CHARACTERIZATION OF LIGNINOLYTIC *AGROBACTERIUM* SP. STRAIN S2 AND IDENTIFICATION OF DEPOLYMERIZATION ENZYMES INVOLVED

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DEDICATION

This thesis is dedicated to my father, who taught me that the best kind of knowledge to have is that which is learned for its own sake. It is also dedicated to my mother, who taught me that even the largest task can be accomplished if it is done one step at a time.

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ABSTRACT

The abundant availability of lignocellulosic biomass has contributed to an interest in the conversion of biomass into bioethanol or biochemical products via a sustainable pre-treatment process to break down the recalcitrant lignin structure. This study attempts to characterize the ligninolytic *Agrobacterium* sp. strain S2 isolated from empty fruit bunch (EFB) and subsequently to identify the lignin depolymerization enzymes involved. The strain was previously isolated from decaying EFB that was left for more than six months at a plantation. Biochemical characterization and quantitate study were administered to study the lignin depolymerization and degrading ability of the strain. The strain grew in minimal media with alkali lignin as the sole carbon source and it reached maximum growth on day three. Several known ligninolytic enzyme assays were performed lignin peroxidase (LiP), laccase (Lac) and manganese peroxidase (MnP) activities had been detected. Gel permeation chromatography (GPC) analysis was administered to confirm the strain's ability to depolymerize or degrade lignin macromolecules. The alkali lignin treated with strain S2 was depolymerized to 2,263 Da on day three and further degraded to 1,004 Da on day 7, achieving 70.48% lignin degradation. After confirming the lignin-degrading ability of the respective strain, whole-genome sequencing was administered. The strain was identified as species that belongs to the *Agrobacterium* genus. The draft genome revealed genes encoding enzymes responsible for degradation of high and low molecular lignins. Catalase peroxidase (CP), superoxide dismutase (SD), non-heme chloroperoxidase (NCP) and cytochrome P450 (CP450) were selected for molecular study because these enzymes were believed to be involved in lignin depolymerization. The enzymes were selected based on the inferred homology via Basic Local Alignment Search Tool (BLAST), a sequence alignment of the target proteins against the databases. Genes encoding the metabolism of peripheral pathways for catabolism of aromatic compounds and central aromatic intermediates were also predicted. TA cloning and heterologous expression of genes encoding enzymes of interest in *E.coli* (JMP109) were administered via recombinant DNA technology and a recombinant CP450 was successfully expressed. The expression of CP450 confirmed the ease of genetic modification and recombinant efficiency in bacteria of smaller genome. CP450 is known to catalyse aromatic O-demethylation, a rate-limiting step in the conversion of aromatic compounds to valuable chemicals. In conclusion, the *Agrobacterium* sp. strain studied has demonstrated promising ligninolytic potential and a variety of enzyme candidates for future study.

ABSTRAK

Disebabkan ketersediaan biojisim lignoselulosa yang besar jumlahnya, pertambahan minat dalam penukaran biojisim kepada bioetanol atau produk biokimia memerlukan proses pra-rawatan yang mampan untuk memecahkan struktur tegar lignin. Kajian ini cuba mencirikan ligninolitik *Agrobacterium* sp. S2 daripada tandan buah kosong (TBK) dan seterusnya mengenal pasti enzim penyahpolimeran lignin yang terlibat. *Strain* itu sebelum ini diasingkan daripada TBK reput yang terbiar selama lebih enam bulan di ladang. Untuk mengkaji penyahpolimeran lignin dan keupayaan degradasi lignin, pencirian biokimia dan kajian kuantitatif telah diselidik. *Strain* tumbuh dalam medium minimum dengan lignin alkali sebagai sumber karbon tunggal dan mencapai pertumbuhan maksimum pada hari ketiga. Ujian enzim ligninolitik berjaya mengesan aktiviti *lignin peroxidase* (LiP), *laccase* (Lac), dan *manganese peroxidase* (MnP). Untuk mengesahkan keupayaan menyahpolimer atau merendahkan makromolekul lignin, analisis menggunakan *gel permeation chromatography* (GPC) telah dilakukan. Lignin alkali yang dirawat dengan *strain* S2 telah dinyahpolimerkan kepada 2,263 Da pada hari ketiga dan degradasi lagi kepada 1,004 Da pada hari ketujuh, mencecah 70.48% degradasi lignin. Selepas mengesahkan keupayaan degradasi lignin oleh *strain* tersebut, penjujukan genom keseluruhan telah dibuat. *Strain* dikenalpasti sebagai spesies yang tergolong dalam genus *Agrobacterium*. Draf genom mendedahkan gen pengekodan enzim yang bertanggungjawab untuk degradasi lignin molekul tinggi dan rendah. *Catalase peroxidase* (CP), superoxide dismutase (SD), non-heme chloroperoxidase (NCP) and cytochrome P450 (CP450) telah dipilih untuk kajian molekul. Enzim-enzim ini dipercayai terlibat dalam penyahpolimeran lignin, dipilih berdasarkan homologi yang disimpulkan melalui via *Basic Local Alignment Search Tool* (*BLAST)* berdasarkan persamaan penjajaran jujukan protein sasaran terhadap pangkalan data. Metabolisme pengekodan gen bagi laluan periferi untuk katabolisme sebatian aromatik dan perantaraan aromatik pusat juga telah diramalkan. Pengklonan TA dan ekspresi heterolog gen yang mengekodkan enzim yang menarik dalam E.coli (JMP109) melalui teknologi DNA rekombinan telah dilakukan. Hasilnya, rekombinan CP450 telah berjaya diekspresikan dan bersedia untuk langkah seterusnya. Ekspresi CP450 mengesahkan kemudahan pengubahsuaian genetik dan kecekapan rekombinan bagi bakteria genom yang lebih kecil. CP450 mempunyai kebolehan memangkinkan O-demetilasi aromatik, langkah penting dalam penukaran sebatian aromatik kepada bahan kimia yang berharga. *Agrobacterium* sp. strain yang dikaji telah menunjukkan potensi ligninolitik yang tinggi.

