724763. BLAST analysis of the sequence obtained revealed a 99% identity with the sequence of *T. reesei* VTT-D-80133, with a single nucleotide difference at position 1184. Changes in this nucleotide result in a different amino acid at position 395. Thus, the amino acid is isoleucine in strain M5, rather than threonine as in strain VTT-D-80133. The amino acid change occurs in the glycosyl hydrolase 6 domain.

Expression and purification of CbhII

A cellobiohydrolase from *Trichoderma reesei*, CbhII, was expressed in the methylotrophic yeast *P. pastoris* X-33 and the properties of the recombinant protein were characterized. The full-length cDNA of *cbhII* was cloned into a pPICZαC expression vector, and the recombinant CbhII protein was expressed extracellularly and partially purified by ultra-filtration. SDS-PAGE and Western blotting profiles showed two protein sizes (~58 kDa and ~ 80 kDa) for secreted recombinant CbhII (Figure 1). The partially purified CbhII protein had a specific activity of 0.486 U/mg against Avicel.

Optimum pH, temperature and enzyme stability

The purified CbhII enzyme displayed an optimum activity at 50°C and was stable between 30°C and 45°C (Figure 2A). The stability and activity of CbhII decreased at a higher temperatures, especially 50°C and 60°C, where its residual activity drop to less than 70%. For pH, the maximum activity of CbhII was observed at pH 5.0 at 50°C (Figure 2B),

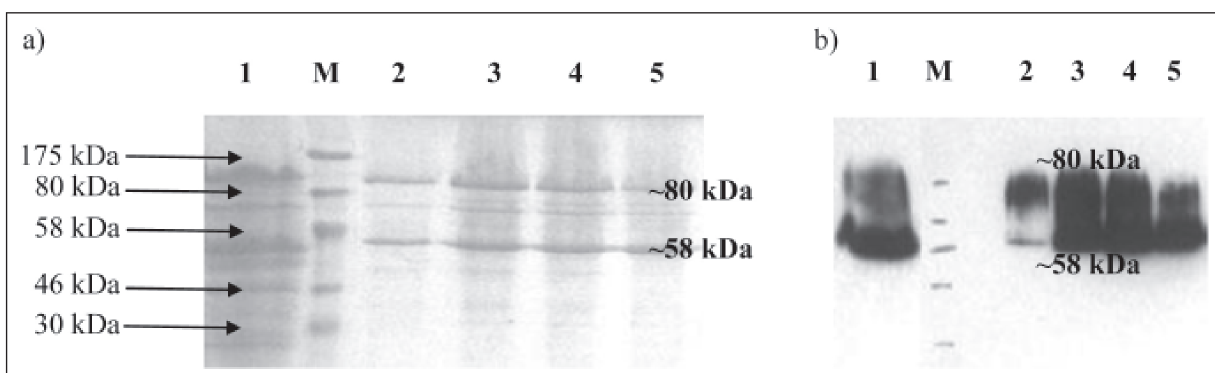

Fig. 1. CbhII protein expression and purification profile. (a) SDS-PAGE analyses of the CbhII protein purification fractions. Lane 1: CbhII crude extract; Lane M: Protein marker (New England Biolabs); Lanes 2-5: CbhII targeted protein elution fractions. (b) Western blotting analyses for detection of purified CbhII using an anti-His antibody. Lane 1: CbhII crude extract; Lane 2-5: Purified CbhII protein.

