Fractionation and hydrolysis of cellooligosaccharides by *Trichoderma reesei* Cellobiohydrolase 7A

Sharifah Annirah Syed Abdul Rahman¹, Dayang Norulfairuz Abang Zaïdel¹,²*

¹School of Chemical and Energy Engineering, Faculty of Engineering, Universiti Teknologi Malaysia, 81310 Johor Bahru, Johor, Malaysia.
²Institute of Bioproduct Development, Universiti Teknologi Malaysia, 81310 Johor Bahru, Johor, Malaysia.

*Corresponding author: dnorulfairuz@utm.my

**Abstract.** This study aimed to investigate the fractionation and hydrolysis of cellooligosaccharides by *Trichoderma reesei* Cellobiohydrolase 7A (*TrCel7A*). The enzyme *TrCel7A* was purified from enzyme mixture (Celluclast®). Fractionation of soluble cellooligosaccharides was performed using size exclusion chromatography (SEC) and hydrolysis of the cellooligosaccharides with degree of polymerisation (DP) from 3 to 6 was conducted by *TrCel7A* at 25 °C for 1 h, and the product concentration was analysed by high-performance liquid chromatography (HPLC). HPLC results showed that glucose was produced for each DP of cellooligosaccharides. However, the highest product that achieved for all DP of cellooligosaccharides was cellobiose, followed by cellotriose (in the case of DP 5 and 6) and glucose. The reaction of cellooligosaccharides with DP 3 to 6 during hydrolysis by *TrCel7A* were modelled to show exactly the cleavage sites for each DP of cellooligosaccharides. The model showed that all cellooligosaccharides was hydrolysed at the first (glucose) and second (cellobiose) glycosidic linkages by *TrCel7A*.

1. Introduction
Lignocellulosic biomass consists of three different parts; cellulose, hemicellulose, and lignin. Cellulose is the major component of plant cell walls and the most abundant polysaccharides when compared to hemicellulose and lignin, with nearly 20 to 50 % based on its dry weight [1]. Cellulose has a great potential as a renewable energy source and it can be applied in various industries such as paper and pulp, construction materials for the polymer, and natural textile fibers. Hydrolysis of cellulose to soluble sugars can be used as a starting material for the production of food, fuel and industrial chemicals [2]. Cellulose is insoluble in water and heterogeneous due to the complex structure of cellulose, which contains both crystalline and amorphous regions. The heterogeneous of cellulose structure directed to a rapid fall in hydrolysis rate as the reaction continues [3]. Hence, the breakdown of cellulose chain is slightly difficult. This matter can be solved by using soluble cellulose-based substrate such as a soluble cellooligosaccharides. Cellooligosaccharides are linear oligomers of glucopyranose moieties linked by β-1, 4-glycosidic bonding. In fact, cellooligosaccharides are a shorter form of cellulose, thus they have a matching chemical structure as cellulose. Cellooligosaccharides with up to a degree of polymerisation (DP) 8 are soluble in water and produced
by the controlled hydrolysis of cellulose followed by fractionation and purification of cellooligosaccharides into different chain lengths (DP) [4]. Industrially, cellulose can be degraded into soluble sugar chain via enzymatic or acid hydrolysis. Researchers have proven that the enzymatic hydrolysis is preferable, and efficient than acid hydrolysis in degrading the complex chain of cellulosic biomass [5]. Enzymatic hydrolysis is performed under a mild condition of reaction such as temperature and pressure, environmental-friendly and produced highly selective of fermentable sugars [6]. Several steps are involved in the enzymatic hydrolysis of cellulose such as adsorption of cellulase on cellulose surface, the breakdown of cellulose to soluble sugars and desorption of resulting sugars from the cellulose. The efficient hydrolysis of cellulose requires a synergistic action of multiple enzymes in cellulase. There are three different enzymes in cells, namely, exoglucanase or known as cellobiohydrolase (CBH), endo-glucanase (EG), and beta-glucosidase (BG). CBH is an exo-processive enzyme that begins hydrolysing from the cellulose chain end, whereas EG is an endo-processive enzyme that randomly cleaves the cellulose chain. Whilst, BG hydrolyses cellobiose to glucose, thus reduce the product inhibition of CBH [7].

