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# Effect of *Humicola insolens* recombinant endoglucanase on the performance of commercial cellulase in oil palm biomass hydrolysis

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## ABSTRACT

**Aims:** The cellulase complexes of *Trichoderma reesei* are relatively low in endoglucanase and β-glucosidase activity compared with exoglucanase. The aim of this study is to determine the effect of *Humicola insolens* recombinant endoglucanase on the activities of commercial cellulases, Celluclast® and Acellerase® BG, during the hydrolysis of pretreated oil palm empty fruit bunch (OPEFB) fibres to simple sugars.
**Methodology and results:** An endoglucanase (CMC3) from *H. insolens* ATTC 16454 was expressed in *Pichia pastoris*. The recombinant protein was purified and verified by SDS-PAGE and Western blot. The enzymatic hydrolysis of OPEFB fibres was carried out at 55 °C for 72 h and 1:40 and 1:100 mixtures of CMC3 and Celluclast® were used. All reaction mixtures were added with commercial β-glucosidase, Accelerase® BG, at a fixed concentration of 116 mg/mL. The sugars produced were analysed by high-performance liquid chromatography. Two sugar peaks were successfully resolved at different retention times and were identified as xylose and glucose. At Celluclast®-to-CMC3 activity ratio of 1:100, the highest reducing sugar concentration was obtained, whereby the glucose and xylose production increased by ~59% and ~27%, respectively.
**Conclusion, significance and impact of study:** Recombinant CMC3 can act synergistically with Celluclast® and Accelerase® BG to increase the production of glucose and xylose from pretreated OPEFB fibre. This study contributes greatly towards the development of efficient cellulase enzyme cocktail for the efficient hydrolysis of OPEFB biomass and the production of simple and fermentable sugars.

*Keywords: Humicola insolens*, cellulose, endoglucanase, hydrolysis

## INTRODUCTION

Malaysia is one of the leading producers and exporters of palm oil in the world. Palm oil production generates a massive amount of residues which can be used for the production of biofuels or fine biochemicals. In a palm oil plantation, only 10% of oil palm is produced, whereas the remaining 90% are considered biomass. The biomass waste generated from palm oil production comprises oil palm empty fruit bunch (OPEFB), oil palm trunk, oil palm frond, mesocarp fruit fibre, palm kernel shells and palm oil mill effluent (Kurnia *et al*., 2016).

OPEFB fibres are woody residues that remain after the fruit bunches are pressed at oil mills and are considered waste after oil extraction. OPEFB fibres, mainly composed of approximately 20%–30% lignin, 20%–30% hemicellulose and 40%–50% cellulose, have not been utilised commercially (Hassan *et al.*, 2010). However, owing to the high cellulose content in OPEFB, this biomass has been studied as a potential feedstock for future renewable energy (Umar *et al*., 2013).

The limitations for sugar production from biomass lie in enzymatic hydrolysis (Knauf and Moniruzzaman, 2004). The high cost of enzymes involved in cellulose hydrolysis process presents a significant barrier and deters large-scale production of sugar from lignocellulosic biomass (Gregg *et al*., 1998; Arantes and Saddler, 2010). Many strategies have been explored for the improvement of biomass hydrolysis. One such strategy is producing a cocktail of enzymes or combining two or more enzymes for the synergistic hydrolysis of cellulose (Berlin *et al*., 2005; Berlin *et al*., 2007; Garcia-Aparicio *et al*., 2007; Kostylev and Wilson, 2014).

In our previous study, we produced recombinant endoglucanase from *H. insolens* in a *P. pastoris* expression system. The crude enzyme showed high activity towards carboxymethyl cellulose (CMC) substrate and is comparable to the endoglucanase of other fungi (Abdul Fattah *et al.*, 2015). In this paper, we purified and

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examined the effect *H. insolens* endoglucanase to improve commercial cocktails for the hydrolysis of pretreated OPEFB for sugar production. The amount of sugars produced after enzymes hydrolysis was evaluated through high-performance liquid chromatography (HPLC) analysis. This study provides a data for subsequent studies in improving hydrolysis of OPEFB biomass and the performance of recombinant endoglucanase enzymes produced in laboratory towards biomass degradation.

