UNIVERSITI PUTRA MALAYSIA

CHARACTERISATION AND EXPRESSION OF RECOMBINANT BETAGLUCOSIDASE 2 FROM Trichoderma asperellum UPM1

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FBSB 2019 31

CHARACTERISATION AND EXPRESSION OF RECOMBINANT BETA-GLUCOSIDASE 2 FROM *Trichoderma asperellum* UPM1

By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

March 2019

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Trichoderma sp. is a fungus capable of producing three categories of cellulase for cellulose degradation into glucose; endoglucanase, cellobiohydrolase and β-glucosidase. However, native production of β-glucosidase from fungi is often at low concentrations and takes a longer time. Furthermore, product inhibition caused by glucose on β-glucosidase reduces resulting yields, making it the rate limiting enzyme and represents an obstacle for commercial cellulose hydrolysis. Studies on β-glucosidases produced by *Trichoderma* sp. have elucidated two variants, which have been classified into glycosyl hydrolase (GH) families 1 and 3, with attention given to GH family 1 (Bgl2), owing to its relatively lower sensitivity to glucose inhibition, a desirable character for bioprocess development for efficient lignocellulosic biomass saccharification. As such, using locally isolated *Trichoderma asperellum* UPM1, this study has sought to characterise the *bgl2* gene isolated and following heterologous expression in *Escherichia coli*, characterise the recombinant enzyme for enzyme activity and glucose tolerance.

Trichoderma asperellum bgl2 (*Tabgl2*) gene sequence isolated was found to be 1398 nucleotides in length, encoding a protein of 465 amino acids in length, with an estimated molecular weight of 52798.31 Daltons. The identity of *Trichoderma* sp. glycosyl hydrolase family 1 β-glucosidase was affirmed by the presence of N-terminal signature of 15 amino acids in length, cis-peptide bonds at A180-P181 and W417-S418, conserved active site motifs with glutamate (E) residues ('TFNEP' and 'VTENG'), 17 corresponding substrate binding and a lone conserved stabilising tryptophan (W) residue. Automated protein structure homology-modelling revealed the common triosephosphate isomerase (TIM) barrel fold, functioning as a monomer while protein phylogeny analyses positioned the isolated protein to a clade with known *Trichoderma* sp. β-glucosidases. Intracellular protein localisation was confirmed by the absence of a signal sequence. Suggestive glucose tolerance was inferred from the presence of 14 of 22 consensus residues from known glucose tolerant amino acid residues

as well as the presence of corresponding residues L167 and P172, crucial in the retention of the active site's narrow cavity, found in glucose tolerant Bgl2 from *Trichoderma reesei*.

Characterisation thus proceeded by codon optimisation of the gene followed by transformation and heterologous expression in *Escherichia coli* using plasmid vector pET-20b(+), targeted for periplasmic expression of recombinant *T. asperellum* Bgl2 (*Ta*Bgl2). Protein expression analysis using SDS-PAGE showed the presence of a ~52 kDA protein in size while the crude enzyme extracts showed a specific activity of 0.0081 U/mg in the periplasmic fraction, 11.6-fold higher than in the periplasmic fraction of the *E. coli* host without IPTG induction. Glucose tolerance was affirmed with 40% of relative activity retained in a concentration of 0.2 M glucose. Thus, the relatively low sensitivity of *Ta*Bgl2 to inhibition by glucose makes this enzyme a potential candidate for further analyses in cellulose hydrolysis.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENCIRIAN DAN PENGEKSPRESAN BETA-GLUCOSIDASE 2 REKOMBINAN DARIPADA *Trichoderma asperellum* UPM1

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Trichoderma sp. adalah kulat yang boleh menghasilkan tiga kategori selulase untuk penguraian selulosa kepada glukosa; endoglukanase, selobiohidrolase dan β-glukosidasa. Walau bagaimanapun, pengeluaran β-glukosidasa daripada kulat secara semula jadi adalah pada kepekatan rendah dan mengambil masa yang lebih lama. Tambahan pula, perencatan produk disebabkan oleh glukosa ke atas β-glukosidasa merendahkan hasil, menjadikannya enzim pembatas kadar dan menjadi halangan kepada hidrolisis selulosa secara komersil. Kajian berkenaan β-glucosidasa yang dihasilkan oleh *Trichoderma* sp. telah menjelaskan dua varian, yang telah diklasifikasikan kepada kumpulan glikosil hidrolase (GH) 1 dan 3, dengan banyak perhatian diberikan kepada β-glucosidasa kumpulan GH 1 (Bgl2), kerana kepekaan yang lebih rendah terhadap perencatan glukosa, sifat yang digalakkan untuk perkembangan bioproses bagi proses penguraian biojisim lignoselulosa yang berkesan. Oleh itu, dengan menggunakan *Trichoderma asperellum* UPM1 yang dipencilkan secara setempat, kajian ini telah berusaha untuk mencirikan gen *bgl2* yang dipencilkan dan selepas ekspresi heterologus dalam *Escherichia coli*, enzim rekombinan dicirikan untuk aktiviti enzim dan toleransi terhadap glukosa.