Cellulases are primarily produced by microorganisms, for instance, bacteria and fungi. Soft-rot and white-rot fungi, for example, *Humicola*, *Trichoderma*, *Schizophyllum*, *Penicillium* and *Fusarium* secrete efficient cellulase systems for hydrolysis of cellulose. To date, the best characterised cellulolytic system was from soft rot fungus *Trichoderma reesei* (*T. reesei*) [8]. The main component of *T. reesei* cellulolytic system is glycoside hydrolase (GH) family 7 cellobiohydrolase, *TrCel7A* (previously known as CBH I). Several studies were conducted on hydrolysis of soluble cellooligosaccharides by *TrCel7A*. A study by Nidetzky *et al.* showed that the initial velocity of cellooligosaccharides degraded by *TrCel7A* increased with DP up to 6 (cellohexaose) and then remained constant [9]. Therefore, this study aimed to investigate the hydrolysis pattern of soluble cellooligosaccharides such as cellotriose (DP 3), cellotetraose (DP 4), cellopentaose (DP 5), and cellohexaose (DP 6) catalysed by *TrCel7A*. The hydrolysis pattern was modelled to show exactly the cleavage sites for each DP of cellooligosaccharides.

2. Materials and Methods
This section described the materials and methods used in this study.

2.1. Solvents and reagents
Glucose, cellobiose, cellotriose, cellotetraose, cellopentaose, cellohexaose and cellulose diacetate (38 wt. % acetyl content) were purchased from Sigma Aldrich, USA. Disodium salt hydrate (BCA) was purchased from Fluka (England). All reagents were of analytical grade and used as received.

2.2. Preparation and purification of enzyme
*Trichoderma reesei* cellobiohydrolase 7A (*TrCel7A*) was purified from commercial enzyme mixture, Cellulast® from *Trichoderma reesei* ATCC 26921 (Sigma-Aldrich). The buffer was exchanged as 50 ml of cellulase mix solution was filtered through 0.2 µm PVDF filter (Macherey-Nagel) to eliminate all insoluble particles that may damage column. Toyopearl HW40 column (378 ml) was equilibrated with 10 mM ammonium acetate buffer pH 5.0 at 4 °C and 50 ml of filtered cellulase mix solution was loaded. The flow rate was 0.5 ml/min, fraction size was 10 ml and 56 fractions were collected. The presence of protein was detected by measuring absorbance at 280 nm on UV-VIS spectrophotometer (Shimadzu, Japan). Fractions 15 to 25 were gathered for further purification step [10]. Ion-exchange chromatography was performed using the ÄKTA Explorer chromatography system and 55 ml Q-sepharose column (GE Healthcare). Before loading sample, the column was equilibrated with 10 mM ammonium acetate buffer pH 5.0 and 50 ml of sample were loaded. Flow rate was 0.5 ml/min except for sample loading step when flow rate was 1 ml/min. The presence of protein was detected by measuring absorbance at 280 nm [10].
2.3. Preparation and fractionation of soluble cellooligosaccharides

Water-soluble cellulose acetate samples (DS 0.5) were prepared by mixing 10 g cellulose acetate with an acetyl content of 38 wt % (DS 2.5) in a mixture of 7.8 ml of methanol and 22.2 ml of acetic acid in a round flask at room temperature for 16 hours. Then, 0.025 moles of sulfuric acid was added and the medium was heated to 72 °C. The mixture was kept at this temperature for 200 minutes. After cooled for 30 minutes, 0.05 moles of sodium acetate trihydrate was added to the mixture for neutralizing the acid and stirred until dissolved. Then, the sample was transferred to 1.5 ml microcentrifuge tubes and the sample was poured in methyl acetate and washed by centrifuged sample at 10000 g for 2 minutes to remove any byproduct formed in the sample. Washing and separation of the sample were performed for three times. After that, 1 ml of milli-Q water was added to the sample, mixed and centrifuged at 10000 g for 2 min to remove salt in the sample. The supernatant was kept and transferred to new 1.5 ml microcentrifuge tubes. The sample was then evaporated at 45 °C for 30 minutes via vacuum concentrator (Eppendorf) [11].

