

BIOSUGAR PRODUCTION FROM OIL PALM MESOCARP FIBER USING  
CRUDE LIGNOCELLULOSIC DEGRADING ENZYMES FROM *Rhizomucor*  
*pusillus* AK2

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A thesis submitted in fulfilment of the  
requirements for the award of the degree of  
Doctor of Philosophy (Biosciences)

Faculty of Biosciences and Medical Engineering  
Universiti Teknologi Malaysia

JANUARY 2018

*Explicitly dedicated to my beloved parents and my entire family for their moral support and whom during my research engagements have been supportive and understanding despite missing me*

## ACKNOWLEDGEMENT

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

*(In the name of Allah, the most gracious and the most merciful)*

All thankfulness is to Allah my creator, whom by his will allowed me to successfully complete my study. My sincere appreciation goes to my supervisor Dr. Nor Azimah Mohd Zain and my co-supervisor Professor Madya Madihah MD Salleh, for their motivation, constructivism and guidance throughout the research errand. I really remain grateful and indebted.

I would like to express my appreciation to the management of Abubakar Tafawa Balewa University for the opportunity giving to me to undertake this study. Special appreciations go to Tetfund (Nigeria), Ministry of Higher Education Malaysia (MOHE) and Universiti Teknologi Malaysia (UTM) for the financial supports.

I am also grateful to my friends, colleagues and lab mates for their tolerance, patience and guidance during the UTM and the laboratory marathon life. The experience and idea sharing have really inspired this achievement. Finally, my parents and family members, words of gratitude are deficient. May Almighty Allah reward you with Jannah.

## ABSTRACT

The oil palm industry is undergoing global expansion as a result of the demand of oil palm. Consequently, similar expansion is occurring in amounts of lignocellulosic residues generated by the industries. The conversion of these residues to biosugar is limited by the uneconomical hydrolysis process and the high cost of lignocellulolytic enzymes. Thus, this study aimed at the production of enzymes for efficient degradation of oil palm mesocarp fiber (OPMF). The sum of 6 fungi isolates, AK1 to AK6, were isolated from OPMF, and screened for lignocellulolytic enzymes production on selective medium. Further screening of these isolates were carried out on untreated OPMF using solid state fermentation (SSF). Design-Expert software version 7.0, was used to operate the 2 level fractional factorial design and Central Composite Design (CCD) for the screening and optimization of significant factors affecting lignocellulose degrading enzymes production. The activity of crude enzyme, Viscozyme and Celluclast were evaluated based on the generation of biosugar from OPMF by determining the effects of pretreatments (2% (v/v) HNO<sub>3</sub>, 2% (w/v) NaOH and 2% (w/v) 1-Butyl-3-methylimidazolium chloride), solid loading (1-4 % w/v) and enzyme cocktail (1:1 (v/v), crude enzyme and Viscozyme, crude enzyme and celluclast, viscozyme and celluclast, and 1:1:1 (v/v) crude enzyme, Viscozyme and Celluclast). Isolate AK2 exhibited potential for lignocellulase enzyme production based on the hydrolysis zones >1.5 mm on selective media and producing CMCCase (25.4 U/g), FPase, (5.5 U/g),  $\beta$ -glucosidase (9.8 U/g), Xylanase (68.4 U/g) and MnP (4.9 U/g) at exceptional level. The isolate was thus identified by 18S RNA gene sequencing using a universal primer ITS1-F and ITS4-R as *Rhizomucor pusillus* AK2 (KY583064). From the 2 level fractional factorial design pH, temperature, inoculum size and moisture content are the significant factors affecting the production of lignocellulolytic enzymes. Xylanase was observed to be the highest activity in the lignocellulolytic enzymes cocktail (111.01 U/g). Therefore, CCD was carried out focussing on xylanase production. At the optimum condition, xylanase (128 U/g), was obtained at pH 4.98, temperature 40.27 °C, inoculum size at 10<sup>8.2</sup> spores/g and moisture at 80.64% using CCD. The regression model of the ANOVA was found to be significant with  $p < 0.0001$  and  $R^2$  of 0.9831. Biochemical characterization of the crude enzymes indicated that the enzyme was stable at pH of 4 to 6 and temperatures of 30 to 60 °C. Enzymatic saccharification was carried out with the crude enzymes comparative to Viscozyme and Celluclast. Maximum sugar production was obtained from celluclast-saccharified OPMF (1.2 g/L) using 1% (w/v) NaOH pretreated OPMF. Maximum reducing sugar generated from enzyme cocktail was 1.8 g/L obtained from 1% (w/v) NaOH pretreated OPMF which translates to polyoses; glucose (2.59 g/L), xylose (2.1 g/L) and arabinose (0.254 g/L) at 1:1:1 (v/v) crude enzyme, viscozyme and celluclast. The performance of the enzyme cocktail customized in this study is superior to that of the individual and cocktail of commercial enzyme. The study also indicates the potential of OPMF as both a substrate for biosugar and lignocellulase enzyme production from *R. pusillus* AK2.