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LIST OF ABBREVIATIONS

ABTS	-	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
BLAST	-	Basic Local Alignment Search Tool
bp	-	Base pair
CP	-	Chloroperoxidase
CP450	-	Cytochrome P450
DNA	-	Deoxyribonucleic acid
EFB	-	Empty Fruit bunch
GPC	-	Gel Permeation Chromatography
IPTG	-	Isopropyl β-D-1-thiogalactopyranoside
LB	-	Luria Broth
LiP	-	Lignin Peroxidase
MnP	-	Manganese Peroxidase
NCBI	-	National Centre for Biotechnology Information
NCP	-	Non-heme Chloroperoxidase
NGS	-	Next-Generation Sequencing
OD	-	Optical Density
PCR	-	polymerase chain reaction
RST	-	Rapid Annotations using Subsystems Technology
SD	-	Superoxide Dismutase
SDS	-	sodium dodecyl sulphate
VA	-	Veratryl alcohol

LIST OF SYMBOLS

uL	-	Microliter
mL	-	Millilitre
α	-	Alpha
β	-	Beta
°C	-	Degree Celsius

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CHAPTER 1

INTRODUCTION

1.1 Background

Globally, biomass production from various wastes is estimated at a staggering amount of 146 billion tons yearly (Guragain & Vadlani, 2021). Four major crop residues consist of corn stover, rice straw, wheat straw and sorghum stover stood at 1413, 1084, 1056 and 81 Mt respectively (Guragain & Vadlani, 2021). Consequently, as a prominent player in agricultural commodities, lignocellulose waste production in Malaysia towers at approximately 168 Mt annually (Bušić et al., 2018). They include but are not limited to oil palm waste, coconut trunk fibres, rice husks, sugarcane, cassava, corn and their respective residues (cassava rhizome and corncobs) as well as municipal waste (Abd Aziz & Kin Mun, 2012; Zafar, 2018). Recognized as the leading palm oil producer and exporter alongside the neighbouring country Indonesia, oil palm wastes in Malaysia account for an astounding amount of 94 % of the abundant agricultural and forestry biomass resources (Zhuohua Sun et al., 2018).

Despite eminent interest in utilizing lignocellulosic biomass for the second generation of bioethanol, the application of biomass feedstock for biofuels and biochemicals is still at a nascent stage, relative to the amount of generated biomass. Current global lands use to grow biofuels feedstocks is only 25 million hectares, which is 0.19% of the world's total land area (Guragain & Vadlani, 2021). The main challenge lies in the pre-treatments technology for converting the lignocellulosic biomass into valuable products (Silva et al., 2021; Zhuohua Sun et al., 2018). Lignocellulose biomass comprises three major polymers of lignin, cellulose and hemicellulose associated in the hetero matrix at varying degrees depending on the source of biomass (Isikgor & Becer, 2015). The chains of polysaccharides cellulose and hemicellulose are the major elements for biofuels, biochemicals and bioethanol production while lignin is a potential polymer for biomaterials production. Amongst

them, lignin is the most complex, posing a significant challenge in pre-treatment technology or lignin valorization: conversion, degradation, depolymerization technology due to the inherent recalcitrance. This can be attributed to complex three-dimensional structure of heteropolymer lignin comprises of diverse stable C-C and C-O linkages within phenyl propanoid monomeric subunits (Isikgor & Becer, 2015).