Partially acetylated cellooligosaccharides was degraded by Celluclast® using 800 μl partially deacetylated cellulose acetate by adding and mixing 1 ml of 50 mM sodium acetate pH 5.0 and 5 μl of cellulase. The sample mixture was incubated at 40 °C for 3 hours via heating block (Eppendorf) and the reaction was stopped. After that, partially acetylated cellooligosaccharides was deacetylated using samples that already dissolved as mentioned in the previous steps. Cellulase was removed by centrifuging at 10000 g for 5 minutes. Supernatant was recovered, kept in a new 1.5 ml microcentrifuge tubes and evaporated at 45 °C using vacuum concentrator (Eppendorf). After dried, 400 μl of sample was added with 50 mM sodium methoxide (dissolved in methanol).The mixture was incubated and shaken constantly at 500 g for 30 minutes at 25 °C. After incubated, 3 μl of 10% acetic acid (neutralizing effect) was added to each tube, mixed and dried in a vacuum concentrator at 45 °C. The solubility of cellooligosaccharides was tested by adding 1 ml of milli-Q water. As a result, the DP profile of the dissolved samples that contain soluble cellooligosaccharides mixture was determined by size exclusion chromatography (SEC). Soluble cellooligosaccharides was fractionated by size exclusion chromatography whereby the samples in tubes were centrifuged at 45 °C using vacuum concentrator (Eppendorf). After dried, 400 μl sample was added with 50 mM sodium methoxide (dissolved in methanol). The mixture was incubated and shaken constantly at 500 g for 30 minutes at 25 °C. After incubated, 3 μl of 10% acetic acid (neutralizing effect) was added to each tube, mixed and dried in a vacuum concentrator at 45 °C. The solubility of cellooligosaccharides was tested by adding 1 ml of milli-Q water. As a result, the DP profile of the dissolved samples that contain soluble cellooligosaccharides mixture was determined by size exclusion chromatography (SEC). Soluble cellooligosaccharides was fractionated by size exclusion chromatography whereby the samples in tubes were centrifuged (10000 g) and the supernatant was preserved for separating protein/Celluclast® and sugar. Subsequently, the samples were filtered through 0.2 μm PVDF filter. Then, SEC was performed using the AKTA explorer chromatography system (GE Healthcare) at 4 °C. The column (HiLoad 26/600 Superdex 30; column volume: 360 ml; a flow rate: 0.5 ml/min) was equilibrated and eluted with water.

2.4. Analysis of the fractionated samples

From the SEC sample, 191 out of 224 fractions (1 ml each fraction) were analysed for the concentration of reducing end groups using the modified BCA method at 560 nm. Two different solutions, reagent A (77.7 mg BCA; 2.171 g sodium carbonate; 0.968 g sodium bicarbonate; 40 ml water) and B (50.5 mg L-serine; 50 mg copper (II) sulfate; 40 ml water) were prepared and mixed at ratio 1:1 (v/v). The reaction was performed as the sample was added to AB reagent at ratio 1:1 and incubated at 75 °C for 30 minutes. Then, the sample mixture was cooled to room temperature. Calibration was prepared using cellobiose at various concentrations; 0, 0.5, 1, 3, 5, 10, 15, 25, 35 μM. Samples were then pipetted into a 96-well plate (F bottom, Greiner bio-one). The concentration of sugar was analysed via ELISA microplate reader (Eppendorf) at 560 nm [12,13,14].