Jujukan gen *Trichoderma asperellum bgl2* (*Tabgl2*) yang dipencilkan didapati sepanjang 1398 nukleotida, diterjemahkan kepada protein sepanjang 465 asid amino, dengan anggaran berat molekul sekitar 52798.31 Daltons. Pengesahan identiti β-glukosidasa kumpulan glikosil hidrolase (GH) 1 daripada *Trichoderma* sp. diperolehi dengan adanya pengenalan N-terminal sepanjang 15 asid amino, ikatan cis-peptida pada A180-P181 dan W417-S418, dua motif yang dipelihara dengan tapak aktif residu glutamate (E) ('TFNEP' dan 'VTENG'), 17 residu pengikat substrat dan satu residu triptofan (W) untuk mengekalkan kestabilan. Pemodelan struktur protein secara automatik berdasarkan homologi mendedahkan model trifosfat isomerase (TIM) yang lazim, berfungsi sebagai monomer dengan pokok filogeni meletakkan protein yang dipencilkan kepada klad merangkumi β-glukosidasa yang dikenalpasti daripada *Trichoderma* sp.

Penempatan protein secara intrasel telah disahkan dengan ketiadaan jujukan isyarat. Toleransi glukosa juga telah dicadangkan berdasarkan kehadiran 14 daripada 22 residu konsensus asid amino β-glukosidasa toleran glukosa beserta dengan kehadiran residu L167 dan P172, penting dalam pengekalan kesempitan rongga tapak aktif, yang terdapat dalam Bgl2 toleran glukosa daripada *Trichoderma reesei*.

Bagi pencirian enzim, pengoptimuman kodon gen diikuti dengan transformasi dan ekspresi dalam *Escherichia coli* menggunakan vektor plasmid pET-20b(+), yang disasarkan untuk ekspresi protein rekombinan *T. asperellum* Bgl2 (*TaBgl2*) ke periplasma. Pengesahan ekspresi protein melalui SDS-PAGE menunjukkan kehadiran jalur protein ~52 kDA sementara ekstrak enzim mentah menunjukkan aktiviti spesifik tertinggi 0.0081 U/mg dalam pecahan periplasmik, 11.6 kali ganda lebih tinggi daripada pecahan periplasmik perumah *E. coli* tanpa induksi IPTG. Toleransi glukosa disahkan dengan 40% aktiviti relatif dikekalkan dalam kepekatan 0.2 M glukosa. Maka, kepekaan relatif *Ta*Bgl2 yang rendah terhadap glukosa menjadikan enzim ini calon berpotensi untuk analisis lanjutan dalam penguraian selulosa.

ACKNOWLEDGEMENTS

In the name of Allah, Most Gracious, Most Merciful

First and foremost, I would like to say Alhamdulillah and thank my Lord, Allah S.WT. for His mercy and guidance for a research which began with an intention has now reached completion. It is by His grace as well that I have received contributions from numerous individuals throughout the progress of my research study.

I begin by expressing my utmost gratitude to my supervisor, Dr Norhayati Ramli for her support, patience, motivation, enthusiasm and most of all, a continuous belief that I can carry the task at hand over the line. Not forgotten as well are my co-supervisors Prof. Dr. Suraini Abd-Aziz and Assoc. Prof. Dr. Farah Diba Abu Bakar for their detailed and constructive comments, insights, suggestions and unconditional supports. Special gratitude is also offered to UniMAP, Perlis and the Ministry of Higher Education for the financial support given in pursuing this study.