Conventionally, the utilization of biomass and lignin depolymerization is governed via physicochemical pre-treatments, albeit these technologies are not without challenges (high input energy, costly, requires stringent conditions and produce toxic intermediates). Therefore, biological processes offering green technology via microorganisms are considered ideal as they mirror the occurrence of lignin degradation in nature, resulting in the continuous development and studies of biological processes (P. Chen et al., 2017; Wang et al., 2013). Microorganism-mediated bio-catalytic processes, for instance, require a relatively mild condition, which translates to low energy requirement and essentially affordable cost (Kim et al., 2016). Utilization of fungi in diverse fields such as bioremediation and biorefinery for biomass delignification has been implanted. (Rybczyńska-Tkaczyk & Korniłłowicz-Kowalska, 2017; R. Xu et al., 2018).

White rot basidiomycetes are the most studied lignin degraders. Lac, MnP and LiP are some of the common lignin oxidizing enzymes secreted by the fungi. However, fungi demonstrate limitations for industrial application due to inferior adaptability to diverse pH, temperature and oxygen limiting conditions. While fungi face challenges in expression at protein level due to the complex and larger genome sizes, bacteria offer convenient molecular genetics and protein expression with smaller genomes (Yang et al., 2021). This allows for more efficient genetic modification to enhance the production of lignin-degrading enzymes, owing to the higher recombination efficiency and greater ease for desired gene expression (Atiwesh et al., 2022). Therefore, the interest has shifted to bacteria, which offer excellent adaptability in various conditions (Bandounas et al., 2011).

Bacterial lignin degradation also demonstrates versatile degradation pathways of aromatic substances, transforming components of highly complex lignin, single

phenols and xenobiotic compounds (Kameshwar & Qin, 2016). Amongst bacteria, the species majorly belong to the actinobacteria and proteobacteria phyla. *Streptomyces viridosporus* T7A, *Nocardia autotrophica*, *Sphingobium* sp. SYK-6, *Pseudomonas putida mt-2*, *Rhodococcus* sp., *Burkholderia cepacia*, *Microbacterium* sp and *Citrobacter* sp were identified as efficient lignin degraders (Taylor et al. 2012). Common ligninolytic enzymes similar to fungi have been reported through biochemical characterization studies (Y. Shi et al., 2013; Z. Xu et al., 2018; Yang et al., 2012). However, genomics studies had shown that bacteria might possess different mechanisms and secrete different ligninolytic enzymes found in fungi. These potential ligninolytic bacteria are largely undiscovered and many ligninolytic enzymes may still emerge. In addition, previous characterization studies on lignin-degrading bacteria mostly used lignin model dimers, such as β-aryl ether lignin, veratrylglycerol-β-guaiacol ether, guaiacylglycerol-β-guaiacyl ether and lignin-derived aromatic compounds to evaluate lignin degradation ability (Ghatge et al., 2018; Li et al., 2020). However, studies on lignin depolymerization, which involve degradation of high molecular weight lignin products are limited and require further evaluation. In recent decades, rapid progress and development of high throughput sequencing technology for omics study allow for the discovery of various lignin-degrading bacteria-enzyme systems from anaerobic and aerobic communities, rotten wood, wastewater treatment plant compost, marine and animal gut microbiota (Silva et al., 2021; Zhuohua Sun et al., 2018). Malaysia's tropical climate and abundant biomass feedstock sourcing from oil palm waste could unearth a variety of ligninolytic bacteria.

A potential strain previously isolated from empty fruit bunch (EFB) was studied (Tahir et al., 2019). The strain is mesophilic, preferring alkaline conditions and showed initial ligninolytic ability when tested with methylene blue dye. For this study, to characterize the ligninolytic potential, the strain growth on the alkali lignin plate and alkali lignin-W minimum media were evaluated. In addition, ligninolytic enzymes' activity and gel-permeation chromatography (GPC) analysis using alkali lignin was administered to study the lignin depolymerization potential. The genome strain, designated as strain S2, was shotgun sequenced. The draft genome sequence was then subjected to genome analysis to identify the genes potentially involved in lignin depolymerization via sequence similarity alignment. Finally, TA cloning and

heterologous expression were performed for the selected candidate genes encoding lignin depolymerization enzymes;- superoxide dismutase (SD), catalase peroxidase (CP), non-heme chloroperoxidase (NCP) and cytochrome P450 (CP450). These genes were selected based on their significant sequence similarity that translates to homologs. Through recombinant DNA technology and protein induction, CP450 recombinant protein was successfully expressed, confirming the ease of genetic modification and recombinant efficiency of this particular strain of bacteria. The scope of study stopped here. However, functional characterization of recombinant CP450 protein on its ability to depolymerize could therefore be pursued for further study.

