UNIVERSITI PUTRA MALAYSIA

CELLULASE PRODUCTION BY THERMOPHILIC Bacillus licheniformis 2D55 AND ITS APPLICATION FOR RECOVERY OF FERMENTABLE SUGAR FROM RICE HUSK

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By

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Chairman : Associate Professor Nor 'Aini Abdul Rahman, PhD
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Fermentable sugar production from various agro-waste biomass via enzymatic saccharification using mesophilic fungi cellulase has been the issue of interest for many researchers. However, research carried out using thermophilic bacterial cellulase for sugar production are limited. Cellulases, chemical pretreatment and enzymatic saccharification required for sugar production are very costly and often result in low sugar yield, thereby affecting the overall economics of the bioconversion process. Hence, the main aim of this study was to isolate, characterize and enhance the production of cellulolytic enzyme complex (CMCase, FPase and β-glucosidase) and Xylanase from agro-waste mixture (a mixture of untreated sugarcane bagasse and pretreated rice husk) by a locally isolated thermophilic *Bacillus licheniformis* 22D55 and utilize the crude thermostable cellulase enzyme for sugar production through enzymatic saccharification of rice husk subjected to high pressure steam pretreatment.

Cellulose degrading bacterium isolated from oil palm empty fruit bunch-chicken manure compost was identified by morphological, biochemical and 16S rRNA test and known as *B. licheniformis* 2D55. In basal medium with microcrystalline cellulose as carbon source, the bacterium produced cellulase at 50°C within 18-24 h. Among the various untreated and sodium hydroxide (NaOH) pretreated agro-waste biomass used, agro-waste mixture comprising of untreated sugarcane bagasse and pretreated rice husk results in overall improvement in the cellulolytic enzyme complex with CMCase at 0.37 U/mL, FPase at 0.29 U/ml, β-glucosidase at 0.006 U/mL and xylanase at 0.98 U/mL. The SEM image reveal deformity in bacteria cell grown on NaOH pretreated sugarcane bagasse only, which resulted in the low performance of the bacteria for cellulase production on the substrate.

Effect of nutritional and physicochemical factors were investigated for enhancing cellulase production. The CMCase, FPase, and β-glucosidase activities increased by 77.4-folds, 44.3-folds, and 10-folds, respectively. The crude enzyme was highly active and stable over broad temperature (50 to 80°C) and pH (3.5 to 10.0) ranges with optimum temperature at 65°C and 80ºC for CMCase and FPase, respectively. While the optimum pH for CMCase and FPase was 7.5 and 6.0, respectively.

An operational condition was developed for high pressure steam pretreatment (HPSP) of rice husk for fermentable sugar recovery through enzymatic saccharification. The pretreatment at 200°C/1.85 MPa for 7 min was found to effectively enhance cellulose content of the rice husk. The scanning electron micrograph (SEM), fourier transform infrared (FTIR) and x-ray diffraction (XRD) analysis expressed effectiveness of the pretreatment. A two-step saccharification of pretreated rice husk at 60°C yielded reducing sugar at 0.581 g/g substrate that was equivalent to 73.5% saccharification.

Therefore, the strain *B. licheniformis* 2D55 has the potential of utilizing agro-waste mixture for higher cellulolytic enzyme production. The thermostable nature of the cellulase contributed to the efficient release of fermentable sugars at higher temperature. The application of high pressure steam pretreatment and two-step enzymatic saccharification provides greener technology and improve the fermentable sugar production. The results of this study could contribute to future research in thermophilic bioprocessing rice husk with the application of thermostable cellulase producing bacterium for improving biomass-sugar conversion.