## MATERIALS AND METHODS

### Substrate preparation

Pretreated OPEFB with 5% (w/v) sodium hydroxide was supplied by Malaysia Palm Oil Board (MPOB). We coarsely crushed and shredded OPEFB to 500 µm particles with a vacuum grinder and then sieved them for 2 h with a sieve shaker to obtain 63 µm particles. CMC sodium salt obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) was used as a standard substrate.

### Enzyme preparation

Celluclast®, a commercial *Trichoderma reesei* cellulase preparation, was purchased from Novozymes (Bagsvaerd, Denmark), and commercial β-glucosidase, Accellerase® BG, was provided by Genencor (Netherlands). Recombinant *P. pastoris* carrying *CMC3* gene from *H. insolens* ATCC 16454 was provided by Molecular Mycology Laboratory, School of Bioscience and Biotechnology, Universiti Kebangsaan Malaysia. CMC3 protein expression was carried out as described by Abdul Fattah *et al*. (2015). The protein in the supernatant was initially purified with dialysis tube (12 000-4 000 MW) in 50 mM sodium phosphate buffer (pH 8.0) overnight at 4 °C. The protein was further concentrated by using Amicon Ultra-15 centrifugal with 10 kDa molecular weight cut-offs (Millipore, USA). The concentrated crude protein was then applied to a 5 mL His-Trap™ HP column (GE Healthcare) by using 500 mM imidazole diluted in 50 mM sodium phosphate (pH 8.0) as a binding buffer in an automated AKTAprime™ Plus Protein Purification system (GE Healthcare, USA). The purified CMC3 was then analysed with 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blot analyses were performed for the verification of the purified protein. Mouse anti-His-tag IgG antibody (Novagen, Germany) and HRP-conjugated anti-mouse antibodies (Promega, USA) were used.

### Measurement of protein concentration

The protein concentration of Celluclast®, Accelerase® BG and purify recombinant endoglucanase of CMC3 were determined by using a Coomassie Plus (Bradford) Assay kit (Thermo Scientific) according to the manufacturer's instructions.

### Enzyme assay and kinetic parameter

The standard endoglucanase activity of Celluclast® and CMC3 was measured quantitatively by colorimetric method using 3,5-dinitrosalicyclic acid (DNS) assay (Miller, 1959) on CMC. The reaction mixture containing 0.2 mL of various concentrations of CMC, 0.8 mL of 50 mM sodium acetate buffer (pH 4.8) and enzymes was incubated for 30 min at 50 °C, and the reaction activities were stopped by the addition of DNS reagent followed by boiling at 100 °C for 5 min for the development of colour. The reaction mixture was then cooled with tap water, and absorption was measured at 540 nm with a microplate reader (Tecan Infinite 200). One unit of enzyme activity is defined as the amount of enzyme required to release 1 µmol of reducing sugars per minute (U/mL) under the standard assay conditions. The Michaelis-Menten constant $K_m$ and $V_{max}$ values were determined from the Lineweaver-Burk graph. The amount of enzyme needed to produce 1 unit of enzyme activity (U/mL) against CMC was then calculated according to the plots.

### Enzymatic hydrolysis

The hydrolysis of pretreated OPEFB with enzymes was carried out at 55 °C in an incubator shaker at 200 rpm for 72 h. The 7.5 mL reaction mixture consisted of 0.15 g of OPEFB sample (2% w/v), enzymes and 50 mM sodium acetate buffer (pH 4.8). The ratio of activities of enzyme loading between Celluclast® and recombinant endoglucanase were expressed in the unit of enzyme activity over CMC as a standard substrate, whereas Accelerase® BG was added at a fixed concentration of 116 mg/mL. The reducing sugars produced in the samples were analysed by HPLC. All experiments were performed in duplicate.

### HPLC analysis

All standard sugars and samples were detected by carbohydrate column LiChroCART® 125-4 Purospher STAR NH2, 5 µm (Merck, Millipore) on HPLC systems. Each HPLC system was equipped with an autosampler 717 plus (Waters, USA), 1525 Binary HPLC Pump (Waters, USA) and evaporative light-scattering detector (ELSD) (Eurosep, France) and operated at 50 °C for evaporating temperature and 60 °C for nebulisation temperature. The filtered and degassed mobile phase consisted of 80% acetonitrile and 20% deionised water. The blank sample used in this experiment was pretreated OPEFB without enzymes.