1.2 Problem Statement

Lignocellulosic biomass is still underutilized relative to their abundance production. The residues from major crops, forests and municipal waste are expected to increase by 2050. 2.6 billion tons of municipal waste production and six billion tons of forest residue are projected by 2050. Currently, only a portion of the amount is being utilized for biochemicals and biofuels production (Asia, 2019; Guragain & Vadlani, 2021). In Malaysia alone, the lignocellulose biomass production stands at 168 Mt annually, with oil palm plantation waste conquering the majority (94%) (Bušić et al., 2018). As a major player in palm oil production and agricultural activities, 13.6 % of the country's land area is allocated for oil palm plantation. Out of palm oil processing yield, only 10% are finished products (palm oil and palm kernel oil) while the remaining 90% are harvestable biomass waste remain underutilized.

The primary challenge in the conversion of lignocellulose biomass is governed by the pre-treatment’s technology, which involves an effective disruption of the recalcitrance lignin. The conventional pretreatments of physicochemical have drawbacks due to their high cost and possible toxic intermediates production (Galbe & Wallberg, 2019). Therefore, biological pretreatments using microbes-mediated systems may offer an alternative for green and improved treatment as they mimic the degradation of biomass in nature. Although studies have reported white-rot and brown-rot fungi as efficient degraders, study on bacterial lignin degradation system is

relatively unexplored. With emerging of “omics” technologies, immense interest has shifted to bacterial enzymes mediated systems as they are superior adapting in various conditions. Earlier report discussed several challenges of fungi biological catalysts for commercialization by listing difficulty in recombinant protein expression and complex genome as one of the challenges. Due to smaller and less complex genome, bacteria will offer more efficient gene expression and recombinant protein expression (Ahmad et al., 2011).

Potential ligninolytic bacteria from various habitats are still largely undiscovered. Bacteria are predicted to secrete other ligninolytic enzymes that are not found in fungi, potentially emerging ligninolytic candidate genes discovery. In addition, most studies for lignin degradation of bacteria focus on the utilization of lignin dimers or artificial substrates, with limited quantitative evidence on lignin depolymerization or the ability of bacteria to degrade lignin macromolecules.

1.3 Research Goal

Therefore, this study attempts to characterize ligninolytic potential of *Agrobacterium* sp. strain S2 and identified the depolymerization enzymes involved.

1.3.1 Research Objectives

The objectives of the research are:

(a) To evaluate the lignin degradation and depolymerization ability of a strain isolated from decaying empty fruit bunch.

(b) To predict candidate genes encoding lignin depolymerization enzymes from the draft genome

(c) To express a recombinant protein through DNA manipulation.

1.4 Research Scope

The scope of this research can be delineated into two primary frameworks. The first work focused on the characterization of the previously isolated strain on its ligninolytic capability through biochemical study and quantitative analysis. Biochemical characterization includes growth of bacterial strain S2 in alkali lignin agar, alkali lignin W-media (broth) and crude enzyme activities study of common ligninolytic enzymes consisting of LiP, MnP and Lac. The quantitative analysis was done through GPC analysis to confirm whether the high molecular weight of lignin particles was successfully degraded to smaller ones, providing evidence on the strain lignin depolymerization capability.

The second framework detailed the genomic analysis of the strain. The strain was shotgun sequenced with Ion Torrent. Genomic analysis revealed the strain as species belonging to *Agrobacterium* genus. The draft genome sequence was then subjected to genome analysis via RAST annotation and sequence similarity alignment using BLAST (against the NCBI, UniProt, EMBL-EBI and Pfam databases) to infer homology. This method allowed for the reliable identification of candidate genes responsible for lignin depolymerization in the genome. Similarly, other putative genes playing roles for peripheral pathways for the catabolism of aromatic compounds and central aromatic intermediates were also discussed.

Based on the presence of homologs inferred from sequence similarity alignment, four selected enzymes of SD, CP450, NCP and CP were pursued. Through recombinant technology, molecular cloning was administered via DNA extraction, primer design, genes amplification (PCR), TA cloning, heterologous expression, induction and protein expression. One recombinant protein, CP450, was successfully expressed and ready for future study.

