HYDROLYSIS OF SOLUBLE CELLOOLIGOSACCHARIDES BY *TRICHODERMA REESEI* CELLOBIOHYDROLASE 7A

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A thesis submitted in fulfilment of the
requirements for the award of the degree of
Master of Engineering (Bioprocess)

Faculty of Chemical and Energy Engineering
Universiti Teknologi Malaysia

JUNE 2017

Specially dedicated to my *husband, mother, late father, late grandparents and family.*

LOVE ALL OF YOU TILL JANNAH!

ACKNOWLEDGEMENT

Thanks to Almighty Allah S.W.T for His blessing in completing this thesis. This study was carried out at the Institute of Molecular and Cell Biology (OMICUM), University of Tartu (UT), Estonia. I appreciate to all of my colleagues, especially to Mihhail Kurasin and Riin Kont for fruitful and enjoyable collaboration, friends, relatives and Yayasan Sultan Iskandar who have helped me to make this work possible. In particular, I express my sincere gratitude to my supervisor and co-supervisor, Dr. Dayang Norulfairuz Bt Abang Zaidel and Assoc. Prof. Priit Valjamae (UT) for their guidance, valuable comments and support throughout the course of this research. Last but not least, a special thanks to my husband (Mr. Ahmad Azmi), my family and for those who pray for my success in submitting this thesis.

ABSTRACT

Cellulose is the main component of plant cell wall and the most abundant biopolymer on the earth. The complexity of cellulose structure and multiple binding modes of cellulose restrict the ease of understanding the mode of action of cellulolytic enzymes. Hence, in this study, soluble cellooligosaccharides were used as substrates. In nature, microorganisms are capable of degrading cellulose by secreting a set of enzymes called cellulases. Most studies have been conducted on the cellulolytic system that is secreted by the soft-rot fungus, *Trichoderma reesei* (*Tr*). This study aims to analyse the hydrolysis pattern of cellooligosaccharides (including cellotriose, cellotetraose, cellopentaose and cellohexaose) when hydrolysed by *Tr* cellobiohydrolase 7A (*Tr*Cel7A) and evaluate the reaction kinetics such as bond cleavage frequencies and bond cleavage probabilities. This work involves purification of enzyme *Tr*Cel7A from enzyme mixture (Celluclast®), analysis of purified enzyme *Tr*Cel7A, preparation of substrate (i.e. cellooligosaccharides), hydrolysis of the cellooligosaccharides with degree of polymerisation (DP) from 3 to 6 by *Tr*Cel7A at 25 °C for 1 hour, and high-performance liquid chromatography analysis of the product concentration**.** Based on kinetic modelling of cellooligosaccharides, the frequencies of hydrolysis of the glycosidic bond in the enzyme-substrate complex and the probabilities that hydrolysis of glycosidic bond took place specifically in the enzyme-substrate complex were calculated. Based on the quantitative data on the bond cleavage frequencies of cellooligosaccharides with DP 4 to 6 showed that the bond cleaved more frequent at glucose linkage (cellopentaose = 0.102 ± 0.021 s^{-1}, cellohexaose = 0.109 ± 0.011 s^{-1}) followed by at cellobiose linkage (cellopentaose = 0.085 ± 0.003 s^{-1}, cellohexaose = 0.053 ± 0.002 s^{-1}) and cellotriose linkage (cellohexaose = 0.040 ± 0.004 s^{-1}). A similar trend was observed for the result of bond cleavage probabilities for most substrates that shows the glucose linkage (cellopentaose = 0.542 ± 0.050 s^{-1}, cellohexaose = 0.540 ± 0.036 s^{-1}) of the substrate chain has the highest probabilities than cellobiose linkage (cellopentaose = 0.458 ± 0.050 s^{-1}, cellohexaose = 0.263 ± 0.021 s^{-1}) and cellotriose linkage (cellohexaose = 0.197 ± 0.022 s^{-1}). Therefore, this research suggested that *Tr*Cel7A catalysed degradation of cellooligosaccharides from the reducing end of the chain. Also, it specified that the position of whole cellooligosaccharides chain is in the active site for the *Tr*Cel7A.

