

LIGNIN BIODEGRADATION BY *Thermomyces lanuginosus* EFB2 AND  
*Aspergillus flavus* EFB4 CO-CULTURE AND PURE CULTURE IN SOLID  
STATE FERMENTATION

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A dissertation submitted in partial fulfillment of the  
requirements for the award of the degree of  
Master of Science (Biotechnology)

Faculty of Biosciences and Medical Engineering  
Universiti Teknologi Malaysia

JANUARY 2013

*Specially dedicated to my inspiration...*

*My beloved parents*

*Abdul Ghani @ Mohd Azmi bin Kasa & Solhah binti Haron... for their utmost love, continuous support and advice, and endless prayers. Thank you Allah for such wonderful parents*

*My dearest siblings*

*Ahlam, Asyraf, and Ajwad Mubarak... for their concern, assistance, and continuous encouragement and motivation*

## ACKNOWLEDGEMENT

Alhamdulillah, thank you to Allah S.W.T, Most Gracious, Most Merciful, whom with His willing and blessing, I am able to complete this research and the dissertation, as well as to complete Master of Science (Biotechnology).

I would like to express my deepest gratitude and thanks to my dedicated supervisor *Dr. Adibah Yahya* for her tremendous encouragement and guidance. The invaluable expertise, ideas and constructive comments shared by her throughout the experimental and dissertation works have contributed a lot to the success of this research.

My sincere thanks also extended to all colleagues from Project Lab, Biochemistry Lab and Research Lab for their extraordinary help, guidance and encouragement throughout the research is carried out. My acknowledgement also goes to all the lab assistants, lab technicians, and other staffs of Faculty of Biosciences and Medical Engineering for their assistance and cooperation.

Special thanks to my beloved parents: *Tn. Hj Abd Ghani bin Kasa* and *Pn. Hj. Solhah binti Hj Haron*, and my siblings: *Ahlan binti Hj Abd Ghani*, *Asyraf bin Hj Abd Ghani* and *Ajwad Mubarak bin Hj Abd Ghani* for always being my strength and providing me with continuous support. To those who indirectly contributed in this research, your kindness and helps also means a lot to me.

## ABSTRACT

The performance of pure culture and co-culture of *T. lanuginosus* EFB2 and *A. flavus* EFB4 in lignin biodegradation were observed in solid state fermentation (SSF) using oil palm empty fruit bunch (OPEFB) as the substrate. Biodegradation of lignin was evaluated based on the activity of crude ligninolytic enzymes (LiP, MnP and Lac), FPase assay and fibre content analysis of OPEFB through gravimetric method after SSF. Results showed that EFB2, EFB4 and EFB2+EFB4 were able to produce all major ligninolytic enzymes showing the ability of both strains to grow and degrade the OPEFB in SSF. Higher activity of MnP (27.17 U/L) and Lac (2.81 U/L) in EFB2+EFB4 compared to EFB2 (5.41 U/L and 2.20 U/L) and EFB4 (22.2 U/L and 2.37 U/L) pure culture demonstrated that co-culture may degrade lignin better than pure culture. MnP was predominant in both fungi as shown by the highest activity produced (27.17 U/L) among all of the lignolytic enzymes. As a pure culture, EFB4 produced significantly higher activity of LiP, MnP and Lac than EFB2. A very low level of FPase activity was detected in all cultures throughout the SSF period. The evaluation of the fibre content in OPEFB showed that EFB2, EFB4, and EFB2+EFB4 caused significant decrease of lignin, cellulose and hemicellulose amount after SSF. A maximum loss of lignin at 7.3 % was recorded on the day 8 of SSF by EFB2+EFB4. Low level of correlation between ligninolytic enzymes activity and the decrease of lignin content was observed. Characterization of LiP, MnP and Lac demonstrated that they were stable at acidic condition and LiP and MnP exhibited optimum activity at pH 4 - 5, while Lac at pH 3. Optimum temperature for LiP and MnP were recorded at 60°C and they were stable at 30°C-50°C over 3 hours of incubation while optimum temperature of Lac is 70°C and stable at 30 - 60°C. Results suggest that co-culture of EFB2 and EFB4 in SSF effectively improved lignin biodegradation in OPEFB.