1.5 Significance of Study

Characterizing the ligninolytic ability of the previously isolated strain through biochemical study and quantitative analysis through GPC will contribute to the knowledge on a variety of bacteria isolated from diverse habitats showing lignin degradation and depolymerization capability. In addition, genome annotation identified the strain as genus *Agrobacterium*. This genus has limited information on its genome features regarding lignin degradation capability relative to other commonly reported bacteria lignin degraders such as *Streptomyces, Rhodococcus* and *Amycolatopsi*. Although there have been several bacteria from genus *Agrobacterium* isolated from cherry tree gall, municipal solid waste and wood decay samples (hardwood) from mountain Qinling, this is first draft genome sequence with reported study detailing on ligninolytic genes in genome *Agrobacterium* isolated from EFB in a tropical climate. This is significant as the EFB source harbours a substantial amount of lignin (29.2%) compared to that in hardwood (15 to 24%), softwood (20 to 27%) and other agricultural waste (12 to 25%).

Additionally, the discovery of a variety of candidate genes responsible for depolymerization and degradation of small lignin molecules will contribute to the knowledge of ligninolytic enzymes machinery and pathways in bacteria that are relatively less studied than fungi. This is important as fungal enzymes have been found to be lacking in terms of feasibility for commercial production due to several challenges. The enzymes are more susceptible to degradation in extreme temperature and pH conditions than their bacterial counterpart. Moreover, bacteria may offer the ease of genetic modification and recombination efficiencies for desired gene expression in comparison to fungi with more complex genomes. The expressed recombinant enzyme CP450 showed that the genetic modification and gene expression were successful. The recombinant enzyme can therefore be subjected for further functional characterization study to reveal the role and understand the lignin degradation pathways (Atiwesh et al., 2022; Yang et al., 2021). Understanding bacterial lignin degradation and the active enzymes are essential for the pursuit of green technology as an alternative to the current physicochemical methods of biomass utilization.

In long terms expectancy, this study may be beneficial to the society and industrial scales as it provides alternative insight and approach to revamp the existing physicochemical pre-treatments. Consequently, this will propel the nascent growth of biomass waste management and utilization, especially in Malaysia and Indonesia. Economically, this study has an attractive market prospect as it is associated to the prominent market demand of biofuels ($23640 million in 2017 and expected to reach $46431 million by 2026) and lignin derived compounds, as illustrated below in Table 1.1 and Figure 1.1.

Table 1-1 Economic prospect of lignin derived compounds

Products	**Market Price ($/kg)**	**Function**	**Demanded quantity/annum**
PHA	1.06	Biomedical application	750,000 tons
Vanilin	15	Food industry	16,000 tons
Lipid	1.22	biodiesel	22.5 billion tons
Muconate	1.7	Adipic acid building block	2.7 million tons

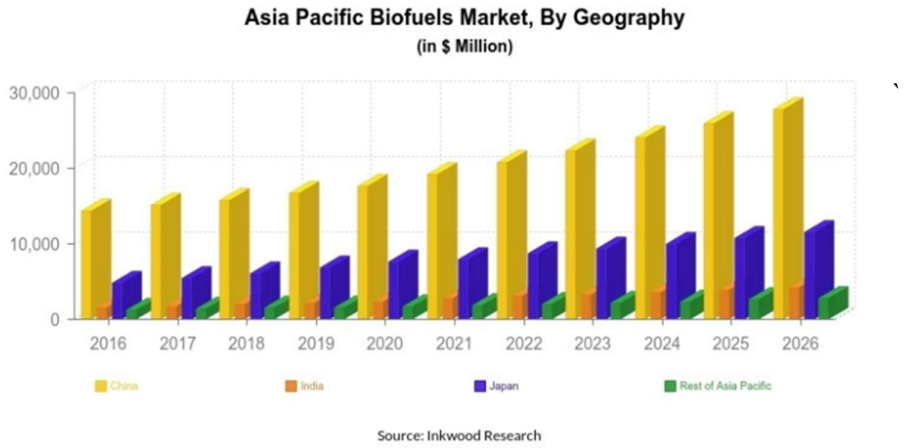

Figure 1.1 Market prospect of biofuels in Asia. Adapted from 2019 Economic Research Institute for ASEAN and East Asia (https://www.eria.org/RPR-FY2013-20.pdf).

In terms of environment, this study advocates a green sustainable reformation through the alternative or integration of biological route with the existing physico-chemical pre-treatments that typically expounds major dismal in environmental aspects.

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