Abstrak tesis yang dikemukakan kepada senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGHASILAN SELULASE OLEH *Bacillus licheniformis* 2D55 TERMOFILIK DAN APLIKASINYA UNTUK PEMEROLEHAN GULA FERMENTASI DARIPADA JERAMI PADI

Oleh

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Penghasilan gula fermentasi daripada pelbagai sisa biomass pertanian melalui pensakaridaan enzim menggunakan kulat selulase mesofilik menjadi isu yang diminati oleh ramai penyelidik. Walaubagaimanapun, kajian menggunakan bakteria selulase termofilik untuk penghasilan gula sangat terhad. Enzim selulase, pra-rawatan kimia dan pensakaridaan enzim yang diperlukan untuk penghasilan gula fermentasi memerlukan kos yang tinggi dan kerap menghasilkan gula yang rendah serta memberi kesan ekonomi keseluruhan kepada proses penukaran. Jerami padi merupakan substrat penghasilan gula yang kurang mendapat perhatian penyelidikan. Oleh itu, kajian ini bertujuan untuk memencil, menciri dan mengoptimum penghasilan kompleks enzim selulase (CMCase, FPase dan β -glucosidase) dan xylanase daripada campuran sisa pertanian (hampas tebu dan jerami padi terawat) oleh bakteria termofilik tempatan yang dipencilkan, *Bacillus licheniformis* 22D55 dan menggunakan enzim selulase termostabil mentah untuk penghasilan gula fermentasi melalui enzim pensakaridaan oleh jerami padi yang dirawat pada wap tekanan tinggi.

Bakteria pengurai selulosa yang dipencilkan daripada kompos tandan sawit kosong dan tinja ayam dikenalpasti melalui ujian morfologi, biokimia dan 16s rRNA dan dikenalpasti sebagai *Bacillus licheniformis* 2D55. Dalam media asas menggunakan mikrokristal selulosa sebagai sumber karbon, bakteria menghasilkan aktiviti CMCase dan FPase sebanyak 0.33 U/mL dan 0.09 U/mL, masing-masing pada suhu 50°C selama 18-24 jam. Di antara pelbagai sisa biomass pertanian tidak terawat dan menggunakan pra-rawatan natrium hidroksida, campuran sisa pertanian hampas tebu tidak terawat dan jerami padi terawat menunjukkan peningkatan keseluruhan penghasilan kompleks enzim selulase dengan CMCase sebanyak 0.37 U/mL, FPase (0.29 U/mL) β-glukosidase (0.006 U/mL) and xilanase (0.98 U/mL). Imej mikroskop

elektron menunjukkan pertumbuhan bakteria terencat di atas hampas tebu terawat oleh natrium hidroksida yang menyebabkan kurang prestasi bakteria untuk menghasilkan selulase daripada substrat.

Kesan faktor nutrisi dan fizikokimia dinilai untuk meningkatkan penghasilan enzim selulase. Penghasilan aktiviti CMCase, FPase, dan β-glukosidase meningkat 77.4-kali ganda, 44.3-kali ganda, and 10-kali ganda, masing-masing, menggunakan parameter optimum. Enzim mentah sangat aktif dan stabil pada julat suhu (50 ke 80°C) dan pH (3.5 ke 10) dengan suhu optima CMCase dan FPase ialah 65°C dan 80°C, masing-masing. Manakala, pH optima untuk CMCase dan FPase ialah 7.5 dan 6.0, masing-masing.

Kondisi pengoperasian pra-rawatan wap tekanan tinggi (PSTT) jerami padi dibuat untuk pemerolehan gula fermentasi melalui pensakaridaan enzim. Satu keadaan operasi telah dibangunkan untuk pra-rawatan wap tekanan tinggi terhadap sekam padi untuk pemerolehan gula fermentasi melalui sakarifikasi enzim. Pra-rawatan pada 200C/1.85MPa selama 7 min telah didapati berkesan menggalakkan kandungan selulosa sekam padi. Analisis scanning electron micrograph (SEM), fourier transform infrared (FTIR) dan x-ray diffraction (XRD) menunjukkan keberkesanan pra-rawatan tersebut. Dua langkah sakarifikasi pra-rawatan sekam padi pada 60°C menghasilkan gula penurun pada 0.581 g/g substrat yang bersamaan 73.5% sakarifikasi.