## ABSTRAK

Industri kelapa sawit sedang mengalami perkembangan global berikutan permintaan minyak sawit yang tinggi. Kesannya, berlaku peningkatan jumlah sisa tanaman sawit oleh industri, terutamanya residu lignoselulosik. Penukaran residu ini kepada biosugar adalah terbatas dari segi ekonomi disebabkan proses hidrolisis yang mahal serta kos enzim yang tinggi. Justeru, enzim lignoselluosa yang efisien perlu dihasilkan bagi tujuan pendegradan serat mesokarp kelapa sawit (OPMF). Sejumlah 6 pencilan kulat, AK1 hingga AK6, diasingkan dari OPMF, dan disaring untuk pengeluaran enzim pendegradan lignoselluosa menggunakan medium selektif. Penyaringan lanjut terhadap pencilan dilakukan terhadap OPMF yang tidak dirawat dengan menggunakan penapaian keadaan pepejal (SSF). Perisian Design-Expert versi 7.0 digunakan untuk mengendalikan reka bentuk faktorial pecahan 2 dan reka bentuk komposit sentral (CCD) untuk penyaringan dan pengoptimuman faktor-faktor penting yang mempengaruhi pengeluaran enzim pendegradan lignoselluosa. Aktiviti enzim mentah, Viscozyme dan Celluclast dinilai berdasarkan penjaan biosugar dari OPMF dengan menentukan kesan prarawatan (2% (v/v) HNO<sub>3</sub>, 2% (w/v) NaOH dan 2% (w/v) 1-Butyl-3-methylimidazolium chloride), pembebanan pepejal (1-4% w/v) dan koktel enzim (1:1 (v/v), enzim mentah dan Viscozyme, enzim mentah dan Celluclast, Viscozyme dan Celluclast 1:1:1 (v/v) enzim mentah, Viscozyme dan Celluclast). Pencilan AK2 mempamerkan potensi pengeluaran enzim lignoselulase berdasarkan jangkauan zon hidrolisis >1.5 mm pada media selektif dan menghasilkan CMC<sub>ase</sub> (25.4 U/g), FPase, (5.5 U/g),  $\beta$ -glucosidase (9.8 U/g), Xilanase (68.4 U/g) dan MnP (4.9 U/g) pada tahap yang terbaik. Pengecaman pencilan *Rhizomucor pusillus* AK2 (KY583064) dilakukan menerusi urutan gen RNA 18S yang mengguna pakai primer semesta ITS1-F dan ITS4-R. Menurut reka bentuk faktorial pecahan 2, faktor pH, suhu, saiz inokulum dan kandungan lembapan adalah faktor penting yang mempengaruhi enzim lignoselulase. Dikalangan koktel enzim lignoselulase, pengeluaran xilanase adalah tertinggi (111.01U/g). Oleh itu, CCD dilakukan bagi memfokuskan peningkatan pengeluaran xilanase. Pada kondisi optimum, xilanase (128U/g) dihasilkan pada pH 4.98, suhu 40.27°C, saiz inokulum 10<sup>8.2</sup> spora/g dan kandungan lembapan 80.64%. Model regresi ANOVA didapati signifikan dengan p <0.0001 dan R<sup>2</sup> dari 0.9831. Pencirian biokimia enzim mentah menunjukkan enzim stabil pada pH 4 hingga 6, dan suhu 30 hingga 60°C. Sakarifikasi enzim dilakukan menggunakan Viscozyme and Celluclast sebagai perbandingan kepada enzim mentah. Pengeluaran gula maksima (1.2g/L) diperoleh hasil sakarifikasi-Celluclast OPMF yang telah dirawat menggunakan 1% (w/v) NaOH. Prarawatan OPMF menggunakan 1% (w/v) NaOH juga menghasilkan gula penurun secara maksimum 1.8g/L dari koktail enzim, yang mengandungi poliosis; glukosa (2.59 g/L), xylose (2.1 g/L) dan arabinose (0.254 g/L) pada 1:1:1 (v/v) enzim mentah, Viscozyme dan Celluclast. Prestasi enzim koktail yang disesuaikan dalam kajian ini adalah lebih baik daripada enzim individu dan koktail komersil. Kajian juga menunjukkan potensi OPMF sebagai substrat untuk pengeluaran biosugar serta enzim lignoselulase dari *R. pusillus* AK2.

