SCREENING, IDENTIFICATION AND CHARACTERIZATION OF LIGNIN-DEGRADING FUNGI FROM SABAH LOCAL BIODIVERSITY FOR BIOCONVERSION OF OIL PALM WASTE

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DECLARATION

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29 November 2022

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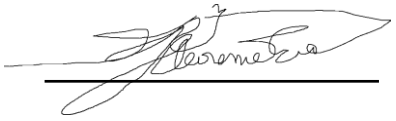

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ABSTRACT

Oil palm empty fruit bunch (OPEFB) is the residue that remains at the industrial sites once the crude oil palm has been extracted from the fruit bunches with approximately 22 to 23 million tons of OPEFB produced annually in Malaysia. OPEFB used to be disposed of as waste, but recently this abundantly available plant-based waste in Malaysia has been discovered as potential raw material for lignin. The biodegradation of lignin in lignocellulosic biomass has gained a lot of attention due to the eco-friendly advantages offered by this pre-treatment method compared to chemical and mechanical pre-treatments. However, studies on the biodegradation of lignin especially in OPEFB remain limited. It was hypothesized that locally isolated fungi were capable of degrading lignin in OPEFB. Hence the objectives of this study were i) to isolate and screen potential OPEFB lignin degraders, ii) to identify potential OPEFB lignin-degraders and iii) to characterize the degraded OPEFB lignin. Initially, 40 fungi portraying lignin degrading capability were isolated from decaying wood and soil samples collected from several locations in Sabah. After primary screening using Remazol Brilliant Blue R (RBBR), only seven fungi isolates exhibited positive and significant lignin-degrading results. The ligninolytic enzyme assay was conducted on the seven potential lignin-degraders to study the predominant lignin-degrading enzymes secreted by the fungi. The results revealed the capability of all seven fungi to secrete a significant amount of lignin peroxidase (LiP) enzyme when incubated in an RBBR liquid media with fungal strain 15B20 exhibiting the highest LiP production (19.81μM). The quantitative enzyme assay was followed by secondary screening incorporating Sundman and Nase assay. Three potential lignin-degraders were chosen based on the significant decolourization rate of Sundman and Nase Assay containing kraft lignin and actual OPEFB. The genomic DNAs of these potential lignin-degraders were extracted and amplified using polymerase chain reaction (PCR) and ITS1 (forward) and ITS4 (reverse) as the primers. Based on the sequence of the PCR products, fungal strains 19A23, 15B20 and 14A11 were identified as *Monascus* sp., *Mucor* sp., and *Aspergillus* sp. respectively. Consequently, lignin-degrader 19A23 (*Monascus* sp) was grown under fifteen different treatment conditions to study the effect of three parameters; the age of inoculum, weight of OPEFB and incubation

time, on the OPEFB lignin degradation yield. The biological pretreatment involving seven days of inoculum age, 1.32g OPEFB and four weeks of incubation resulted in the highest degradation yield of 77.1%. In the FTIR analysis of treated OPEFB, a significant peak between 850cm^{-1} to 750cm^{-1} was detected indicating the C-H bending in the aromatic ring, which was related to the vibration of lignin.

ABSTRAK

SARINGAN, PENGENALPASTIAN DAN PERINCIRIAN SAMPEL KULAT YANG DIDAPATI DARI BIODIVERSITI TEMPATAN SABAH SEBAGAI PENDEGRADASI LIGNIN UNTUK BIOKONVERSI SISA MINYAK KELAPA SAWIT YANG OPTIMUM.