Oleh itu, *Bacillus licheniformis* 2D55 berpotensi menggunakan campuran biomas sisa pertanian untuk penghasilan enzim selulolitik yang lebih tinggi. Keadaan termostabil selulase membantu kecekapan penghasilan gula fermentasi pada suhu yang lebih tinggi. Penggunaan pra-rawatan wap tekanan tinggi dan dua langkah pensakaridaan enzim menawarkan teknologi hijau dan meningkatkan penghasilan gula fermentasi. Hasil dari kajian ini menyumbang kepada kajian akan datang dalam bioproses termofilik jerami padi oleh bakteria selulase termostabil untuk meningkatkan penukaran biomas kepada gula fermentasi.

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

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β	Beta
BLAST	Basic Local Alignment Search Tool
BLASTN	Nucleotide-nucleotide BLAST
BSA	Bovine serum albumin
CFU/ml	Colony –forming unit per millilitre
CMC	Carboxymethyl cellulose
CMCase	Carboxymethyl cellulase
dBa	Decibel
DNA	deoxyribonucleic acid
DNS	3,5-dinitrosalicylic acid
dNTPs	Deoxynucleotides
EDX	Electron diffraction x-ray
FPase	Filter paperase
FPU/ml	Filter paper unit per millilitre
FTIR	Fourier transform infrared
g	Gram
g/L	Gram per litre
g/ml	Gram per millilitre
HCL	Hydrochloric acid
HPLC	High performance liquid chromatography
KH_2PO_4	Potassium dihydrogen phosphate
K_2HPO_4	Potassium hydrogen phosphate
KI	Potassium iodide

kg	Kilogram
L	Litre
$MgSO_4.7H_2O$	Magnesium sulphate
min	Minutes
$NaH_2C_6H_5O_7$	Sodium citrate
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
$(NH4)_2SO4$	Ammonium sulphate
$NO_3C_6H_5O_7.2H_2O$	Sodium acetate
°C	Degree centigrade
OD	Optical density
PEG	Polyethylene glycol
PCR	Polymerase chain reaction
%	Percentage
rpm	Rotations per minute
SA	Specific activity
SEM	Scanning electron microscope
U/mL	unit per millilitre
w/v	Weight per volume
XRD	X-ray diffraction

CHAPTER 1

INTRODUCTION

Fuel has been a significant factor that shapes the economic life style of human for generations. The global increase in world population results in huge energy demand. Increase in fuel prices, shortage of fossil fuel and major concern over environmental pollution has directed research attention towards fermentable sugar (substrate for biofuel) production from food sources such as cereals, starch, sugar beets and sugarcane. Unlike biofuel, burning fossil such as gas, coal and oil results in the generation of CO_2 which has been a major contribution to global warming (Eriksson and Gray, 2017; Stips *et al.*, 2016). However, the unsustainability of sugar production from food crops due to increase in food prices has guided research toward production of sugars from agro-waste biomass. Sugar production from agro-waste biomass is renewable, sustainable, and ensures food security. Likewise agro-waste biomass is cheap, highly abundant and is the only alternative major raw material for fuels and chemical production at present and in future (Simpson-Holley *et al.*, 2007).

Various agro-waste biomass such as sugarcane bagasse, date palm biomass, rice staw, corn cob and wheat straw have been applied in the production of sugars (Alrumman, 2016; Gupta and Parkhey, 2014) . However, less attention have been focused on rice husk as substrate for sugar production