ABSTRAK

Selulosa adalah komponen utama pada dinding sel tumbuhan dan biopolimer paling banyak di bumi. Struktur selulosa yang rumit dan pelbagai jenis ikatan selulosa mengehadkan kefahaman tentang tindakan enzim selulolitik. Oleh itu, dalam kajian ini, *cellooligosaccharides* telah digunakan sebagai substrat. Secara semula jadi, mikroorganisma mampu mengurai selulosa dengan merembeskan satu set enzim yang dikenali sebagai *cellulase*. Kebanyakan kajian telah dijalankan ke atas sistem selulolitik yang dirembeskan oleh kulat yang mereput secara lembut, *Trichoderma reesei* (*Tr*). Kajian ini bertujuan menganalisa hidrolisis *cellooligosaccharides* (termasuk *cellotriose*, *cellotetraose*, *cellopentaose* dan *cellohexaose*) apabila dihidrolisis oleh *Tr cellobiohydrolase* 7A (*Tr*Cel7A) dan menilai tindak balas kinetik seperti kekerapan belahan ikatan dan kebarangkalian belahan ikatan. Kerja-kerja ini melibatkan penulenan enzim *Tr*Cel7A daripada campuran enzim (*Celluclast*®), analisis enzim *Tr*Cel7A yang telah ditulenkan, penyediaan substrat (iaitu *cellooligosaccharides*), hidrolisis *cellooligosaccharides* dengan darjah pempolimeran (DP) dari 3 hingga 6 oleh *Tr*Cel7A pada 25 °C selama 1 jam, dan analisis kromatografi cecair berprestasi tinggi untuk kepekatan produk. Berdasarkan model kinetik, kekerapan hidrolisis ikatan glikosidik pada kompleks enzim-substrat dan kebarangkalian hidrolisis ikatan glikosidik yang berlaku khususnya dalam kompleks enzim-substrat telah dikirakan. Berdasarkan data kuantitatif terhadap kekerapan belahan ikatan *cellooligosaccharides* dengan DP 4 hingga 6 menunjukkan bahawa ikatan terbelah lebih kerap di rantaian glukosa (*cellopentaose* = 0.102 ± 0.021 s^{-1}, *cellohexaose* = 0.109 ± 0.011 s^{-1}) diikuti oleh rantaian *cellobiose* (*cellopentaose* = 0.085 ± 0.003 s^{-1}, *cellohexaose* = 0.053 ± 0.002 s^{-1} dan rantaian *cellotriose* (*cellohexaose* = 0.040 ± 0.004 s^{-1}). Kecenderungan yang sama dapat dilihat bagi keputusan kebarangkalian ikatan hidrolisis untuk kebanyakan substrat yang menunjukkan bahawa rantaian glukosa (*cellopentaose* = 0.542 ± 0.050 s^{-1}, *cellohexaose* = 0.540 ± 0.036 s^{-1}) pada substrat mendapat kebarangkalian tertinggi berbanding rantaian *cellobiose* (*cellopentaose* = 0.458 ± 0.050 s^{-1}, *cellohexaose* = 0.263 ± 0.021 s^{-1}) dan rantaian *cellotriose* (*cellohexaose* = 0.197 ± 0.022 s^{-1}). Oleh itu, kajian ini mencadangkan bahawa *Tr*Cel7A memangkinkan degradasi *cellooligosaccharides* dari hujung rantai yang menurun. Juga, dinyatakan bahawa kedudukan seluruh rantai *cellooligosaccharides* berada pada tapak aktif *Tr*Cel7A.

