



***PRODUCTION AND OPTIMIZATION OF CARBOXYMETHYL
CELLULASE AND FILTER PAPERASE OF LOCALLY ISOLATED
Streptomyces lucitanus C5/2 UNDER SUBMERGED FERMENTATION
OF NAPIER GRASS***

SHAHIRAH BINTI MANAP

IPTPH 2017 1



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By

SHAHIRAH BINTI MANAP

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

January 2017

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Abstract of thesis Presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

**PRODUCTION AND OPTIMIZATION OF CARBOXYMETHYL CELULASE
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SHAHIRAH BINTI MANAP

January 2017

Chairman : Assoc. Prof. Rosfarizan Mohamad, PhD
Institute : Tropical Forestry and Forest Product

Lignocellulosic biomass materials are known as a sustainable, renewable feedstock for biofuels production due to domestically abundant and low-production-cost derived from non-food sources. However, due to complex molecular structures of the cellulose, it becomes difficult to hydrolyze into fermentable sugar as the feedstock. The bioconversion of cellulose to fermentable sugar can be catalyzed by a group of enzymes known as cellulolytic enzymes, which is commonly produced by microorganisms (fungi, bacteria, and protozoa). Cellulolytic enzymes system mainly comprised of endoglucanase (carboxymethyl cellulase), exoglucanase, and β -glucosidase enzymes which react together to perform complete hydrolysis process. Grass species are one of cheap lignocellulosic biomass that can be used as energy source in many bioprocesses and industrial applications. Napier grass, scientific name *Pennisetum purpureum* sp is one of the potential carbon sources, which can be utilized as a substrate for the production of bio-product. The potential of Napier grass as a fermentation substrate was investigated throughout this study.

Isolation and identification of the cellulolytic producing bacteria were carried out by initially searching the bacteria from various environmental sources (cattle waste compost, decayed wood and ponds). The cellulolytic enzymes biosynthesis by the selected bacteria isolate using Napier grass as a substrate in submerged fermentation was carried out in batch cultivation using 250 mL shake-flask. The investigations were followed by statistical optimization using response surface methodology (RSM) approach to obtain the optimized cultural conditions and medium composition for maximum activities of carboxymethyl cellulase (CMCase) and filter paperase (FPase). For the assessment of crude enzyme produced, enzymatic hydrolysis of Napier grass was performed by using untreated and treated (1.4M H₂SO₄ and 7% NaOH) Napier grass to investigate the total reducing sugar production.

A total of twenty- five (25) bacterial isolates were grown on the selective media and only six (6) bacterial isolates showed positive results by exhibiting clear zone on carboxymethyl cellulose (CMC) agar plates, which indicate the enzymes production. The C5/2 isolate was selected as the best cellulolytic enzymes producer due to the highest enzyme activities obtained during the quantitative screening and molecularly identified as *Streptomyces lucitanus* by 16S rDNA sequencing.

The optimum values of the fermentation parameters for CMCase and FPase biosynthesis based on the statistical optimization was determined at initial pH of 7.8, agitation speed 170 rpm, inoculum size 19.7% (v/v) and Napier grass concentration 4.30% (w/v) with maximum activities 7.362 U/mL and 2.895 U/mL, respectively which were close to the predicted values. The production of CMCase and FPase shows improvement, 66% and 30%, respectively by application of the optimum values of parameters during the cultivation. The total reducing sugar produced from enzymatic hydrolysis by using treated Napier grass (1.4M H₂SO₄; 6.541 mg/mL, 7% NaOH; 27.449 mg/mL) as a substrate is higher compared to the untreated Napier grass (1.776 mg/mL).

It can be concluded that the newly isolated *S. lucitanus* C5/2 was able to produce appropriate amount of CMCase and FPase using raw Napier grass as the substrate and significantly improved enzyme activities after statistical optimization by RSM was employed. In addition, the crude cellulolytic enzyme produced by *S. lucitanus* C5/2 has proven to possess potential application in the hydrolysis of lignocellulosic biomass as Napier grass was been used for production of fermentable sugar. From this investigation, Napier grass shows a good fermentation substrate that it can be fully utilized for the production of bio-product.