Tandan kosong buah kelapa sawit (OPEFB) merupakan sisa yang dilupuskan di kawasan perindustrian sejurus selepas minyak sawit mentah diekstrak dari buah kelapa sawit dimana sekitar 22 ke 23 juta tan OPEFB dihasilkan setiap tahun di Malaysia. Kebelakangan ini, sisa berasaskan tumbuhan yang banyak terdapat di Malaysia ini didapati mempunyai potensi sebagai bahan mentah lignin. Biodegradasi lignin semakin mendapat perhatian disebabkan oleh kelebihannya yang mesra alam berbanding dengan pra-rawatan kimia and fizikal. Namun, kajian mengenai biodegradasi lignin terutama sekali dalam OPEFB masih terhad. Hipotesis yang dikaji dalam kajian ini adalah, kulat tempatan mempunyai keupayaan untuk mendegradasi lignin OPEFB. Oleh yang sedemikian, objektif kajian ini adalah, i) untuk mengumpul dan membuat saringan terhadap pendegradasi lignin OPEFB yang berpotensi, ii) untuk mengenal pasti spesis pendegredasi lignin OPEFB yang berpotensi dan iii) membuat perincian ke atas lignin OPEFB yang telah didegradasi. Pada mulanya, 40 kulat yang mempunyai potensi dalam pendegradasian lignin telah diperolehi daripada sampel kayu reput dan tanah yang dikumpul dari beberapa lokasi di Sabah. Selepas proses saringan pertama iaitu penyahwarnaan Remazol Brilliant Blue R (RBBR), hanya tujuh kulat mempamerkan keputusan yang positif dan ketara. Ujian enzim ligninolitik dijalankan terhadap tujuh kulat tersebut untuk mengenal pasti enzim pendegradasi lignin utama yang dihasilkan, dan ujian tersebut mendapati ketujuh-tujuh kulat yang terpilih menghasilkan enzim lignin peroksidas dalam kuantiti yang tinggi apabila diinkubasi di dalam RBBR berbentuk cecair, dengan fungi 15B20 menunjukan penghasilan enzim LiP yang tertinggi (19.81μM). Ujian enzim ligninolitik seterusnya diikuti oleh saringan kedua, ujian Sundman dan Nase. Tiga kulat dipilih sebagai pendegradasi lignin yang paling berpotensi berdasarkan kadar penyahwarnaan Sundman dan Nase yang mengandungi Kraft lignin dan OPEFB sebenar. DNA pendegredasi lignin yang berpotensi ini telah diekstrak dan

diperkuatkan menggunaan tindak balas berantai polimeras (PCR) dan ITS1 serta ITS4 sebagai primer. Berdasarkan jujukan produk PCR, kulat 19A23, 15B20 dan 14A11 masing-masing telah dikenalpasti sebagai Monascus sp.*, Mucor* sp.*,* dan *Aspergillus* sp. *Pendegradasi lignin yang berpotensi, 19A23 (Monascup* sp.*) dibiarkan tumbuh di atas OPEFB dengan 15 kondisi yang berlainan untuk mengkaji kesan tiga parameter; usia inokulasi, berat OPEFB dan tempoh inkubasi ke atas hasil degradasi lignin OPEFB. Pra-rawatan biological lignin in mendapati kajian nombor 9 yang mengimplementasikan usia inokulasi selama tujuh hari, berat OPEFB sebanyk 1.32g dan tempoh inkubasi selama empat minggu menunjukan tahap degradasi lignin OPEFB yang tertinggi iaitu sebanyak 77.1%. Analisis FTIR ke atas OPEFB yang dirawat menghasilkan puncak yang ketara di antara 850cm^{-1} to 750cm^{-1} menunjukkan pembengkokkan C-H dalam gelang aromatik, yang berkaitan dengan getaran lignin.*

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LIST OF SYMBOLS

&	-	And
:	-	Ratio
α	-	Alpha
β	-	Beta
+	-	Positive
-	-	Negative
%	-	Percentage
°C	-	Degree Celcius
ml	-	Millilitre
g	-	Gram
rpm	-	Revolution per minute
µM	-	Micro molar
ng	-	Nanogram
µl	-	Micro liter

LIST OF ABBREVIATIONS

OPEFB	-	Oil palm empty fruit bunch
LCBM	-	Lignocellulosic biomass
LMEs	-	Lignin modifying enzymes
LNPS	-	Lignin nanoparticles
bp	-	Base pair
FTIR	-	Fourier-transform infrared
PCR	-	Polymerase chain reaction
ITS	-	Internal transcribed spacer

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CHAPTER 1

INTRODUCTION

1.1 Background of study

Oil palm (*Elaeis guineensis*) production has contributed greatly to the agricultural industry in Malaysia. Approximately 5.8 million hectares of land are under oil palm cultivation with over 17 million tonnes of palm oil produced yearly (Parveez *et al.,* 2021). Oil palm empty fruit bunch (OPEFB) is the residue that remains at the industrial sites once the crude oil palm was extracted from the fruit bunches. OPEFB used to be disposed of as waste, but recently this abundantly available plant-based waste in Malaysia has been discovered as potential raw material for lignin.