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LIST OF ABBREVIATIONS

AmAc	-	ammonium acetate
BC	-	bacterial cellulose
BMCC	-	bacterial microcrystalline cellulose
BG	-	*beta*-glucosidase
CBM	-	cellulose binding molecule
CD	-	catalytic domain
CBH	-	cellobiohydrolase
DP	-	degree of polymerisation
DS	-	degree of substitution
EG	-	endo-glucanase
GH7	-	family 7 glycoside hydrolase
GH	-	glycoside hydrolase
HPLC	-	high-performance liquid chromatography
HCl	-	hydrochloric acid
MCC	-	microcrystalline cellulose
*p*NPL	-	*para*-nitrophenol cellobioside
*p*NPL	-	*para*-nitrophenol-*beta*-D-lactoside
SEC	-	size-exclusion chromatography
H_2SO_4	-	sulfuric acid
T. reesei	-	*Trichoderma reesei*

LIST OF SYMBOLS

cm	-	centimetre
^{o}C	-	degree Celsius
hr	-	hour
g	-	gram
g/L	-	gram per litre
g	-	gravity
kDa	-	kilo Dalton
µg	-	microgram
µl	-	microlitre
µM	-	micromolar
mg	-	milligram
ml	-	millilitre
mm	-	millimetre
mM	-	milimolar
min	-	minute
M	-	molar
nm	-	nanometre
%	-	percent
rpm	-	rotation per minute
v/v	-	volume per volume
wt	-	weight

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Recently, there has been a growing interest in carbohydrate degraded by lignocellulosic biomass. It is because they play a vital role in diverse fields, namely, chemical, renewable and ecological-based product, and global energy. According to Perlack *et al.* (2005), conversion of biomass to transportation fuels may perhaps counterweight about 30% of the existing petroleum used in the United States. Currently, biomass from renewable sources is the most abundant source of energy in the United States and European Union. 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 (Pauly and keegstra, 2008). Cellulose, the most recalcitrant carbohydrate substrate, is also produced by some animals and microorganisms including bacteria and algae. 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. Moreover, hydrolysis of cellulose to soluble sugars can be a starting material for the production of food, fuel and industrial chemicals (Chheda *et al*., 2007). Apart from that, 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 (Väljamäe *et al*., 1999). 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) (Akpinar *et al*,. 2004).

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 (Harmsen *et al.*, 2010). It is due to the fact that the enzymatic hydrolysis is performing under a mild condition of reaction such as temperature and pressure, environmental-friendly and produce highly selective of fermentable sugars (Karmakar and Ray, 2011). 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 (Rosgaard *et al.*, 2007). 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 is from soft rot fungus *Trichoderma reesei* (*T. reesei*) (Martinez *et al*., 2008). The main component of *T. r*eesei cellulolytic system is glycoside hydrolase (GH) family 7 cellobiohydrolase, *Tr*Cel7A (previously known as CBH I). Several studies were conducted on hydrolysis of soluble

cellooligosaccharides by *Tr*Cel7A. A study by Nidetzky *et al.* (1994) showed that the initial velocity of cellooligosaccharides degraded by *Tr*Cel7A increased with DP up to 6 (cellohexaose) and then remained constant.

Therefore, this study aims to analyse the hydrolysis pattern of soluble cellooligosaccharides such as cellotriose (DP 3), cellotetraose (DP 4), cellopentaose (DP 5), and cellohexaose (DP 6) catalysed by *Tr*Cel7A. Furthermore, the kinetics of enzymatic hydrolysis was studied to understand the mechanisms of *Tr*Cel7A in hydrolysing cellooligosaccharides. Kinetics equation was derived based on Michaelis-Menten equation in order to quantify the kinetics parameter such as the bond cleavage frequency (observed rate constant) and the probability of bond cleavage that occurs specifically inside the enzyme-substrate complex.

1.2 Problem Statement

Initially, the cellobiohydrolase (CBH) in cellulase is understood to release cellobiose residues processively from the non-reducing end of the cellulose chain. However, recent studies shown that cellulose is attacked by CBH from the reducing and the non-reducing end of the cellulose chain (Puls and Stork, 1995). As a consequence, the ease of understanding the mode of action of cellulolytic enzymes is restricted (Jalak and Väljamäe, 2014).