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**LIST OF ABBREVIATIONS**

2LFFD	-	Two level Fractional Factorial Design
ANOVA	-	Analysis of Variance
BGL	-	$\beta$ -glucosidase
BLAST	-	Basic Local Alignment
BMIM[Cl]	-	1-Butyl-3-methylimidazolium chloride
BSA	-	Bovine Serum Albumin
CaCl <sub>2</sub>	-	Calcium chloride
CCD	-	Central Composite Design
CMC	-	Carboxymethyl cellulose
CMCase	-	Carboxymethyl cellulase
DNA	-	Deoxyribonucleic acid
DNS	-	Dinitrosalicylic acid
FeSO <sub>4</sub> .7H <sub>2</sub> O	-	Iron (II) Sulfate Heptahydrate
FESEM	-	Field Emission Scanning Electron Microscope
FPase	-	Filter paper activity
FTIR	-	Fourier Transform Infra-Red
EDTA	-	Ethylenediaminetetraacetic Acid
EtBr	-	Ethidium bromide
mL	-	Millilitre
g	-	Gram
H <sub>2</sub> SO <sub>4</sub>	-	Sulphuric acid
HCL	-	Hydrochloric acid
H <sub>2</sub> O <sub>2</sub>	-	Hydrogen peroxide
HNO <sub>3</sub>	-	Nitric acid
HPLC		High Performance Liquid Chromatography

IUPAC	-	International Union of Pure and Applied Chemistry
$\text{KH}_2\text{PO}_4$	-	Monopotassium phosphate
L	-	Liter
M	-	Molar
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	-	Magnesium sulphate
min	-	Minute
mL	-	Milliliter
mm	-	Millimeter
MMT	-	Million metric tons
mM	-	Millimolar
MnP	-	Manganese Peroxidase
MW	-	Molecular weight
NaOH	-	Sodium hydroxide
nm	-	Nanometer
OPMF	-	Oil palm Mesocarp fiber
PDA	-	Potato Dextrose Agar
pNPG	-	p-nitrophenyl $\beta$ -D-glucoside
PCR	-	Polymerase Chain Reaction
RID	-	Refractive Index Detector
RSM	-	Response Surface Methodology
SSF	-	Solid State Fermentation
U/g	-	Unit of enzyme per gram
U/mL	-	Unit of enzyme per millilitre
v/v	-	Volume per volume
w/t	-	Weight percent
w/v	-	Weight per volume
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	-	Zinc Sulfate Heptahydrate
$^{\circ}\text{C}$	-	Degree Celsius
%	-	Percentage
$\mu\text{l}$	-	Microliter
$\mu\text{m}$	-	Micrometre

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## CHAPTER 1

### INTRODUCTION

#### 1.1 Background of the study

Oil palm is one of the most economically and highly potential oil crops, a native of West Africa, belonging to the family of *Palmaceae*, species of *Elaeis guineensis*. Due to the favourability of the weather towards this crop, its cultivation covers large hectares farm in Malaysia. The overall plantation area in 2007 was 4.17 million hectares, slightly increased to 4.48 million hectares in 2008 (Wahid and Chan, 2007) to 5.74 million hectares in 2016 (Malaysian Palm Oil Board, 2017).

The waste generated in the processing mills are usually in the form of oil palm mesocarp fibre, empty fruit bunches (EFB), palm kernel shells (PKS) and palm oil mill effluent (POME). Evaluation on the annual generation of the biomass showed that, roughly, 77.24 million tons of biomass is yearly generated by oil palm mills. Out of which comprises 44.48 million tons of palm oil fronds, 6.93 million tons of empty fruit bunches, 13.97 million tons of trunk, 7.29 million tons of palm fibre and 4.21 million tons of shell, the increase in these higher values is even expected till 2020 (Ng *et al.*,

2012). Oil palm mesocarp fibre (OPMF) is a lignocellulose waste consisting of cellulose, hemicellulose and lignin. Fundamentally, cellulose and hemicellulose polymers are pools of sugars, which can be explored after degradation of lignin which is a tough compound physically sealing the sugar polymers. Thus, the degradation of cellulose and hemicellulose into monomers sugar requires enzyme complexes system that are hydrolytic and lignolytic in nature to catalyze in synergy.