Lignin is an aromatic biopolymer and one of the major components of lignocellulose that plays a prominent role in providing structural support to plants (Shrotri *et al*., 2017). Lignin was developed from three basic phenylpropanoid monomers consisting of guaiacyl, syringyl and *p*-hydroxyphenyl subunits (Priyanga and Kannahi, 2018). Although this component has been constantly perceived as waste in the past, lignin has gained considerable attention as a potential raw material for valuable products over the years due to its abundant availability and inexpensive supply (Yu and Kim, 2020).

Lignin has been utilized in developing products such as lignin-based polymers, lignin-based carbon fiber and biomedical materials (Yu and Kim, 2020; Patil *et al*., 2016; Iravani and Varma, 2020). However, due to the heterogeneity, rigid and complex structure, lignin is recalcitrant towards degradation (Hong *et al*., 2015). Hence, during the past few decades, numerous pretreatment methods have been

developed to degrade lignin into smaller and simpler components or possibly convert them into value-added products.

The three major pretreatments of lignocellulosic biomass (LCBM) include physical, chemical and biological pretreatments, each possessing its own benefits and drawbacks in the degradation of lignin. However, a biological method for lignin degradation has drawn great attention in recent years due to its distinct and more eco-friendly advantages compared to physical and chemical pre-treatments. Biological pre-treatment for lignin degradation or also known as lignin biodegradation is a method that applies microorganisms such as fungi and bacteria in degrading lignin into simpler components. Hammel and Cullen (2008) mentioned that manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase are the main enzymes catalyzing the lignin biodegradation process. Biological pre-treatment of lignin offered low energy demand, minimal chemical requirement and low disposal cost (Wang *et al.*, 2018).

1.2 Problem Statement

The abundance of oil palm empty fruit bunch (OPEFB) that has been continuously produced by oil palm plantations creates an alarming environmental issue. Researchers have discovered that the structural properties of lignin in OPEFB can be utilized to develop products such as lignin-based polymeric materials derived from the lignin monomers and these monomers can be obtained through the degradation process. Biological pretreatment for lignin degradation has received increasing attention over the years due to its eco-friendly approach and distinct advantages compared to physical and mechanical pre-treatments. However, studies on lignin-degrading fungi that can particularly degrade lignin in OPEFB are still limited. Even though there are numerous types of fungi available commercially, only a small fraction of that have been reported to have an appreciable degree of lignin degradation capability. In a study conducted by Saito *et al.* (2018), it was reported that the white-rot fungi *Phanerochaete chrysosporium* significantly degraded lignin. Thus, these fungi were used as a benchmark for OPEFB lignin degradation. These fungi might not be able to adapt to the local environment where the OPEFB were abundantly produced. This condition would affect the OPEFB lignin degradation

capability of *Phanerochaete chrysosporium* hence the need to find a more efficient OPEFB lignin-degrader.Therefore, fungal samples isolated from Sabah local biodiversity were screened for their OPEFB lignin degradation abilities.

1.3 Research Hypothesis

It was assumed that locally isolated fungi might have developed certain characteristics that enable them to degrade OPEFB efficiently due to their adaptation to the same environment as the oil palm. It was hypothesized that locally isolated fungi have a higher rate of decolorization in primary and secondary screening and higher OPEFB lignin degradation yield compared to *P.chrysosporium,* exhibiting apparent changes in the structure of OPEFB lignin after pre-treatment compared to the untreated OPEFB.

1.4 Research Objectives

i) To isolate and screen OPEFB lignin-degrading fungi from Sabah biodiversity using primary and secondary screening methods;

ii) To identify potential OPEFB lignin degraders through morphological and gene sequence analyses; and

iii) To characterize the degraded OPEFB lignin using FTIR and Klason lignin analyses.

1.5 Significance of Study

The findings from this study could provide potential lignin-degraders isolated from Sabah local biodiversity that can degrade lignin specifically in OPEFB. Apart from that, the knowledge of the predominant enzyme secreted by the potential-lignin degraders can serve as a reference for the enzymatic hydrolysis of OPEFB. This study serves as the first step in transforming OPEFB from waste into an alternative material for value-added products.