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

**PENGHASILAN DAN PENGOPTIMUMAN KARBOKSIMETIL SELULASE
DAN FILTER PAPERASE OLEH *Streptomyces lucitanus* C5/2 DENGAN
MENGUNAKAN KAEDAH PENAPAIAAN SEPARA TENGGELAM
RUMPUT NAPIER**

Oleh

SHAHIRAH BINTI MANAP

Januari 2017

Pengerusi : Profesor Madya Rosfarizan Mohamad, PhD
Institusi : Perhutanan Tropika dan Produk Hutan

Bahan biojisim lignoselulosa dikenali sebagai bahan mentah yang boleh diperbaharui dan digunakan untuk penghasilan biofuel. Walau bagaimanapun, disebabkan oleh struktur molekul selulosa yang kompleks ia menyukarkan proses hidrolisis kepada gula sebagai bahan mentah. Penukaran selulosa kepada gula boleh dimungkinkan oleh sekumpulan enzim dikenali sebagai enzim selulolitik yang dihasilkan oleh mikroorganisma (kulat, bakteria dan protozoa). Enzim selulolitik sistem terdiri daripada endoglukanase, eksoglukanase, dan β -glukosidase di mana setiap enzim ini berinteraksi di antara satu sama lain untuk proses hidrolisis yang sempurna. Spesies rumput merupakan salah satu daripada biojisim lignoselulosa yang murah. Rumpun Napier atau nama saintifiknya *Pennisetum purpureum* sp. merupakan salah satu sumber karbon yang berpotensi digunakan sebagai substrat untuk penghasilan bio-produk. Oleh itu, potensi rumput Napier sebagai substrat di dalam proses penapaian dikaji sepanjang kajian ini dijalankan.

Pengasingan dan pengenpastian bakteria yang boleh menghasilkan enzim selulase telah dijalankan dengan memencilkan bakteria daripada pelbagai sumber alam sekitar (kompos sisa lembu, kayu reput dan kolam). Biosintesis selulase oleh bakteria yang terpilih dengan menggunakan rumput Napier sebagai substrat dalam penapaian separa tenggelam telah dijalankan di dalam kultur sekelompok dengan menggunakan 250 mL kelalang kon. Penyelidikan diikuti dengan pengoptimuman media dan kondisi proses penapaian dengan menggunakan Kaedah Permukaan Gerakbalas (RSM) untuk mendapatkan aktiviti enzim yang maksimum. Seterusnya, untuk penilaian terhadap enzim mentah yang terhasil, hidrolisis enzim telah dijalankan dengan menggunakan rumput Napier yang dirawat dengan 1.4 M H_2SO_4 , 7% NaOH dan rumput yang tidak dirawat untuk penghasilan gula.

Hasilnya, sebanyak dua puluh lima (25) bakteria pencilan hidup di atas media terpilih dan hanya enam (6) daripadanya menunjukkan hasil positif dengan menunjukkan zon jelas di atas piring agar karboksimetil selulosa dimana ianya membuktikan penghasilan enzim. Pencilan C5/2 telah dipilih sebagai pengeluar selulase terbaik kerana menghasilkan aktiviti enzim yang tinggi semasa analisis kuantitatif dan dikenal pasti sebagai *Streptomyces lucitanus* oleh penjujukan 16S rDNA.

Nilai-nilai optimum yang diperolehi berdasarkan pengoptimuman statistik untuk biosintesis CMCase dan FPase telah ditentukan pada pH 7.8, kelajuan guncangan 170 rpm, saiz inokulum 19.7% (i/i) and biojisim rumput Napier sebanyak 4.30% (j/i) dengan maksimum aktiviti enzim sebanyak 7.362 U/mL dan 2.895 U/mL, masing-masing. Dengan menggunakan nilai parameter yang optimum, penghasilan CMCase dan FPase menunjukkan kadar peningkatan sebanyak 66% dan 30% masing-masing. Jumlah keseluruhan gula yang terhasil melalui proses hidrolisis enzim daripada rumput Napier yang dirawat dengan 1.4 M H₂SO₄ adalah sebanyak 6.541mg/mL and 7% NaOH sebanyak 27.449 mg/mL adalah lebih tinggi berbanding rumput Napier yang tidak dirawat iaitu sebanyak 1.776 mg/mL.