2.5. Hydrolysis and analysis of cellooligosaccharides

Soluble cellooligosaccharides (DP 3 to 6) were hydrolysed enzymatically and the patterns of hydrolysis products were analysed. Each hydrolysis was performed in 1.5 ml microcentrifuge tubes in 50 mM ammonium acetate buffer pH 5.0 at 25 °C, containing BSA 0.1 g/L. All oligosaccharides were hydrolysed separately at three different substrate concentration; 200 μM, 300 μM, 400 μM. Hydrolysis was started by the addition of the TrCel7A to bring the final concentration in hydrolysis mixture to 0.1 μM, except in the case of cellotriose hydrolysis where TrCel7A used was brought to a final concentration of 1 μM. The reaction volume was 900 μl. The reaction was stopped after 1 hour when
100 μl of 1 M ammonium hydroxide was added to the reaction mixture. Then, the reaction mixture was filtered through molecular weight 5000 cut-off Vivaspin 500 filter and centrifuged for 10 minutes at 10000 g (Eppendorf). About 800 μl of the filtrate was neutralized with freshly filtrated 80 μmol of acetic acid (10%) through filter Nylon Aerodisc 0.45 μm (Gelman Sciences) and evaporated via vacuum concentrator (Eppendorf). Each concentration of substrate was dried except for substrate containing 200 μM of cellotetraose and cellopentaose. The background for each substrates with different concentration was also prepared by adding the stop buffer first before mixed with the enzyme solution, ensuring no formation of the product.

The concentrations of formed glucose, cellobiose and cellotriose were analysed by HPLC. HPLC was carried out using a Prominex HPLC system (Shimadzu) equipped with an Aminex HPX-87P (BioRad, 5 μm, 250 mm x 7.8 mm) column and a refraction index detector RID-10A (Shimadzu). The column temperature was kept at 80 °C, the flow rate was 0.6 ml/min, and the eluent was water [15,16].

2.6. Modeling the hydrolysis pattern of cellooligosaccharides by TrCel7A
The reaction of cellooligosaccharides with DP 3 to 6 during hydrolysis by TrCel7A were modeled to show exactly the cleavage sites for each DP of cellooligosaccharides. In fact, the reaction occurred in the active site tunnel of TrCel7A when cellooligosaccharides chain were attacked by TrCel7A. These models were drawn using CorelDRAW Graphics Suite X8 software and developed based on the experimental measurement of the product concentration from HPLC analysis.

3. Results and discussion
3.1. Production of cellooligosaccharides
Partially acetylated cellulose that soluble in water was formed from partial deacetylation and depolymerisation of cellulose diacetate as a starting material and it had a DS of 0.5. At this stage, this method assumed that the DP of resulting sample was around 150 due to partial depolymerisation of cellulose diacetate with a DP of 250 that occurred simultaneously with partial deacetylation. This finding was supported by Gomez-Bujedo et al and Malm et al. that obtained water-soluble cellulose acetate at a DS comprised between 1 and 0.5 [11,18]. The result shows that not only partial deacetylation occurred, but simultaneous depolymerisation was also observed in this reaction. These processes might happen because of the hydrolysis of the glycosidic bond in the catalytic acid media. Then, limited hydrolysis was performed by mixing the resulting sample from production of watersoluble partially acetylated cellulose with Celluclast® to further decrease its DP. However, the reaction with cellulases does not affect DS value. Thus, partially acetylated cellooligosaccharides with DP as low as 1 to 6 were achieved from initial DP of 250. This can be explained by the fact that the substrate crystallinity has an impact on the DP of the substrate [19]. Further fraction was shown in the Figure 1. Depolymerisation occurs as a result of substrate chain being attacked by endo-glucanases and exo-glucanases (also known as cellobiohydrolases). Endo-glucanases is favourable to attack less ordered regions of the cellulose chain, quickly reduce the DP of substrate. In contrast, exo-glucanases which produce cellobiose by hydrolysing from the end of the substrate chain is unable to cause a major effect to alter the DP during hydrolysis. However, there appears to decrease the cellulose DP irrespective of the substrate being hydrolysed. This is associated with the increased recalcitrance of the residual (crystalline) cellulose [14]. Next, total deacetylation was conducted to fully remove the acetyl group in the partially acetylated sample by using sodium methoxide that dissolved in methanol. As a result, various DP of soluble cellooligosaccharides (DP 1 to 6) with DS of 0 was successfully produced as it was observable that cellooligosaccharides formed was soluble when tested by mixing with water.