Part of a research project entails the privilege of working as a team and to this, I extend my gratitude to several individuals of note, being Nik Ida Mardiana Nik Pa, Diana Mohd Nor, Siti Suhailah Sharuddin, Nurhasliza Zolkefli and Noor Shaidatul Lyana Mohamad Zainal for their companionship and assistance.

Beyond the walls of the lab, I would also like to take this opportunity to thank several individuals particularly Ahmad Muhaimin Roslan, Mohd Nor Faiz Norrrahim, Aliya Dalila Mohd Ruslan, Tuan Mohd Taufik Tuan Hussin, Norzahiruddin Amin Husin and others who have seen fit to have me in their circles and in more ways than they could imagine, contributed to my cause throughout my journey.

Last but never the least, my greatest gratitude I extend to my parents, Mohamad Sobri Mahmud and Elizabeth Dalmacio, my siblings, Mohamad Feroz, Mohamad Nazreen, Muhammad Zafri Raymi and other family members for their unconditional love, constant prayers and supports. I cannot be prouder still to have them see my tears of joy today replace those of fears in the past.

In a study of such lengthened duration, I acknowledge that this list shall always be far from exhaustive, and to this I pray for forgiveness from those I did not mention by name and include them still in my heart-felt gratitude.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members for the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

°C	Degree Celsius
%	Percentage
ρNPG	ρ-nitrophenyl β-D glucopyranoside
φ	Phi torsion angle
ψ	Psi torsion angle
× g	Relative centrifugal force
µg	Microgram
µg/mL	Microgram per millilitre
µL	Microliter
µm	Micrometre
µmole	Micromole
µM	Micromolar
Å	Angstrom
Bgl2	β-glucosidase 2
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
CAZy	Carbohydrate-Active Enzymes
CBH	Cellobiohydrolase
CBM	Carbohydrate binding module
cDNA	Complementary deoxyribonucleic acid
CMC	Carboxymethylcellulose
DEPC	Diethyl pyrocarbonate

DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DP	Degree of polymerisation
EDTA	Ethylenediaminetetraacetic acid
g	Gram
g/L	Gram per litre
GH	Glycosyl hydrolases
Glu	Glutamate
GSP	Gene specific primer
h	Hours
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilobases
kDa	Kilodalton
kPa	Kilopascal
L	Litre
LB	Luria Bertani
min	Minutes
M	Molarity
mA	Milliampere
MBP	Maltose binding protein
mg	Milligram
mL	Millilitre
mL/L	Millilitre per litre
mm	Millimetre

ng/µL	Nanogram per microliter
nm	Nanometre
NusA	N-utilising substance-A
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
pHis	Poly-histidine
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Reverse transcription
s	Seconds
SB	Super Broth
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sp.	Species
spp.	*Species pluraris* (multiple species)
TaBgl2	*Trichoderma asperellum* Bgl2
TAE	Tris base-acetic acid-EDTA
TB	Terrific Broth
TIM	Triosephosphate isomerase
TrBgl2	*Trichoderma reesei* Bgl2
U	Unit of enzyme activity
U/mg	Specific activity
USA	United States of America

UV	Ultraviolet
V	Volt
v/v	Volume per volume
WT	Wild-type
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

Trichoderma sp. is a genus of fungi that plays a significant role to the environment within its vicinity (Błaszczyk et al., 2014). As such, over the years, *Trichoderma* sp. has been the subject of several studies, ranging from phylogeny, distribution, defence mechanism, host interaction, production and secretion of enzymes, sexual development and also responses to changes in the environment. Thus, given the extensive interest placed, *Trichoderma* has been one of the most studied fungi, with numerous qualities highlighted for purpose of application (Schuster and Schmoll, 2010). These include its usage as biocontrol agents (Benítez et al., 2004), bioremediation agents (Rhodes, 2014) and as cellulase producers (Strakowska et al., 2014).

Beginning from the Second World War, studies into cellulase production has grown with further support given during the 1970s as interest in microbial cellulases and their possible application in conversion of biofuel production from biomass increased (Montenecourt, 1983). Since then, numerous fungi species within the genus have been studied such as *Trichoderma atroviride*, *Trichoderma virens, Trichoderma asperellum* and *Trichoderma virens* (Strakowska et al., 2014). Over time, growth in the bioethanol market and research into textile and feed production has positioned *Trichoderma reesei* in particular as a significant production platform within industries and thus will continue to be the focus of researches in the future (Brotman et al., 2010; Paloheimo et al., 2016).