To enable the accessibility of the enzymes to the fibres for degradation, various works suggested pretreatment using chemicals (Sun and Cheng, 2002; Auxenfans *et al.*, 2017). However, such harsh treatments result in losses of sugars from cellulose and hemicellulose, and the lignin can be degraded into inhibitory products (Mussatto and Roberto, 2004). The size reduction process which is a form of physical pretreatment enables enzymatic conversion of biomass (Ang *et al.*, 2015a). Besides physical process, biological treatment for lignin degradation is an oxidative process, involving three major enzymes, lignin peroxidases (LiP), manganese peroxidases (MnP) and laccases (Lac). LiP and MnP oxidize the substrate by two successive one-electron oxidation steps with cation radical formation as intermediate (Sánchez, 2009).

Cellulases and xylanases are two major hydrolytic enzymes responsible for degradation of lignocellulosic resources into biosugar. Cellulases include endoglucanase, exoglucanase and  $\beta$ -glucosidase that act synergistically to degrade cellulose to glucose. Xylanase is an enzyme that catalyses the hydrolysis of xylan backbone to produce pentose sugars as xylose and arabinose, and hexose sugar as mannose and galactose (Chávez *et al.*, 2006). The glucose and xylose produce as a result of cellulases and xylanases action become available as substrate for producing biofuel.

The cost of enzyme production together with the raw material are the two leading contributors to the overall biosugar and biofuel cost such as bioethanol (Wingren *et al.*,



2003; Galbe and Zacchi, 2007). In the year 2010, the demands in industrial enzymes in the global market were valued at \$3.6 billion; which grow at a compounded annual growth rate of 9.1% to reach \$6 billion by 2016 (Dewan, 2012). World record on sugar, estimated in 2010 that the production of sugar was 159.7 million metric tons (MMT) below the need estimate of 161.7 MMT, and 178 MMT less than 181 MMT in 2017 (Licht, 2017). Global consumption continues to expand between 1.5 to 2%, driven largely by population growth (Nyberg, 2006). Since the key obstacle hindering biosugar and biofuel production from biomass is the general absence of low cost technology for lignocellulosic degrading enzyme production and the development of an economically viable hydrolysis process (Kuhad *et al.*, 2016). Development of an economically viable lignocellulosic degrading enzyme for the hydrolysis process is a key solution to identifying competent strains that can degrade lignocellulosic biomass considering the vast majority of the fungal kingdom remains unexplored for industrial applications (Seppälä *et al.* (2017).

Filamentous fungi are good producers of extracellular lignocellulolytic enzymes and can be cultivated very easily (Adhyaru *et al.*, 2015). Research findings have indicated that the fermentation method can markedly influence enzyme production (Elisashvili *et al.*, 2001). Solid state fermentation has been ranked as the most suitable method for fungi cultivation and lignocellulolytic enzyme production, because SSF growth condition is similar to their natural niche (Pandey *et al.*, 1999). The productions of lignocellulosic degrading enzymes in the form of cellulase, xylanase, ligninase from oil palm biomass such as oil palm trunk (Ang *et al.*, 2015b) Palm kernel (Kheng and Omar, 2005), palm oil mill (Prasertsan *et al.*, 1997), oil palm empty fruit bunch (Ottenheim *et al.*, 2014) have been intensively studied. None of the available literature reported the production of lignocellulose degrading enzymes using treated or untreated mesocarp fibre. This is a clear indication that the biomass in question has been pint-sized in literature and requires exploration to unfold its biotechnological potentials. As a lignocellulose they may serve as an inducer for producing large titres of low cost enzymes that can be applied in producing biosugar at increased solid loading.

## 1.2 Statement of the problem

Global record on sugar forecast has shown that in 2010 the production of sugar was 159 MMT below the consumption value of 161.7 million metric tons (MMT) while in 2011 the production was 165.4 MMT against the consumption 162.7 MMT. In 2016 production was 174.7 below the consumption value of 180. The production in 2017 was 178 MMT below consumption 181 MMT in food and biofuel industries (Licht *et al.*, 2017). This imbalance of consumption against the production is posing a threat in world sugar production that necessitates a drastic measure to upset the production and the consumption. Conversely, oil palm production has been on increase as a result of global demand, which caused a significant increase in the area of cultivation (Yacob, 2008). For that reason, it is believed that enormous sugar polymer rich lignocellulose biomass will consistently be generated in the mills.