## ABSTRAK

Prestasi kultur tulen dan ko-kultur *T. lanuginosus* EFB2 dan *A. flavus* EFB4 dalam penguraian lignin telah dilihat dengan “Solid state fermentation (SSF)” menggunakan tandan kosong kelapa sawit (OPEFB) sebagai substrat. Tahap penguraian lignin diukur berdasarkan aktiviti enzim penguraian lignin (LiP, MnP and Lac), FPase, dan analisis kandungan serat dalam OPEFB selepas SSF menggunakan kaedah gravimetrik. Keputusan menunjukkan EFB2, EFB4 dan EFB2+EFB4 boleh menghasilkan semua enzim penguraian lignin yang utama dan ini menunjukkan kebolehan kedua-dua strain untuk tumbuh dan mengurai OPEFB dalam SSF. EFB2+EFB4 menunjukkan aktiviti MnP (27.17 U/L) dan Lac (2.81 U/L) yang lebih tinggi berbanding kultur tulen EFB2 (5.41 U/L and 2.20 U/L) dan EFB4 (22.2 U/L and 2.37 U/L) menandakan ko-kultur boleh mengurai lignin lebih baik daripada kultur tulen. MnP adalah dominan dalam kedua-dua fungsi dan ini ditunjukkan melalui aktivitinya yang paling tinggi (27.17 U/L). Sebagai kultur tulen, EFB4 menghasilkan aktiviti LiP, MnP dan Lac yang lebih tinggi berbanding EFB2. Aktiviti FPase dikesan sangat rendah dalam semua kultur sepanjang masa SSF. Penilaian kandungan serat dalam OPEFB menunjukkan EFB2, EFB4 dan EFB2+EFB4 menyebabkan pengurangan jumlah lignin, selulosa dan hemiselulosa selepas SSF. Sebanyak 7.3% kehilangan lignin dicatat pada hari ke 8 SSF oleh EFB2+EFB4. Korelasi yang rendah antara aktiviti enzim dan pengurangan kandungan lignin telah dilihat. Pencirian LiP, MnP dan Lac menunjukkan bahawa enzim stabil dalam keadaan berasid serta aktiviti optimum LiP dan MnP pada pH4 – 5 manakala Lac pada pH 3. Suhu optimum LiP dan MnP dicatat pada 60°C dan mereka stabil pada suhu 30°C-50°C selepas 3 jam inkubasi manakala suhu optimum Lac adalah pada 70°C dan ia stabil pada suhu 30 - 60°C. Keputusan menunjukkan ko-kultur EFB2 dan EFB4 dalam SSF memberi kesan yang baik dalam penguraian lignin dalam OPEFB.

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**LIST OF ABBREVIATIONS**

°C	-	Degree Celsius
β	-	Beta
μ	-	Micro liter
g	-	Gram
mg	-	Milligram
ml	-	Milliliter
mm	-	Millimeter
nm	-	nanometer
L	-	Liter
M	-	Molar
mM	-	Millimolar
Min	-	Minute
rpm	-	Rotation per minute
U	-	Unit activity of enzyme
v/v	-	Volume per volume
w/v	-	Weight per volume
kDa	-	Kilo Dalton
psi	-	Pounds per square inch
OPEFB	-	Oil palm empty fruit bunch
EFB2	-	<i>Thermomyces lanuginosus</i>
EFB4	-	<i>Aspergillus flavus</i>
PDA	-	Potato Dextrose Agar
BSA	-	Bovine Serum Albumin
DNS	-	Dinitrosalicylic acid
HCl	-	Hydrochloric acid