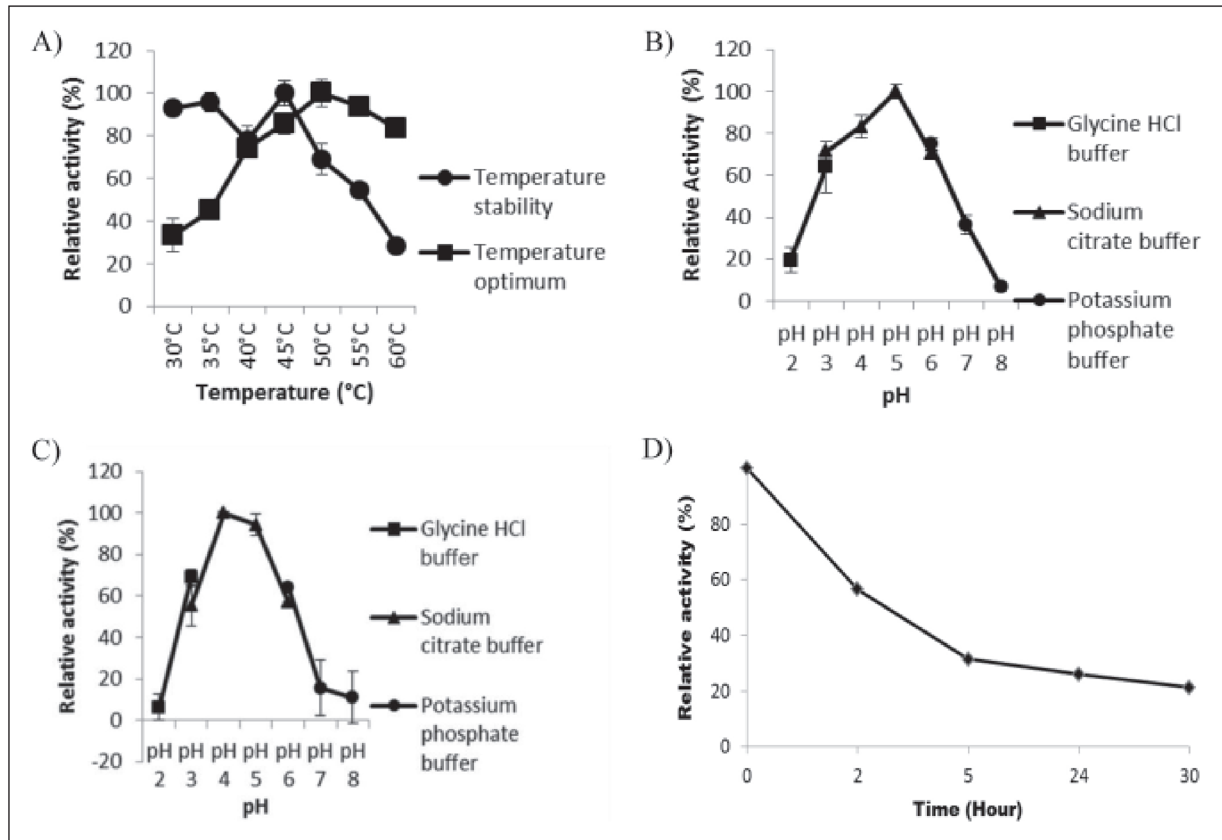

Fig. 2. Biochemical properties of purified recombinant CbhII. (A) Temperature profile and temperature stability of CbhII; (B) pH profile; (C) pH stability; (D) Temperature stability of CbhII incubated at 50ºC for a course of time points.

and this enzyme was most stable in the range of pH 3.0 to 6.0 (Figure 2C). Its catalytic activity was weak at the highly acidic pH range of 2.0 to 4.0 and also at the alkaline pH of 8.0. Upon incubation at 50°C for a longer period of time up to 30 hours, CbhII was observed to have less than 50% of residual activity after 5 hours (Figure 2D).

OPEFB Cellulose Hydrolysis

An optimal balance of conditions and a good enzyme synergy ratio for the cellulase enzymes are the main factors needed to improve multi-enzyme performance, lower processing costs and reduce enzyme usage (Zhou *et al*., 2009). In this work, the enzyme ratios 3:1, 1:1 and 1:3 were studied (Goyal *et al*., 1991). Figure 3 shows that the highest yield of reducing sugars were produced at an EglA/CbhII ratio of 1:3. Figure 4 shows a scanning electron microscopy (SEM) image of OPEFB fiber morphology following enzymatic hydrolysis by CbhII and EglA compared with unreacted OPEFB substrate as a control. We observed that OPEFB fibers are rougher and have more pores on their surface after treatment with enzyme hydrolysis. Therefore, the cellulose microfibrils built from the glucose polymer chains have been degraded into simple sugars by the reaction enzymes, thereby creating a rougher surface and exposing the indigestible lignin and hemicellulose fibers.