Lignocellulosic wastes could be harnessed as potential raw materials for economic production of high added value products such as biosugar, biocatalysts, and biofuel that currently remains the subject of considerable attention (Iqbal and Kamal, 2012). The high cost of converting lignocellulose by enzymatic hydrolysis is a recurring problem that limits the production of biosugar and to subsequently bioethanol from a renewable biomass (Hess, 2008). Low hydrolysis rates remain the main obstacle in lignocellulosic biosugar production with enzymes, which could be improved through using more digestible feedstocks in combination with increases in lignocellulase activity. To fully realize the potential of sustainable biosugar production, there is a great need to identify novel organisms, enzymes and molecules with activities that can be harnessed for a range of breakdown and lignocellulosic conversion applications (Monciardini *et al.*, 2014, Seppälä *et al.*, 2017). Thus, the need for the exploration of a new type of fungus that can produce enzymes to convert lignocellulosic biomass to biosugar.

### 1.3 Research objectives

This study aims at finding a new source of biosugar and competent lignocellulosic degrading fungi for biosugar production. In order to achieve the mentioned objectives, the following need to be addressed:

- I. To characterize the composition of lignin, cellulose and hemicellulose of oil palm mesocarp fibre (OPMF).
- II. To isolate, screen and identify lignocellulosic degrading fungi from oil palm mesocarp fibre using 18S rDNA gene sequencing.
- III. To screen the significant parameters influencing the production of lignocellulosic degrading enzymes using 2-Level fractional factorial design.
- IV. To optimize the significant parameters influencing the production of lignocellulosic degrading enzymes by using Response Surface Methodology (RSM).
- V. To compare the performance of crude lignocellulolytic degrading enzyme cocktail produced by *Rhizomucor pusillus* AK2 and commercial enzyme in saccharification of oil palm mesocarp fibre for biosugar production.

### 1.4 Scope of research

The research was conducted within the following limits:

- I. The characterization of OPMF was conducted to determine the content of lignin, cellulose and hemicellulose.

- II. The media used for the fungi isolation was Potato dextrose agar (PDA) agar. Qualitative screening to determine the potential lignocellulase enzymes producers was based on hydrolysis zones on Carboxymethyl cellulose (CMC) and Xylan agar plates. Quantitative screening was conducted based on CMCase, FPase,  $\beta$ -glucosidase, xylanase and manganese peroxidase activities via solid state fermentation of OPMF. Identification of the potential isolates based on 18S rRNA gene sequencing was based on universal primer ITS 1 (F) and ITS 4 (R).
- III. The parameters screen for the 2-Level fractional factorial design was based on physiological factors, namely; pH, temperature, incubation days, inoculum size and moisture content. The available software used for the experiment was Design expert software (version 7.0).
- IV. Optimization of significant factors was carried using Central composite design (CCD) of Response surface methodology (RSM) based on the data generated by Design expert software (version 7.0).
- V. Evaluation of the crude lignocellulosic degrading enzyme cocktail produced by *Rhizomucor pusillus* enzyme was evaluated alongside two commercial enzymes celluclast and viscozyme based on the production of biosugar from OPMF.

## 1.5 Significance of study

Residues from oil palm mill such oil palm mesocarp fibre (OPMF) is a lignocellulose waste containing cellulose and hemicellulose. Fundamentally, cellulose and hemicellulose polymers are pools of fermenting sugars that can be used by various industries such as biofuel, pharmaceuticals and food and beverages. Enzymatic conversion of the OPMF to the biosugar does not inflict any pollution problem. OPMF seems to be a bioresource which can be entirely converted into biosugar, a concept of “waste to wealth approach”. The growing interest on use of OPMF for bioconversion to

biosugar is justified as the substrate is of lower cost and renewable. The study gives insight into the better combination of parameters from the use of Central Composite Design (CCD) statistical optimization in achieving enzymes production, thus paving a way for reduced enzyme cost a major factor contributing to higher biosugar and biofuel cost. This study provides a better understanding of the synergy between cellulases and hemicellulases application via the saccharification of OPMF substrates, thereby enable effective biosugar production at elevated solid loading in enzymatic hydrolysis.

Furthermore, every palm oil processing company is aiming at avoiding unnecessary waste accumulation. The application of OPMF in fermentation and saccharification will improve the revenue of the palm oil mill industry. By and large contribute to the nation's economy in addition attaining environmental sustainability.

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