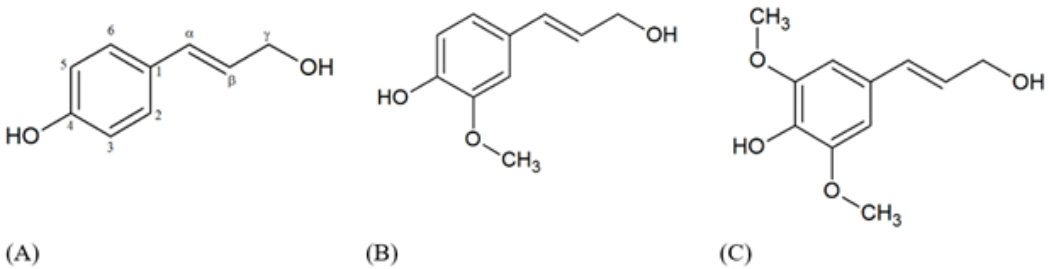
CHAPTER 2

LITERATURE REVIEW

2.1 Lignin

2.1.1 Structure Of Lignin

Lignin is a complex amorphous polymer found in vascular plants, comprising three precursors known as coniferyl alcohol, sinapyl and *p*-coumaryl as depicted in **Figure 2.1** which are incorporated into lignin in the form of guaiacyl (G), syringyl (S) and *p*-hydroxyphenyl (H) subunits, respectively (Martinez *et al*, 2005; Abhilash & Thomas, 2017). Phenylpropanoid monomers in lignin are either linked by ether or carbon-carbon (C-C) linkages (Ma *et al.,* 2017). The complex structure of lignin made it recalcitrant to any kind of degradation. However, the formation of lignin in plants is cell-specific and it shows distinct sub-cellular localization and monomer composition which restricted the understanding of the general lignification mechanism.



(A) (B) (C)

Figure 2.1 : **Basic structural units of lignin. (A) *p*-coumaryl alcohol, (B) Coniferyl alcohol, (C) Sinaphyl alcohol**

Source : Gomes *et al*. 2020

Lignin constitutes approximately 20-35% of the dry weight of lignocellulose (Bugg *et al.*, 2011 and is known to be the second most abundant biopolymer earth next to cellulose. Lignin is a vital component in the plant cell wall (Liu *et al.*, 2018), apart from enhancing the cell wall rigidity, it also prevents plants from degrading by forming a barrier that inhibits the penetration of destructive lignocellulosic enzyme through the cell wall (Barros *et al.,* 2015; Hong *et al.*, 2012). Studies have also reported the role of lignin in transporting water and protecting plants against pests and pathogens (Yang *et al.*, 2018).

2.1.2 Types Of Lignin

Lignin is divided into two groups known as native lignin and technical lignin. Native lignin is defined as the original form of lignin present in lignocellulose (Shrotri *et al.,* 2017). To date, two native lignin were derived from the lignocellulosic biomass through two distinct methods. Milled wood lignin was isolated from lignocellulose via the Björkman process. This process involves treating finely milled wood with a neutral organic solvent such as the aqueous dioxane solutions under mild conditions to isolate lignin (Lu *et al.*, 2017). The second native lignin was known as Brauns' lignin which was obtained through the milling process and by extracting lignocellulosic biomass (LCBM) with ethanol (Abejón *et al.*, 2018). Brauns' lignin was reported to portray very identical chemistry to native lignin (Holtzapple, 2003). However, the yield of this process is relatively low, with only less than 10% of lignin isolated from the lignocellulosic biomass.

Technical lignin, or industrial lignin, is a term used to describe lignin-by products (Berlin & Balakshin, 2014). In this particular group of lignin, significant structural changes have occurred especially the cleavage of α-O-4 and β-0-4 due to physical and chemical pre-treatment. Hence, the lignin belonging to this group has distinctive properties compared to the native lignin found in plants. Technical lignin comprises three major lignin including kraft, sulfite and soda lignin (Macfarlane *et al.,* 2014; Lu *et al.*, 2017). Kraft lignin undergoes the Kraft process that involves immersing LCBM in an aqueous solution that contains a mixture of sodium hydroxide (NaOH) and sodium sulfide (Na_2S) while exerting high temperature (150 to 180°C)