β-glucosidase is a cellulase system component that can react synergistically with endoglucanase and cellobiohydrolase to degrade cellooligo-saccharides or cellobiose into glucose. Figure 5 shows that adding commercial β-glucosidase (Novozyme 188) to the EglA-CbhII enzyme mixture at a 1:3 ratio (Xiao *et al*., 2004; Sun and Chen, 2007) increased the total reducing sugar produced by nearly two fold. Furthermore, elevation of activity by 3-fold was observed when 10 mM manganese chloride was added to the hydrolysis reaction containing CbhII-EglA (Figure 5). OPEFB hydrolysis with both β-glucosidase and manganese ions in the CbhII-EglA enzyme mixture yielded five times more reducing sugars relative to the reaction containing only CbhII and EglA. This result demonstrates that this enzyme mixture supplemented with an enzyme cofactor significantly increased the production of reducing sugars from OPEFB cellulose.

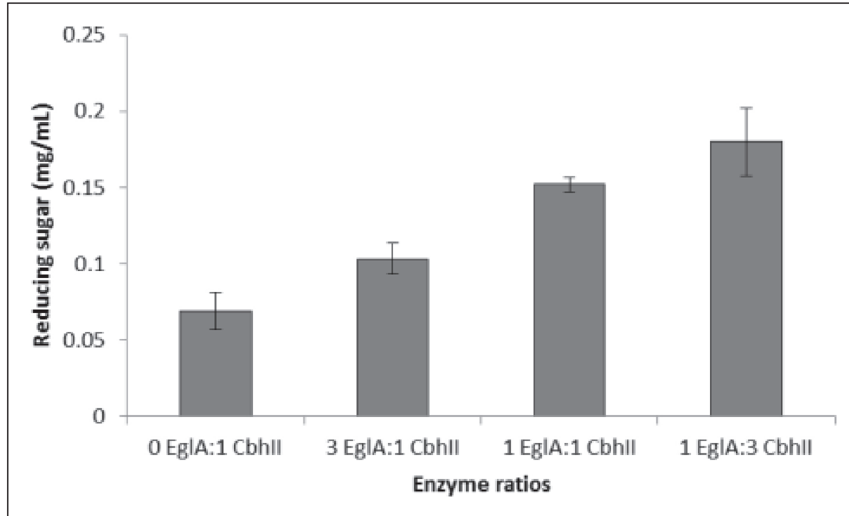

Fig. 3. Reducing sugar production profile using different ratios of recombinant enzyme mixture of EglA *A. niger* and CbhII *T. reesei* on OPEFB cellulose

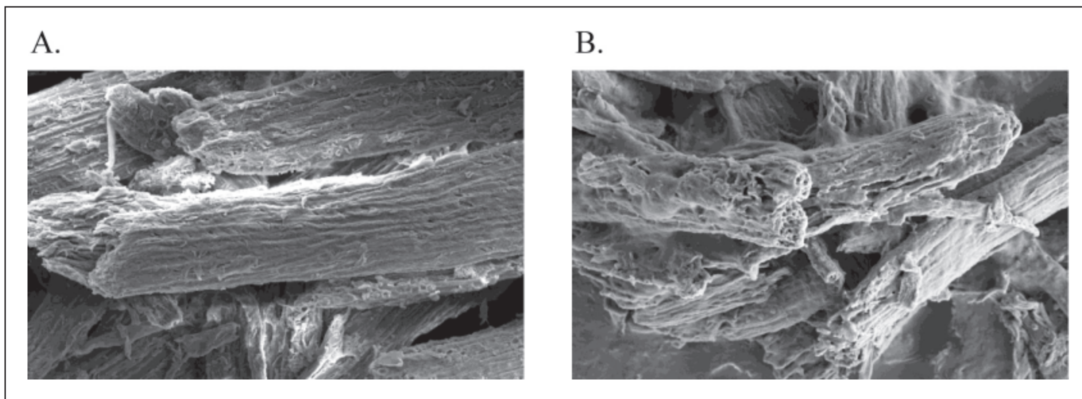

Fig. 4. Scanning electron microscope image of the morphology of OPEFB before and after enzymatic hydrolysis. Panel A) Morphology of the control OPEFB fiber structure. Panel B) Morphology of the OPEFB fiber structure after hydrolysis by an EglA-CbhII enzyme mixture with a 1:3 ratio for 24 hours at 45ºC.