Rice is ranked as largest cultivated cereal crop and a staple food for more than half of the world's population (Johar *et al.*, 2012). Rice consumption in Malaysia is expected to increase from 2.30 million metric tonnes to 2.69 million metric tonnes by 2020 (Shafie *et al.*, 2014). About 1.2 million tonnes of rice husk was generated in 2015 and the Malaysian government has projected 7 million tonnes per year paddy residue by the year 2020 (Shafie *et al.*, 2014). In addition, the growth population was expected to reach 41.50 million by 2040 from 28.60 million in 2010 (DOSM, 2016). Consequently, it was then projected that in order to meet the demand of the population growth and the self-sufficiency level (SSL) of 90 % by the year 2060, an additional 1,320,000 tons per year of rice must be produced (Siwar *et al.*, 2014). Thus, resulting in higher generation of rice husk. However, rice husk disposal system through open burning and burying causes environmental pollution with great health risk. Therefore, rice husk will be suitable raw material for sugar production due to its abundance, low-cost, low commercial value.

Pretreatment followed by enzymatic saccharification are the majorly required for the bioconversion of agro-waste to sugars. For several decades fungi cellulase from *Trichoderma* and *Aspergillus* species have been the central focus of both academic and industrial researchers (Howard *et al.*, 2003; Narra *et al.*, 2014; Rana *et al.*, 2014). Therefore, saccharification reactors are conducted at low temperatures because fungi cellulase is limited by low thermostability, high enzyme usage and prolong hydrolysis thereby resulting in low hydrolysis and high cost of sugar production. However, processing of agro-waste biomass at elevated temperatures offers several advantages such as improved hydrolysis, low risk of potential contamination, better substrate solubility thus improving the overall economics of the process (Rastogi *et al*., 2010). Hence, developing thermostable cellulase enzymes would be the best strategy for rapid release of sugars. But the lack of robust cellulase that can efficiently function at elevated temperature and broad pH range remains a bottle neck in thermophilic digestion of agro-waste biomass. Thus, thermophilic cellulase producing bacteria are better reservoir for thermostable enzymes with great potential for developing viable conversion of agro-waste biomass to sugars.

Quite few thermophilic cellulase degrading bacteria such as *Geobacillus* sp., *Brevibacillus* sp., *Bacillus* sp. C1, *Bacillus valismortis* and *Bacillus stearothermophilus* have been isolated from gold mine, cow dung, garden soil and forest soil (Assareh *et al.*, 2012; Gaur and Tiwari, 2015; Rastogi *et al.*, 2009; Sadhu *et al.*, 2013). However, the harsh conditions such as active degradation, extreme temperature, pH, pressure, inhibitors and toxic metal experienced during composting makes it a potential source of robust bacteria since the environment is similar to those of industrial processes. Furthermore, thermophilic bacteria are also advantageous due to high growth rate, high stability, versatility, genetic amiability, resistance to relatively harsh condition and production of cellulosome (Multi-enzyme complex) (Maki *et al.*, 2009; Maki *et al.*, 2011b).

It is worth to note that sensitivity analysis and techno-economic modelling has suggested that carbon source is a key factor in cellulase production and could account for about 50 % of total enzyme cost (Humbird *et al.*, 2011; Ryu and Mandels, 1980). Therefore, selecting appropriate carbon source is very important. Carbon substrates such as sugars; (*e.g.*, lactose, sucrose cellobiose) and pure cellulose (*e.g.*, caboxymethyl cellulose-CMC, avicel, microcrystalline-MCC cellulose and solka floc) have been reported as good inducers of cellulase synthesis (Jyotsna *et al.*, 2015; Sethi *et al.*, 2013). The utilization of these substrates on industrial scale is uneconomical due to the high cost of substrate. Likewise single substrate agro-waste like rice straw, sugarcane bagasse, date palm leaves, wheat straw, rice bran have been reported for carbon substrate (Alrumman, 2016; Gaur and Tiwari, 2015; Yang *et al.*, 2014). However, using single substrate might not be sustainable in real biorefinary condition due to shortage in feedstock as a result of seasonal variation (Ebadian *et al.*, 2011; Rentizelas *et al.*, 2009). In addition different agro-waste carbon substrate induces cellulolytic enzymes at various levels. Therefore, combining agro-waste as cocktail carbon feed stock could help mitigate this effect and are uncommonly reported.