Apart from that, less kinetic studies was performed on hydrolysis of soluble cellooligosaccharides using individual enzymes isolated from mixture of enzyme, Celluclast® (for example, *Tr*Cel7A in the absence of any synergistic enzymes) (Vršanská and Biely, 1992; Nidetzky *et al.*, 1994). Soluble cellulose-based substrates are important for efficient conversion of cellulose to soluble sugars. It is because the structural limitation of the cellulose such as instant surface area, crystallinity, and the presence of hemicellulose and lignin content might delay the hydrolysis of cellulose (Pan *et al*., 2006). In view of this, this study focuses on

hydrolysis of soluble cellooligosaccharides (DP 1 to DP 6) by *Tr*Cel7A to study the hydrolysis pattern and the kinetics of this enzyme.

1.3 Significant of the Study

This study will be a significant endeavour in production of cello-oligosaccharides, glucose and other soluble sugars from cellulosic wastes. Thus, the cost for producing fuels and chemicals can be reduced. Moreover, this research will provide a database regarding hydrolysis pattern and kinetics equations of *Tr*Cel7A that can be further developed in the future research to increase the understanding on the mechanism of the enzyme-substrate reaction. Furthermore, this study will serve as a future reference to produce non-digestible oligosaccharides (NDO) in food industries since cellooligosaccharides are neither absorbed in the human gastrointestinal tract nor hydrolysed by human enzymes. In addition, they possess important physicochemical and physiological properties. Thus they are potential to increase the health of consumers due to several advantages, for example reduce diabetes and obesity, treat cancer and inflammatory disease, and increase the adsorption of mineral in our body (Mussatto *et al*., 2007).

1.4 Research Objectives

(i) To analyse the hydrolysis pattern of different DP of soluble cellooligosaccharides (cellotriose, cellotetraose, cellopentaose, and cellohexaose) catalysed by *Tr*Cel7A.

(ii) To evaluate the kinetics parameter such as the bond cleavage frequency and the probability for hydrolysis of cellooligosaccharides bond.

1.5 Scopes of Study

Several scopes were covered to fulfil the objectives of this study which covers:

(i) Preparative works that include the purification of *Tr*Cel7A and preparation and purification of cellooligosaccharides which is limited to DP 1 to 6. *Tr*Cel7A was purified from the commercially available crude cellulase preparation, Celluclast® using ion-exchange chromatography. Ion-exchange chromatography was performed using the ÄKTA Explorer chromatography system at 4 ºC using 0.5 M ammonium acetate buffer pH 5.0 as an eluent. Besides, several analyses of purified enzyme *Tr*Cel7A that was obtained in this study was performed which includes *p*NPL hydrolysis, BC hydrolysis, measurement of soluble sugar concentration by the anthrone-sulfuric acid method and gel electrophoresis. Apart from that, cellooligosaccharides was purified using cellulose diacetate as starting material. Preparation of cellooligosaccharides includes partial deacetylation and depolymerisation of cellulose diacetate, limited hydrolysis of partially acetylated cellulose with Celluclast® and total deacetylation followed by separation of hydrolysis products using size-exclusion chromatography (SEC). The DP of soluble cellooligosaccharides that involves in hydrolysis is limited to DP 3 to 6.

(ii) Measurement of enzyme kinetics involves hydrolysis of soluble cellooligosaccharides which are limited to DP 3 to 6. The concentration for each DP of cellooligosaccharides attempts to cover three different concentrations: 200 µM, 300 µM, and 400 µM. Hydrolysis were performed was followed by measuring the increase in the number of reducing groups via a modified BCA method from the separation of products by HPLC. This part of the research also includes the derivation of appropriate kinetic equations based on Michaelis-Menten equation to obtain kinetics parameters such as the bond cleavage frequency and the probability for bond cleavage for cellooligosaccharides with DP 3 to 6.

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