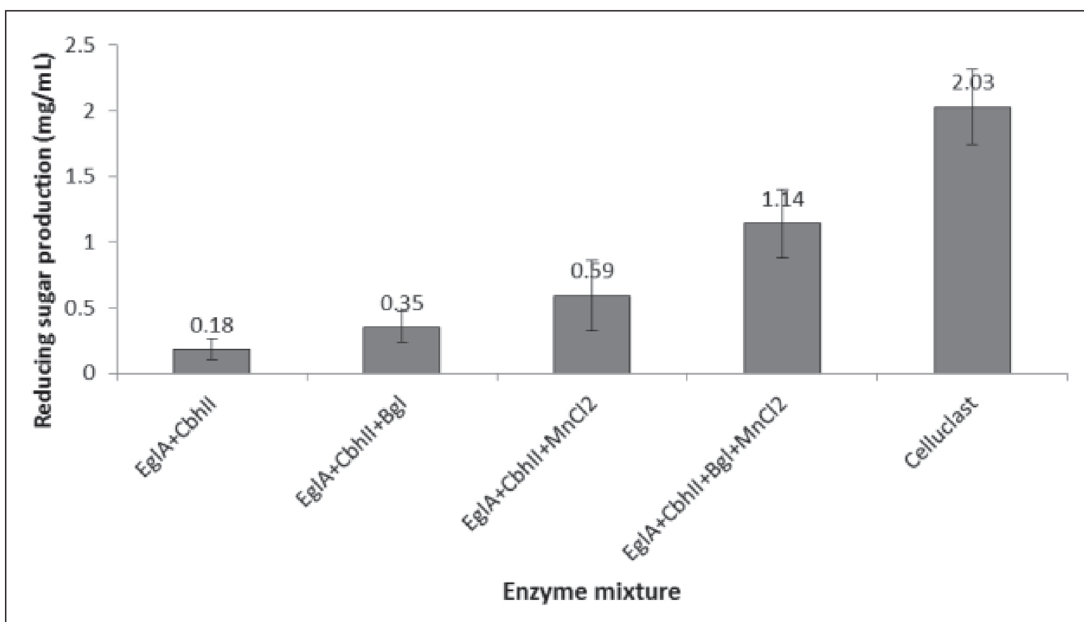

Fig. 5. Enhancement effect of reducing sugar production by enzymatic hydrolysis of EglA-CbhII on pretreated OPEFB and comparison to Celluclast™.

DISCUSSION

T. reesei, a filamentous, mesophilic fungus, is well known for its efficient production of cellulolytic enzymes and is the most widely studied cellulolytic fungus. This fungus can be induced to produce at least five different endoglucanases, two different cellobiohydrolases and two β-glucosidases to facilitate the complete degradation of cellulose into glucose (Foreman *et al*., 2003). In cellulase-inducing conditions, the proportion of extracellular proteins produced by *T. reesei* that are cello-biohydrolases is approximately 80-85% (Miettinen-Oinonen *et al*., 2005).

The cellobiohydrolase CbhII from *T. reesei* was expressed in *S. pombe* (Okada *et al*., 1998) and *A. oryzae* (Takashima *et al*., 1998) and varied in its optimum reaction temperature and specific activity. This might be due to the different expression hosts used. In this study, CbhII expressed in *P. pastoris* generates two distinct bands in a Western blot, corresponding to the predicted size along with another larger band at 80 kDa. The larger protein fragment might be glycosylated CbhII, as yeast are capable of post-translational modification (Wonganu *et al*., 2007). The expression of *T. reesei* CbhII in *S. pombe* created two glycosylated molecule species with sizes of 70 and 72 kDa, each of which is larger than the native enzyme (58 kDa) (Okada *et al*., 1998). Glycosylation can also affect enzyme activity at a particular temperature and pH. This is because the carbohydrate moiety can affect the packing and stability of the glycosylated enzyme by forming new interactions between the enzyme's sugars and amino acids (Shental-Bechor *et al*., 2008). Jeoh *et al.* (2008) has reported that high levels of N-glycosylation on the recombinant cellobiohydrolase, Cel7A, reduced enzyme activity and increased non-productive binding of cellulose. The expression level of recombinant CbhII in crude extracts was approximately 100 mg/l, which is comparable to the expression of recombinant *Volvariella volvacea* endoglucanase in *P. pastoris* crude extract observed by Ding *et al.* (2002), who recovered approximately 65-100 mg/l.