Dapat disimpulkan bahawa *S. lucitanus* C5/2 baru dapat menghasilkan enzim selulolitik dengan menggunakan rumput Napier mentah sebagai substrat dan aktiviti enzim bertambah baik selepas pengoptimuman dengan menggunakan kaedah RSM. Di samping itu, selulase mentah yang dihasilkan oleh *S. lucitanus* C5/2 telah terbukti mempunyai potensi aplikasi dalam hidrolisis biojisim lignoselulosa dimana rumput Napier telah digunakan untuk penghasilan gula mudah menapai. Dari kajian ini, didapati bahawa rumput Napier telah menunjukkan keupayaan sebagai substrat penapaian yang baik dan ia boleh digunakan sepenuhnya untuk penghasilan bio-produk.

ACKNOWLEDGEMENTS

Alhamdulillah, all praise to ALLAH S.W.T who guide my safety, give me strength and endless love. I would like to express a great gratitude to my supervisor, Assoc. Prof. Dr. Rosfarizan Mohamad, who have always had enormous patience and helped me in every way possible. I also would like to express my appreciation from bottom of my heart, to my co-supervisors, especially to Ms. Wan Siti Atikah Wan Omar and not forget to Dr. Wan Zuhainis Saad for their invaluable guidance, constant encouragement and constructive suggestions throughout this study.

My gratitude also goes to my amazing friends and lectures at UiTM and Bioprocess Lab UPM, Sasi, Naidah, Shira, Wawa, Kak.Farina, Abg. Shahrul, Fatehah, Ms. Suhaila, Hj. Badrul and Ms. Shahida, who are directly or indirectly supporting and helping me.

Last but not least, special thanks extended to my family members especially to my beloved father, Hj. Manap bin Omar and mother Pn. Samsiah binti Othman for their invaluable help, enormous support, endless loves, cares, patience and understanding. All prayers from them have brought success to my life.
Thank you for all.

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 of the Supervisory Committee were as follows:

Rosfarizan Mohamad, PhD

Associate Professor

Faculty of Biotechnology and Biomolecular Sciences

Universiti Putra Malaysia

(Chairman)

Wan Zuhainis Saad, PhD

Associate Professor

Faculty of Biotechnology and Biomolecular Sciences

Universiti Putra Malaysia

(Member)

Wan Siti Atikah Wan Omar

Faculty of Applied Sciences

Universiti Teknologi MARA

(External-Member)

ROBIAH BINTI YUNUS, PhD

Professor and Dean

School of Graduate Studies

Universiti Putra Malaysia

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Signature: _____

Name of _____

Member of _____

Supervisory Committee: _____

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LIST OF ABBREVIATIONS

CCD	Central Composite Design
CMC	Carboxymethyl Cellulose
CMCase	Carboxymethyl Cellulase
DCW	Dry Cell Weight
DNS	Dinitrosalicylic acid
FFD	Full Factorial Design
FPase	Filter Paperase
NCBI	National Centre of Biotechnology Information
NIRS	Near Infrared Reflectance Spectrophotometer
RSM	Response Surface Methodology
SmF	Submerged Fermentation
SSF	Solid State Fermentation
TAPPI	Technical Association of Pulp and Paper Industry
TS	Total Solid