The recalcitrant nature of lignocellulose restrict hydrolysis. Rice husk biomass is majorly consist of cellulose (32 – 47 %), hemicellulose (19-27 %) and lignin (5-24 %) (Binod *et al.*, 2010). In the structure of rice husk biomass, cellulose act as a linear crystalline polymer of β-D-glucose unit that is rigid. While lignin act as a cement that binds cellulose fibres to hemicellulose, hemicellulose links cellulose fibre and lignin, giving the whole cellulose-hemicellulose-lignin complex a rigid structure that makes it more difficult and resistant to degradation (Chandra *et al.*, 2007; Hendriks and Zeeman, 2009; Palonen, 2004). Hence, pretreatment becomes necessary to disrupt the structural matrix of the lignocellulose by disintegration of cellulose fibres from hemicelluloses and lignin. Over the years, pretreatment methods have mainly focused on acid, alkali, ionic liquids and organosolvent chemicals (Ang *et al*., 2012; Dagnino *et al.*, 2013; Omidvar *et al.*, 2016; Zhang *et al*., 2016). However, this method present great limitation to agro-waste conversion due its unsustainability, high cost of chemicals and environmental toxicity. Therefore, alternative green, eco-friendly, inexpensive, and sustainable method of high pressure steam pretreatment is suggested. High pressure steam allows exposure of biomass to steam at high temperature (160-240°C), and pressure (0.7 and 4.8 MPa) at low residence time (2-20 min). Unlike chemical pretreatment, high pressure steam does not require extreme washing, neutralization and catalyst. Therefore, high pressure steam pretreatment represent great significance to this study. The application of high pressure steam have been reported for pretreatment of oil palm mesocarp fibre (OPMF) (Yunos *et al.*, 2012) and oil palm biomass (Baharuddin *et al.*, 2013; Baharuddin *et al.*, 2012). However, no research have been aimed at applying high pressure steam for the pretreatment of rice husk for sugar production.

Improving process condition for efficient enzymatic saccharification is of upmost importance towards improving sugar yield (Alvira *et al.*, 2010). Similarly, efficient and rapid enzymatic saccharification remains one of the technical and economical bottlenecks in the overall bioconversion of lignocellulosic biomass to fermentable sugars (Berlin *et al.*, 2007; Walker and Wilson, 1991; Zhang *et al.*, 2009). A variety of method have been suggested to increase hydrolysis yield of sugars including; gradual substrate loading (Rosgaard *et al.*, 2007), addition of surfactant (Börjesson *et al.*, 2007), immobilization (Tsai and Meyer, 2014; Zhang *et al.*, 2016) and enzyme recycling (Ouyang *et al.*, 2013). However, conceptualizing the idea that enzymes bound to substrate after hydrolysis could be recycled by recycling the recovered solids in subsequent steps lead to exploitation of this method for improving sugar production.

Therefore, in this study the feasibility of isolating local thermophilic bacterium that can utilize insoluble agro-waste biomass for the production of cellulolytic enzyme complex (CMCase, FPase, and β-glucosidase and xylanolytic enzymes (xylanase) was investigated. The study also examined enhancement of the crude enzyme production, characterization and subsequent fermentable sugar production using the crude enzyme via enzymatic saccharification studies.

Thus the objectives of this study are:

1. To isolate and characterize thermophilic cellulose-degrading bacteria for utilization of agro-waste biomass for cellulase production.
2. To enhance cellulase production by the isolated bacterium (*B. licheniformis* 2D55) through formulation of nutritional and physiological conditions using one factor at a time and characterize the endoglucanase and exoglucanase enzyme.
3. To develop efficient pretreatment of rice husk under high pressure steam and to improve the production of fermentable sugars through efficient enzymatic saccharification using crude cellulase produced by thermophilic *B. licheniformis* 2D55.

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