The chromatographic technique was used to be able to identify the hydrolysis pattern. Soluble cellooligosaccharides (DP 1 to 6) from the total deacetylation process were measured and quantified. 191 out of 224 fractions (1 ml each) were analysed for the concentration of reducing end groups using the modified BCA method at 560 nm. Fractionation of cellooligosaccharides by size-exclusion
chromatography is shown in Figure 1. A good separation is obtained which produces six different peaks in the chromatogram which specifies cellooligosaccharides with DP 1 to 6. These peaks were released between 247 to 305 ml of elution buffer. The data demonstrates that increased DP of cellooligosaccharides thus increased the concentration of reducing group in the substrate. According to Zhang and Lynd cellooligosaccharides with DP 1 to 6 are soluble in water and cellooligosaccharides with DP 7 to 12 are partially soluble [14]. However, cellooligosaccharides with higher DP than DP 12 are insoluble in water. The issue of solubility of cellooligosaccharides does not influence the measurement of the reducing sugars. However, only soluble hydrolysis products can be investigated in the chromatography. In this step, cellooligosaccharides with DP 3 to 6 were selected for hydrolysis using purified TrCel7A.

![Figure 1. Fractionation of soluble cellooligosaccharides by size-exclusion chromatography. Note: G1=Glucose, G2=Cellobiose, G3=Cellotriose, G4=Cellotetraose, G5=Cellopentaose, and G6=Cellohexaose](image)

3.2. Analysis of cellooligosaccharides using HPLC
Enzymatic hydrolysis of cellooligosaccharides with DP 3 to 6 by purified TrCel7A resulted in the conversion of the cellooligosaccharides to soluble sugars in 1 hour at 25 °C. It is noted that different concentrations (200 μM, 300 μM and 400 μM) of the cellooligosaccharides with DP 3 to 6 were concentrated, except for 200 μM of cellotetraose (DP 3) and cellopentaose (DP 4) during hydrolysis. Figure 2 displays the chromatogram peaks of background and hydrolysis products for cellooligosaccharides with DP 3 to 6. This study only shows the chromatogram selected from a single concentration (400 μM) for DP 3 to 6 to compare the peaks of background and hydrolysis product. Besides, the height and area of the corresponding peaks for background and non-background (product) were not similar to each other, although using the same concentration (400 μM) for every DP of cellooligosaccharides. Moreover, when the soluble cellooligosaccharides (DP 3 to 6) were subjected to hydrolysis by TrCel7A, these substrates were completely hydrolysed to glucose and cellobiose. Cellotriose also was formed as a result of hydrolysis of the substrate with DP higher than 4. Based on the chromatogram, it can be noticed that the background for each DP of cellooligosaccharides did not
produce any product due to the inactivity of the enzyme \textit{Tr}Cel7A as the stop buffer (ammonium hydroxide) was initially added to the reaction before enzyme.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Glucose, cellobiose, and cellotriose (if any) formation from hydrolysis of cellooligosaccharides with DP 3 to 6 by \textit{Tr}Cel7A. (a) Cellotriose (DP 3) (b) Cellotetraose (DP 4) (c) Cellopentaose (DP 5) (d) Cellohexaose (DP 6). Note: A=Background, B=Hydrolysis products.}
\end{figure}
As shown in Figure 2(a), the amount of product cellobiose from hydrolysis of cellotriose (DP 3) by *TrCel7A* could not be detected due to non-separated peak. Theoretically, the amount of cellobiose should be equal to glucose as *TrCel7A* processively cleaves the substrate chain by two units of glucose. Table 1 shows an average concentration of the hydrolysis product formed for DP 3 to 6 of cellooligosaccharides with different concentrations; 200 μM, 300 μM, and 400 μM. It is apparent from the data shown that glucose was produced for each DP of cellooligosaccharides. However, the highest product that achieved for all DP of cellooligosaccharides was cellobiose, followed by cellotriose (in the case of DP 5 and 6) and glucose. This sugar production pattern was in agreement with Medve *et al.* and Nidetzky *et al.* that showed cellobiose as the main product of hydrolysis of cellooligosaccharides by *TrCel7A* [9,16]. Nevertheless, cellobiose concentration for DP 3 was not able to quantify due to an aggregated peak as shown in Figure 2(a). Besides, hydrolysis with even DP (DP 4 and 6) produces not only cellobiose, but glucose and cellotriose (in the case of DP 6). Nidetzky *et al.* claimed that only cellobiose should be produced from the attack of *TrCel7A* on the cellooligosaccharides with an even DP [9]. However, a lack of specificity of cellobiose being released from cellooligosaccharides chain might be due to the endoprocessive action of *TrCel7A* [19]. Furthermore, in this study, the presence of product with a DP higher than 3 or larger than substrate was not detected during hydrolysis by purified *TrCel7A*.