### Standard sugar analysis

A mixture of five standard sugars, namely, xylose, arabinose, glucose, galactose and cellobiose, was pumped at a flow rate of 1 mL/min and a run time of 35 min with injection volume 25 µL to develop a three-point calibration curve for each standard sugar. The external calibration curves were created by analysing standard

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**Table 1:** Summary of recombinant endoglucanase CMC3 purification.

| Purification Steps | Volume (mL) | Total Protein (mg) | Activity (U/mL) | Total Activity (U) | Specific Activity (U/mg) | Purification Factor[b] | Yield (%)[c] |
|---|---|---|---|---|---|---|---|
| Crude | 208 | 13 | 3.74 | 779 | 60 | 1.0 | 100 |
| Ultrafiltration | 15 | 2.85 | 50.6 | 758 | 266 | 4.4 | 97 |
| Chromatography | 7 | 1.93 | 78.6 | 550 | 286 | 4.8 | 71 |

[a]Activity, rate of enzyme hydrolysis of 1 µmol of reducing sugar per minute under standard assay conditions; [b]Purification factor, specific activity of purify sample/specific activity of crude extract sample; [c]Yield, total activity of purified sample/total activity of crude extract sample x 100%.

**Table 2:** Analyses of xylose and glucose concentration by HPLC.

| Mixture | Enzyme Mixture | Ratio of Activity (U) Celluclast® CMC3 | Concentration of Accelerase BG (mg/mL) | HPLC Analysis[a] | |
|---|---|---|---|---|---|
| | | | | Xylose (mg/mL) | Gluose (mg/mL) |
| 1 | Celluclast® + Accelerase BG | 1 | 116 | 1.215±0.148 | 1.220±0.127 |
| 2 | Celluclast® + CMC3 + Accelerase® BG | 1:40 | 116 | 1.097±0.047 | 1.430±0.03 |
| 3 | Celluclast® + CMC3 + Accelerase® BG | 1:100 | 116 | 1.55±0.028 | 1.945±0.08 |

[a]The mean concentration of xylose and glucose (mg/mL) produced after 72 h hydrolysis.

sugar solutions at the concentration of 1.75, 2.5 and 5.0 mg/mL. Calibration curves were acquired by plotting $\log_{10}$ area vs $\log_{10}$ concentration (Agblevor *et al*., 2004; Agblevor *et al.*, 2007).

**Analysis of pretreated OPEFB**

Enzymatic hydrolysis of OPEFB was collected after 72 h of incubation. Approximately 1 mL of samples was centrifuged at 10 000 rpm for 10 min. The supernatant obtained was filtered through a 0.25 µm syringe filter into the auto-sampling vial and was run at a flow rate of 1 mL/min for 35 min with injection volume 25 µL into the column.

**RESULTS AND DISCUSSION**

Recombinant endoglucanase CMC3 was successfully expressed and purified with His-Trap™ HP column (IMAC). The protein fused with an affinity polyhistidine tag expressed in *P. pastoris* system was detected and captured by an immobilised metal ion packed in the column. The enzyme eluted from the column was further analysed by SDS-PAGE and Western blot analysis. Figure 1 (A) shows a single band of purified CMC3 with a molecular weight of ~58 kDa. The eluted enzyme showed hydrolysis activities on carboxymethylcellulose substrate and was verified and confirmed with a Western blot analysis (Figure 1B). Approximately 71% of the recombinant CMC3 was recovered after the purification at a purification factor of 4.8-fold (Table 1). The enzyme activity was also enhanced after subsequent steps of purification. CMC3 was active towards CMC substrates, but no activity was detected when CMC3 reacted with the OPEFB fibres. However, Cellucast® 1.5 L was active on CMC and OPEFB substrates. Both enzymes were stable and showed optimum activity at 50-60 °C.

The chemical reactions of recombinant CMC3 and Celluclast® against CMC were shown in Lineweaver–Burk plot and used for the determination of the kinetic constant, $K_m$ and $V_{max}$ values (Figure 2). For the CMC3 enzyme, $K_m$ and $V_{max}$ values were 9.3 mg/mL and 1202 µmol/min/mg, respectively. Meanwhile, the $K_m$ and $V_{max}$ values of Celluclast® enzyme were 1.25 mg/mL and 211 µmol/min/mg, respectively, which were lower than those of recombinant CMC3. Celluclast® showed higher affinity to CMC compared with recombinant CMC3 enzyme. The reason may be that Celluclast® contains five different endoglucanases (Gruno *et al*. 2004; Druzhinina and Kubicek, 2017) that can bind to CMC more tightly than CMC3.