Degradation of the β-1,4 linkages present within cellulose are done by enzymes known as cellulase. In nature, complete hydrolysis is obtained by the synergistic action of three separate types (1) endoglucanases, (2) cellobiohydrolases and (3) β-glucosidases (Zhang and Zhang, 2013; Zhang et al., 2006). Beginning at the surface of solid substrates, primary hydrolysis is undertaken by endoglucanase and exoglucanase for release of soluble cellodextrins. Secondary hydrolysis then involves the breakdown of cellodextrins such as cellobiose into glucose by β-glucosidases (Zhang et al., 2006). It is noted however that in commercial cellulase producer such as *T. reesei*, β-glucosidase production is at much lower concentrations than endoglucanases and cellobiohydrolases (Rani et al., 2014).

Throughout the process of cellulose saccharification, both substrate and product inhibition were found to occur. In substrate inhibition, this occurs on a two-domain structure found on the exoglucanase, known as cellobiohydrolase, of which for a given fixed enzyme load, increase in substrate concentration results in increase of time/distance necessary for cellobiohydrolases to bind via lateral diffusion to the chain ends resulting from endoglucanase activity (Väljamäe et al., 2001). Product inhibition instead can be attributed to the presence of cellobiose and monosaccharides liberated. Cellobiose affects by steric hindrance at the active

site of cellobiohydrolases (Yue et al., 2004) while β-glucosidases are affected by the presence of glucose (Hsieh et al., 2014). Glucose inhibition on β-glucosidase in particular has made it the rate-limiting enzyme, with impaired yields being one of the main obstacles for commercialisation of cellulose hydrolysis (Sørensen et al., 2013).

Within biotechnology industries, utilisation of β-glucosidases with glucose tolerance and stimulation can improve efficiency of substrate degradation and result in reduction of production costs. Thus, interest towards the use of glucose-tolerant β-glucosidases has increased in recent years. While the majority of β-glucosidases are sensitive to glucose, tolerance coupled with stimulatory effect of the carbohydrate have been observed exclusively among glycosyl hydrolase (GH) family 1 β-glucosidases (Y. Yang et al., 2015). With relative tolerance ranging from tenfold to 1000-times fold higher than GH family 3 (GH3) β-glucosidases, it is thus suggested that GH family 1 (GH1) β-glucosidases are more suitable for plant cell-wall saccharification in biotechnological applications (Giuseppe et al., 2014).

Based on the work by Marx et al. (2013), comparative secretome analysis of *T. asperellum* to that of *T. reesei* following solid-state fermentation on sugarcane bagasse have shown higher hemicellulase and β-glucosidase enzyme activities, along with higher diversity and increased abundance of main and side chain hemicellulases and β-glucosidases. In addition to demonstrated production of cellulase on oil palm empty fruit bunch (Ibrahim et al., 2013) and sago pith residue (Linggang et al., 2012) specific to local isolate *T. asperellum* UPM1, *T. asperellum* therefore presents an enticing alternative for cellulase production.

Given the low expression of native β-glucosidase from fungi as well as severity of glucose inhibition on β-glucosidase activity and subsequent hydrolysis yield, it is therefore hypothesised that isolation of glucose tolerant β-glucosidase from GH family 1 and expression in recombinant *E. coli* would reduce or circumvent the problems altogether. Thus, this research work aims to clone and characterise a GH1 β-glucosidase isolated from the local strain *Trichoderma asperellum* UPM1, thereafter designated as *Ta*Bgl2. Subsequent heterologous expression of the enzyme in *Escherichia coli* would allow for glucose tolerance to be determined experimentally, thereby initiating an exploration of an alternative enzyme for use in cellulose hydrolysis.

The objectives of this research are:

1. To clone the gene encoding glycosyl hydrolase family 1 β-glucosidase derived from *Trichoderma asperellum* UPM1 (*Tabgl2*) and characterise *Tabgl2* and its corresponding enzyme via *in silico* analyses.

2. To express the recombinant β-glucosidase (*Ta*Bgl2) in *Escherichia coli* and biochemically characterise the enzyme's specificity and resistance to glucose inhibition.

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Enzymatic characterization of heterologously expressed fungal β-glucosidase Bgl2 in *Escherichia coli*. Symposium on Applied Engineering and Sciences (SAES) 2017, 14 – 15th November 2017, Serdang, Selangor, Malaysia

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