| Substrate DP | Products (µM) | Ratio G₂/ G₁ |
|--------------|---------------|--------------|
|              | G₁ | G₂ | G₃ |               |
| 3            | 35.3±3.2 | n.d | n.d | n.d |
| 4            | 18.1±3.8 | 106.6±11.3 | n.d | 5.9 |
| 5            | 30.0±2.0 | 94.4±9.7 | 35.4±10.0 | 3.2 |
| 6            | 39.1±3.8 | 96.0±1.9 | 67.6±3.7 | 2.5 |

Note: n.d= not determined, G₁=glucose, G₂=cellobiose, G₃=cellotriose, DP=degree of polymerisation.

It indicates that transglycosylation does not happen during the reaction as confirmed by Nidetzky *et al.* and Vršanská and Biely [9,20] The production of significant amounts of products less than DP 3 (glucose, cellobiose and cellotriose) by *TrCel7A* proves that this enzyme was able to bind productively and non-productively onto the cellooligosaccharides surface. The result in Table 1 also displays the ratio of cellobiose to glucose (range from 5.89 uM to 2.46 uM) to determine the processivity of the hydrolysis in this study. The ratio decreases with an increasing of substrate DP. Indeed, the processive reaction of *TrCel7A* generates several cellobioses from cellooligosaccharides chain and the action of *TrCel7A* results in a gradual decrease of the DP of cellooligosaccharides as it acts on the chain ends [21]. Hence, higher processivity relates to higher production of cellobiose. Thus, it can be concluded that *TrCel7A* hydrolysed cellooligosaccharides according to processive mechanism that cleaves more than one glycosidic bond with every association with the substrate surface (in the case of longer chain with DP higher than 3) before dissociate from enzyme substrate complex [22].

### 3.3. Reaction model of cellooligosaccharides

Figure 3 illustrates the reaction model of primary attack of *TrCel7A* on cellooligosaccharides chain at several binding sites based on the information given in Table 1. *TrCel7A* has contained its active site residues in a 50 Å long tunnel, which covers 5 units of cellobiose (equal to 10 units of glucose) for binding with the substrate [23]. Due to the shorter chain length of soluble cellooligosaccharides used in this study (DP up to 6), only -5 to +3 sites in the active site tunnel of *TrCel7A* are shown for hydrolysis of this substrate. The cleavage only occurs between subsite +1 and -1 in the active site tunnel of the cellooligosaccharides chain. Hence, the +1/+2 sites are known as the product sites. Practically, the +3 site has no interaction with the residue of enzyme.
| Reaction Models |
|-----------------|
| **(a) Cellotriose (DP 3)** |
| bond cleavage | Binding site |
| -2 -1 +1 +1 | (-2,1) |
| bond cleavage | |
| -3 -1 +2 +1 | (-1,2) |