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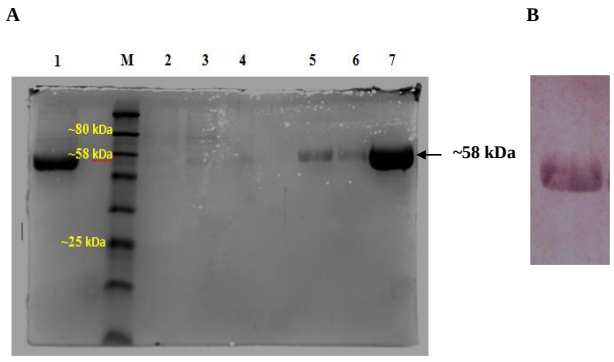


**Figure 1:** SDS-PAGE and Western blot analysis of purified CMC3. (A) Lane M, protein marker; Lane 1, CMC3 crude extract; Lane 2-6: unbound protein, Lane 7: purified CMC3 ~58 kDa; (B) Western blot of purified recombinant CMC3.

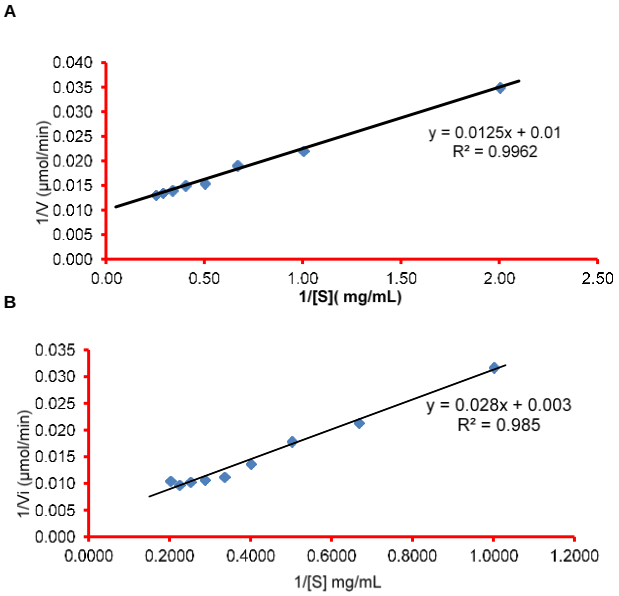


**Figure 2:** Lineweaver-Burk plot of chemical reactions recombinant CMC3 and Celluclast® against CMC substrate. (A) Lineweaver-Burk graph of Celluclast®, (B) Lineweaver-Burk graph of recombinant CMC3.

The degradation of crystalline cellulose to glucose requires at least three cellulases, such as endoglucanase (EC 3.1.2.4), cellobiohydrolase (exoglucanase) (EC 3.1.2.91) and β-glucosidase (EC 3.1.1.21) (Nazir *et al*., 2009; Sajith *et al*., 2016). Celluclast®, a commercial cellulase derived from *T. reesei*, which is well-known for its β-glucosidase activity (Merino and Cherry, 2007). To eliminate the product inhibition by cellobiose and increase the synergistic reaction between endoglucanase and exoglucanase, we added β-glucosidase to the mixture at a constant concentration. β-glucosidase can hydrolyse cellobiose and cellodextrin into simple sugars, thereby increasing the hydrolysis activities and removing the inhibition effect (Berlin *et al.*, 2005; Kristensen, 2008; Ng *et al.*, 2011; Teugjas and Väljamäe, 2013).