CHAPTER 1

INTRODUCTION

Lignocellulosic biomass is currently of interest by many industries and researchers to use as a prime source for fermentable sugar production due to most abundantly available and cheap feedstock. The lignocellulose consists of three main components: cellulose, hemicellulose, and lignin, which build up by a chain of sugar molecules. These chains can be hydrolyzed to produce monomeric sugars. The bioconversion of cellulose to sugar can be catalyzed by a group of enzymes called cellulolytic enzymes that mainly produced by microorganisms (fungi and bacteria). A cellulolytic enzymes system comprises of at least three main components; endoglucanase (1,4- β -D-glucan-4-glucanohydrolase, carboxymethyl cellulase), exoglucanase (1,4- β -D-glucan-glucanohydrolase, avicelase) and β -glucosidase (Dashtban et al., 2010; Demain et al., 2005). These three components work together for a complete enzymatic hydrolysis. The endoglucanase responsible for randomly hydrolyze the β -1,4 bonds in cellulose molecules, while exoglucanase helps to release the non-reducing end of a cellulose chain and lastly β -glucosidase hydrolyses cellobiose to glucose.

For many years, numerous cellulolytic producing microorganisms have been isolated and identified by researchers (Gupta et al., 2012; Huang et al., 2012; Kim et al., 2012; Liang et al., 2014). Many studies have put more interest on fungi because it can produce cellulolytic enzymes abundantly and easy to extract (Maki et al., 2009). However, in recent years, researchers have more interest on the isolation and characterization of cellulolytic enzymes producing bacteria in order to obtain more effective cellulolytic enzymes producing bacteria from various sources (Doi, 2008). Researchers have more focus on bacteria because of their fast growth, expression of multienzyme complexes and resistance to extreme environment (Liang et al., 2014).

Grass species are one of the cheap lignocellulosic biomass where people have not realized the importance of them as a carbon source for a bio-product production. As a lignocellulosic perennial crop, the potential of conversion to energy in the form of biofuels and another by-product is highly promising. Recent research shows some interests to use perennial grasses such as switchgrass and ryegrass as substrates for bioethanol production (Liong et al., 2012). Napier grass (*Pennisetum purpureum* sp.) is known as a C4 perennial grasses that currently used as the feedstock for cow and cattle farming. However, the knowledge about this grass as an energy source is limited. The potential of Napier grass as a substrate for cellulolytic enzymes production by bacterium was investigated throughout this study.

The production of cellulolytic enzymes from Napier grass using fermentation process by bacterial strain could bring an economic impact to an industry especially in bio-ethanol industry. For bio-ethanol production, cellulolytic enzymes are widely used as a hydrolytic enzyme to break down cellulose into fermentable sugar. Therefore, the production of cellulolytic enzymes in large quantity is highly demanding.

The optimization of the medium compositions and fermentation conditions is an important step in producing a maximum production of the enzyme. The optimization by statistical approach using response surface methodology (RSM) gives an alternative technique to analyze optimized conditions and enable the researcher to design the experiment, building models, and evaluate the effect of factors and response through the investigation (Hao et al., 2006; Norfarina et al., 2010). Response surface methodology (RSM) approach has been successfully applied to optimize the medium compositions and fermentation conditions for the cellulolytic enzyme production using fungi by submerged fermentation (Anuradha et al., 2012; Carvalho et al., 2014).

The scope of this study was focused on the improvement of cellulolytic enzymes biosynthesis by locally isolated bacterium using Napier grass as a substrate. The Napier grass hydrolysate containing fermentable sugar was also prepared using crude cellulolytic enzyme obtained from the bacterium cultivation using Napier grass as the substrate. Hence, the objectives of this study are:

- i. To screen, isolate and identify the cellulolytic enzymes producing bacteria from various sources.
- ii. To characterize the Napier grass as potential substrate for cellulolytic enzymes production.
- iii. To optimize the medium composition and culture conditions for the biosynthesis of carboxymethyl cellulase and filter paperase by the isolated bacterium using statistical approach.
- iv. To evaluate the performance of Napier grass hydrolysis using crude cellulolytic enzyme for fermentable sugar production.

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LIST OF PUBLICATIONS

- Shahirah, M., Wan Zuhainis, S., Wan Siti Atikah, W.O., Siti Suhaila, H., and Rosfarizan, M. (2014). Cellulolytic Enzyme Producing Bacteria for Biological Pretreatment of Lignocelulose. National Postgraduate Seminar (10th September 2014), Faculty of Biotechnology and Biomolecular Sciences Universiti Putra Malaysia.
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