| **(b) Cellotetraose (DP 4)** |
| bond cleavage | Binding site |
| -2 -1 +1 +1 | (-3,1) |
| bond cleavage | |
| -2 -1 +2 +1 | (2,2) |

| **(c) Cellopentaose (DP 5)** |
| bond cleavage | Binding site |
| -4 -3 -1 +1 +1 | (-4,1) |
| bond cleavage | |
| -3 -2 -1 +1 +2 | (-3,2) |

| **(d) Cellohexaose (DP 6)** |
| bond cleavage | Binding site |
| -5 -4 -3 -1 +1 +1 | (-5,1) |
| bond cleavage | |
| -4 -3 -2 -1 +1 +2 | (-4,2) |
| bond cleavage | |
| -3 -2 -1 +1 +2 +3 | (-3,3) |

Figure 3. Reaction models of cellooligosaccharides with DP 3 to 6 from hydrolysis by *TrCel7A* with their binding sites (in rectangle). (a) Cellotriose (DP 3), (b) Cellotetraose (DP 4), (c) Cellopentaose (DP 5) (d) Cellohexaose (DP 6). Note: the semi-oval shape is glucose unit, the connector is glycosidic linkage.
Besides, the +4 site is positioned outside the active site tunnel and does not possess carbohydrate-protein interactions. Based on the graph shown in Figure 2(a) to 2(c), hydrolysis of cellotriose, cellotetraose and cellopentaose were predicted to cleave at two binding sites productively. On the other hand, the cleavage for cellohexaose (Figure 2(d)) involves three productive binding sites. As shown in those figures, hydrolysis of some cellooligosaccharides involves the primary and/or secondary productive binding of TrCel7A on the substrate chain. For all cellooligosaccharides, the primary productive binding by TrCel7A occurs at the first (glucose) and second (cellobiose)glycosidic linkages. In contrast, for cellohexaose, the primary productive binding by TrCel7A only happens in the third glycosidic linkage (cellotriose). For cellopentaose, there were two possibilities of primary productive binding of TrCel7A at the binding site (-4,1) and (-3,2) that release celloctaetraose and celloctaetriose as products. However, in the case of celloctaetraose (product of primary attack of cellopentaose) at (-4,1), thus secondary productive binding takes place for further hydrolysing celloctaetraose into two molecules of celloctaetiose. For cellohexaose, primary productive binding by TrCel7A at three binding sites (-5,1), (-4,2) and (-3,3) thus release celloptaetraose, celloctaetetaose and celloctaetriose as products. As a result, secondary productive binding of celloptaetraose (product of primary attack of cellohexaose) at (-4,2) yields celloctaetrioise and celloctaetiose. Moreover, secondary productive binding of celloctaetraose (product of primary attack of cellohexaose) at (-4,2) produces another two molecules of celloptaetiose. Therefore, this study proves that the whole cellooligosaccharides chain is in the active site of the TrCel7A (between -5 to +3 sites) and these substrates entering the active site tunnel of TrCel7A from the -7 site end.

4. Conclusion
In conclusion, the hydrolysis of soluble cellooligosaccharides with DP 1 to 6 catalysed by TrCel7A was successfully analysed. It is shown that the TrCel7A hydrolysed cellooligosaccharides chain from its reducing end. Thus, it indicates that the reducing end of cellooligosaccharides chain enters the active site tunnel of TrCel7A from its -7 side end, showing that the whole cellooligosaccharides chain was situated in the active site of the enzyme TrCel7A during hydrolysis.

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