NaOH	-	Sodium hydroxide
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	-	Ammonium sulfate
KH <sub>2</sub> PO <sub>4</sub>	-	Monopotassium phosphate
CaCl <sub>2</sub> .2H <sub>2</sub> O	-	Calcium chloride dihydrate
MgSO <sub>4</sub> .7H <sub>2</sub> O	-	Magnesium sulfate heptahydrate
FeSO <sub>4</sub> .7H <sub>2</sub> O	-	Ferrous sulfate heptahydrate
MnSO <sub>4</sub> .H <sub>2</sub> O	-	Manganese sulfate monohydrate
ZnSO <sub>4</sub> .7H <sub>2</sub> O	-	Zinc sulfate heptahydrate
CoCl <sub>2</sub> .6H <sub>2</sub> O	-	Cobalt (II) chloride hexahydrate
SSF	-	Solid state fermentation
LiP	-	Lignin peroxidase
MnP	-	Manganese peroxidase
Lac	-	Laccase
MnSO <sub>4</sub>	-	Manganese sulfate
H <sub>2</sub> O <sub>2</sub>	-	Hydrogen peroxide
FPA	-	Filter Paper Assay
ABTS	-	2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
CuSO <sub>4</sub> .5H <sub>2</sub> O	-	Copper sulfate pentahydrate
Na <sub>3</sub> C <sub>6</sub> H <sub>5</sub> O <sub>7</sub> .2H <sub>2</sub> O	-	Sodium citrate
Na <sub>2</sub> CO <sub>3</sub>	-	Sodium carbonate
KCl	-	Potassium chloride
H <sub>2</sub> SO <sub>4</sub>	-	Sulfuric acid
NDF	-	Neutral Detergent Fiber
ADF	-	Acid Detergent Fiber
ADL	-	Acid Detergent Lignin

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## **CHAPTER 1**

### **INTRODUCTION**

#### **1.1 Background of Research**

The abundance of solid waste produced from agro-industries every year caused pollution to the environment. In oil palm industry, approximately 26 tons of oil palm empty fruit bunches (OPEFB) was discharged from the mills for every 100 tons of fresh fruit bunches processed and this is increasing year by year. (Lawrence *et al.*, 2010). They were either incinerated or dumped in nature and that have caused serious pollution problems. While the unused OPEFBs were accumulated and occupied large area consumption, their natural degradation were very slow as they contained high content of lignin (25-35%) (Sreekala *et al.*, 1997). Lignin is the second most abundant aromatic polymer in nature with three-dimensional structure composed of phenyl propanoid units linked through several carbon-carbon and ether bonds (Wong, 2008). Such complex structure of lignin is designed in plant cell wall to protect plant cells from microbial attack (Higuchi, 1990).

In order to overcome the problems, the use of some lignin degrading fungi such as *Thermomyces lanuginosus* EFB2 and *Aspergillus flavus* EFB4 is one of the strategic ways. This is because lignin degrading fungi have developed the necessary enzymes to break lignin apart. Three main lignolytic enzymes are involved in the lignin biodegradation, namely lignin peroxidase (LiP) (EC 1.11.1.4), manganese peroxidase (MnP) (EC1.11.1.3), and laccase (Lac) (EC 1.10.3.2). *Thermomyces lanuginosus* EFB2 and *Aspergillus flavus* EFB4 were previously isolated from composted lignobiomass material and capable of growing using OPEFB as the main source of substrate (data not yet published). The ability of both fungal strains to degrade OPEFB has been observed in submerged (SmF), shake flask fermentation. However, their ability to grow and degrade EFB or other types of lignobiomass in solid state fermentation (SSF) is yet to be studied.

SSF process is more preferable to be used in the degradation of lignobiomass compared to SmF due to various advantages it renders. SSF is more economical process because various cheap agro-industrial wastes such as OPEFB can be exploited to be used as the substrates to induce the production of lignolytic enzymes to degrade lignin. In addition, SSF processes are simple, use low volume equipment, easier to handle in regarding sterility and regulation demand, and are yet effective by providing higher activity of enzymes (Pandey *et al.*, 2000; Pandey, 2003; Pandey, 2008).