We analysed the standard sugar mixtures and hydrolysate sample after 72 h of incubation by HPLC to quantitatively determine the monomeric sugar produced after the reactions. Five standard sugar mixtures (xylose, glucose, arabinose, galactose and cellobiose) were successfully separated with various retention times. The ELSD is nonlinear for area versus concentration; therefore, the curves of calibration were built as $\log_{10}$ area versus $\log_{10}$ concentration (Agblevor *et al.*, 2004; Agblevor *et al.*, 2007). The analysis from the blank sample containing 50 mM sodium acetate buffer and OPEFB showed that no sugar peaks appeared (Figure 3A). The unknown peaks and a hump in the blank sample were detected at retention time (RT) of 19-30 min and at RT of 3.5-4.5 min (Figure 3A). The unknown peak may have originated from acetonitrile mobile phase and sodium salt that were present in the buffer solution containing pretreated OPEFB. It was not considered a sugar compound because the peaks appeared at the same retention time when the solution was run alone. Figures 3B, 3C and 3D show the chromatogram of hydrolysate mixture of Celluclast® and Accelerase® BG, Cellucast:CMC3 (ratio 1:40) and Accelerase® BG and Celluclast®:CMC3 (ratio 1:100) and Accelerase® BG, respectively. The analyses of chromatogram sample hydrolysate successfully resolved two peaks of sugars, which were identified as xylose and glucose. Xylose was resolved at RT around 9.6 min, whereas glucose was resolved at around 16.2 min. The presence of xylose sugar suggested the present of xylanase activities in the commercial Celluclast® cocktail (Viikari *et al.*, 2007) that can hydrolyse amorphous structure of xylan polysaccharide. However, the unknown peak that appeared in a blank OPEFB sample was also detected in all hydrolysate samples at RT of around 24-30 min.

Table 2 shows the quantitative analysis of xylose and glucose concentration in all three reaction mixtures by HPLC based on calibration graph of a standard sugar mixture. The concentrations of both monomeric sugars were determined according to the total sugar produced in 7.5 mL reaction mixture and then compared with the reaction without CMC3. The amount of xylose and glucose increased by 27% and 59%, respectively, when the 1:100 mixture of Celluclast® and CMC3 was used. However, the supplementation of CMC3 activities at a low concentration (ratio 1:40 unit) showed no improvement with respect to the amount of monomeric sugar produced. The results suggest that recombinant endoglucanase CMC3 partly improves the hydrolysis of OPEFB fibres by commercial enzymes.

Endoglucanase hydrolyse internal celluloses binds randomly to create new chain end and susceptible points for *T. reesei* exocellulase digestion. The supplementation of endoglucanase in Celluclast® cocktail increased the synergistic action of endocellulases and exocellulases to produce dimer cellobioses, which was further hydrolysed into glucose by β-glucosidase. The degradation of