The optimum temperature and pH of partially purified recombinant CbhII created in this study was 50°C and pH 5.0. Most fungal cellobiohydrolases have an optimum activity at an acidic pH and at a temperature approximately 50°C, with the exception of thermophilic enzymes, which show a higher optimum temperature (Hong *et al*., 2003). Enzymes that are stable at an acidic pH are suitable for use in the animal feed industries, as the digestive tract of poultry has a pH of approximately 4.8 (Collins *et al*., 2005). Acidic cellulases also could be applied in old newspaper and magazine deinking processes. CbhII showed low activity towards the micro-crystalline cellulose Avicel, which may be due to the insensitivity of the CbhII enzyme to these insoluble microcrystalline substrates. Han *et al.* (1995) reported that filter paper and Avicel could only be hydrolyzed efficiently with the synergistic action of endo- and exoglucanases. Toda *et al.* (2005) also reported that the cellobiohydrolase Ex-1 from *Irpex lacteus* demonstrated very low reactivity (20.9 mU/mg) towards Avicel.

OPEFB, with its high cellulosic content, can be utilized as biomass for the production of biofuel. Hamzah *et al.* (2011) reported that OPEFB consists of up to 43.8% of cellulose, could potentially be hydrolyzed to soluble sugars. To minimize the cost of the enzymatic hydrolysis of cellullose, the identity of effective enzymes, the determination of their properties, and the optimization of their relative ratios could be tailored to specific pretreated substrates, thereby reducing enzyme usage without sacrificing hydrolysis rates and yields. Thus, it is very important to obtain multi-enzymes of superior quality with an increased range of capabilities to degrade various pretreated biomass substrates under different hydrolysis conditions. In this study, the total yield of reducing sugars was highest at the ratio of one part endoglucanase (EglA) to three parts exoglucanase (CbhII). Higher ratio of CbhII cellobiohydrolase to EglA endoglucanase is needed to produce the best yield among those tested ratios may be due to the smaller molecule of EglA endoglucanase could easily enters within the cellulosic fibers to chew up the fiber internally and hence loosening the fibrous chains. This provides more fiber chain ends as the substrates for CbhII cellobiohydrolase catalysis to release cellobiose as the reducing sugars. Hence, one part of EglA is sufficient to provide substrate *in situ* for three parts of CbhII to react in order to give the best yield of reducing sugars. The total reducing sugar yield of 0.18 mg/ml was similar to the yield of cellulase secreted by *T. reesei* (Goyal *et al*., 1991) Supple-mentation of β-glucosidase (Novozyme 188) at 1 part to 3 parts cellulase further increased the yield of reducing sugar to 0.35 mg/ml, indicating that this enzyme is capable of assisting in the efficient hydrolysis of cellobiose to glucose and thus liberating more reducing sugar. Furthermore, the β-glucosidase conversion of cellobiose could alleviate the problem of cellobiose enzyme inhibition when it is present in excess. Cellobiose is a known cellulase inhibitor that can inhibit cellulases 14 times more efficiently than glucose (Holtzaple *et al*., 1984). Previous studies have shown that the addition of Mn^{2+} ions increases the activity of EglA by 2.7-fold (Quay *et al*., 2011) without significantly decreasing the activity of CbhII. The addition of enzyme cofactors further enhances reducing sugar production to 1.14 mg/ml. The yield was still low

compared to the commercial cellulase mixture, Celluclast™ (Figure 5) with the same enzyme unit used in the assay. This is because there were only two cellulase enzymes used in the mixture of this study whereas Celluclast™ is a multicomponent enzyme produced by *T. reesei* composing of several cellulolytic and xylanolytic enzyme components (Sorensen *et al.*, 2007; Virkki *et al.*, 2008). Although there is some increase of activity after supplementation with β-glucosidase and enzyme cofactors, finding the right enzyme ratio for the hydrolysis reaction should drive the reducing sugar yield higher. Indeed, this study demonstrates the potential for designing an enzyme cocktail tailored for the hydrolysis of a specific substrate.

CONCLUSION

CbhII protein has been successfully isolated, cloned and expressed in *P. pastoris* yeast expression system and tested for downstream OPEFB hydrolysis application. However, the yield of the enzyme mixture containing endoglucanase EglA and cellobiohydrolase CbhII supplemented with β-glucosidase and cofactors was still low compared to Celluclast™. Our future work is to propose an enzyme mixture with additional cellulase components for a better OPEFB hydrolysing performance.

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