Furthermore, biodegradation of lignobiomass by co-cultures has been reported to result in much higher rate of degradation due to higher production of lignolytic enzyme in comparison with that of pure cultures through synergistic interactions (Hu *et al.* 2012; Chen *et al.*, 2011b). Thus, by studying the co-culture activities of *T. lanuginosus* EFB2 and *A. flavus* EFB4 in SSF using EFB as the sole source of substrate will facilitate in providing an effective and eco-friendly method for the biodegradation of lignin. Besides, enzyme characterization studies allow control of the SSF condition within the range where enzymes are at active and stable condition. This also ensures the efficiency of process in terms of lignin biodegradation.



## 1.2 Problem Statement

OPEFB, a residue left after the fruit bunches pressed at oil palm mill is the highest lignocellulose waste generated from oil palm industry estimated about 82 million tons annually (MPOB, 2000). The large amount and the bulky nature of OPEFB caused a high land-fill disposal cost. The mills, therefore, burn the OPEFB down to ash which is used as potash fertilizer or distributed directly in the field as mulch. Particulates such as tar and soot droplets of 20-100 microns and a dust loads emitted from the furnaces sometimes cause air pollution to the nearby communities (Igwe and Onyegbado, 2007). In addition to that, gases such as SO<sub>2</sub>, CO<sub>2</sub>, CO and NO<sub>2</sub> were also emitted and caused additional methane emission into the atmosphere (Amal *et al.*, 2008). Its results in public outcries, and hence is prohibited by the Environment Protection Act (1974). In abiding to the regulations, these residues are becoming more expensive to dispose. Thus, unused OPEFBs are accumulated and occupied large area which also caused pests attraction.

With the above concerns in mind, one should conclude that strategic ways to overcome this problem have to be studied. Understanding and improving the production and activity of lignin-degrading enzymes in biodelignification became of significance and could be useful in providing a better biodelignification method for the efficient treatment of the lignocellulosic wastes. In addition, at present, much attention has only been currently drawn to the white rot fungi such as *Phanerochaete chrysosporium* (Wu *et al.*, 2005), *Trametes versicolor* (Erden *et al.*, 2009), *Pleurotus ostreatus* (Liu *et al.*, 2009), and *Phlebia radiata* (Makela *et al.*, in press) and many other white rot fungi. However, less literature was reported to study *Thermomyces lanuginosus* and *Aspergillus flavus* in lignin biodegradation.

### 1.3 Objectives

The objectives of this study were:

1. To analyse the performance of pure culture *Thermomyces lanuginosus* EFB2 and *Aspergillus flavus* EFB4 in lignin biodegradation of OPEFB.
2. To analyse the performance of co-culture *Thermomyces lanuginosus* EFB2 and *Aspergillus flavus* EFB4 in lignin biodegradation of OPEFB
3. To characterize the effect of pH and temperature on the optimum activity and stability of the crude ligninolytic enzymes produced by pure and co-culture of *Thermomyces lanuginosus* EFB2 and *Aspergillus flavus* EFB4

### 1.4 Scope of Work

The scope of this research was focused on the study of lignin biodegradation in oil palm empty fruit bunch by locally isolated fungal strains *Thermomyces lanuginosus* EFB2 and *Aspergillus flavus* EFB4 in solid state fermentation. Their potential to degrade lignin in pure culture and in co-culture condition was evaluated by crude enzymatic activity (ligninolytic and hydrolytic enzymes) assay and the amount of lignin loss in the EFB after SSF. The crude ligninolytic enzymes produced were characterized based on their optimum pH and stability, and optimum temperature and stability.

## **1.5 Significant of Research**

The study of lignin biodegradation from lignobiomass using fungi is very important as it could be used to provide an efficient and improved method to control the abundance of solid waste in the environment. Besides, this study also has the potential to be used in a variety of industrial and biotechnological applications. These applications include biopulping, biobleaching of paper pulps, and biotransformation of lignocellulosic biomass to feed. In biopulping, the use of fungi can reduce the usage of energy and chemicals in the process of paper-making. Furthermore, by studying the lignin biodegradation, pretreatment of lignobiomass to remove lignin using chemicals can be omitted. This is mostly required in the bioconversion of cellulose to sugar for biofuel production such as bioethanol.

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