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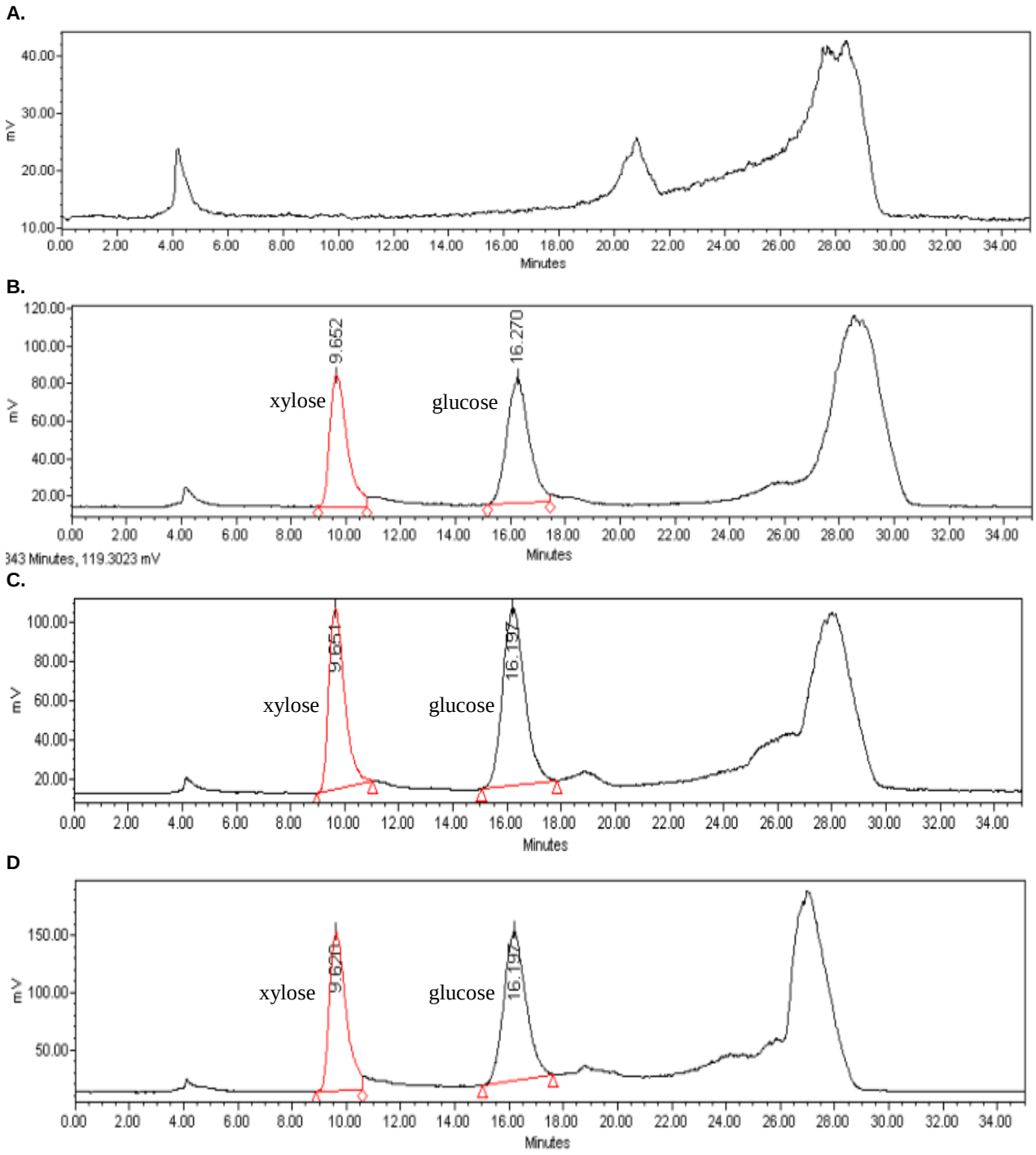


**Figure 3:** Chromatogram of pretreated OPEFB after enzymatic hydrolysis. (A), pretreated OPEFB without enzyme (blank sample), (B) Reaction pretreated OPEFB with Celluclast® and Accelerase® BG, (C) reaction pretreated OPEFB with Celluclast® and recombinant CMC3 (ratio 1:40) and Accelerase® BG, (D) reaction pretreated OPEFB with Celluclast® and recombinant CMC3 (ratio 1:100) and Accelearase® BG.

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cellulose by cellulases may disrupt the hydrogen bonding between xylan and cellulose. Breaking one of these hydrogen bonds enables xylanase to hydrolyse xylan chains and subsequently produce xylose. According to Wyman *et al.* (2005), xylan forms hydrogen bond with cellulose, lignin and other hemicelluloses. Thus, the removal of these substituents generally increase the rate of degradation by xylanase enzymes. This finding suggests that the performance of Celluclast® to hydrolyse cellulose and xylose can be improved by adding endoglucanase instead of adding β-glucosidase in an enzyme cocktail.

Filamentous fungus *T. reesei* is among the best protein secretors, making it attractive for industrial exploitation (Paloheimo *et al*., 2016). However, *T. reesei* cellulase complexes exhibit lower levels of endoglucanase and β-glucosidase activities (Gruno *et al*., 2004; Merino and Cherry, 2007) compared with that of exoglucanase activity. In this study, we showed that adding *H. insolens* endoglucanase into the cocktails of commercial cellulases improves the hydrolysis capacity of biomass into sugar. Further experiment involving the addition of hemicellulases to the reaction mixture for the improvement of sugar hydrolysis should be performed for the optimization of OPEFB biomass hydrolysis.

## CONCLUSION

The addition of recombinant endoglucanase CMC3 from *H. insolens* ATCC 16454 into Celluclast® and Acellerase® BG enzyme mixture improved glucose and xylose production by up to ~59% and ~27%, respectively, during the hydrolysis of OPEFB fibres. Therefore, recombinant CMC3 from *H. insolens* supplements commercial enzymes, such as Celluclast® during the breaking down of OPEFB fibres into simple and fermentable sugars.

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