BIOCONVERSION OF LIGNIN BY OXIDATIVE ENZYMES FOR LIGNIN DEPOLYMERIZATION FROM TROPICAL BACTERIA ISOLATES

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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 conversion of lignocellulosic biomass into bioethanol or biochemical products requires a crucial pre-treatment process to break down the recalcitrant lignin structure. Biological pre-treatment using microbial enzymes appears to be the most promising alternative to depolymerize the lignin fragment, which can simultaneously facilitate conversion into valuable chemical products. Thus, this research focuses on bioconversion of Alkali Lignin (AL) for lignin depolymerization, using several enzymes from bacterial isolates. Two bacteria isolates, *Streptomyces sp.* strain S6 and *Bacillus subtilis* strain S11y, were selected as the potential strains for the source of candidate enzymes responsible for lignin depolymerization. Sequencing of the genomic DNA of these strains revealed four successful candidate genes with lignin depolymerizing ability, in which two genes were identified as dye-decolorizing peroxidase (DyP2, ~41 kDa) and multicopper oxidase (CuO1, ~44 kDa) from *Streptomyces sp.* strain S6, and also two genes identified as Cu/Zn superoxide dismutase (SOD2, ~22 kDa) and monofunctional heme catalase (Kat2, ~55 kDa) from *Bacillus subtilis* strain S11y. These genes were successfully expressed as recombinant enzymes and confirmed to have the ability to degrade AL polymer. Differential UV-vis spectrum (Δε-spectrum) of AL treated with the candidate enzymes demonstrated increased absorbance at ~295 nm and 350 nm after treatment, indicating increased free and conjugated phenol structure due to depolymerization. These enzymes also showed activity for oxidation of AL, reducing ~100-240 Da of the high-molecular-weight fraction of AL within 24 h treatment. Analysis of reaction components of all enzymes with AL by ultra-high-pressure liquid chromatography-mass spectrometry and gas chromatography-mass spectrometry showed that the enzymes generated various low-molecular-weight products of diverse groups, such as vanillyl alcohol, vanillin, dihydro-ferulic acid, salicylic acid, benzoic acid, 2,4-dimethyl-benzaldehyde, and oxalic acid. Based on the depolymerization products, the reaction mechanisms performed by each enzyme were also successfully elucidated, which involved several types of reactions, including β−O−4, Cα-Cβ, Cβ-Cγ, Aryl-Cα bond cleavages, O-demethylation, polymerization, decarboxylation, benzylic oxidation, and aromatic ring oxidative cleavage. Each enzyme appeared to generate radicals formed on the lignin surface, leading to several bond cleavages and structural modification in AL after enzymatic treatment, proving their ability to depolymerize polymer lignin. Successful evaluation of lignin depolymerizing enzymes can be applicable for lignin pre-treatment process in green energy production as well as generation of valuable chemicals in bio-refinery.

ABSTRAK

Penukaran biojisim lignoselulosa kepada bioetanol atau produk biokimia memerlukan proses pra-rawatan yang penting untuk menguraikan struktur lignin yang degil. Pra-rawatan biologi menggunakan enzim mikrob menunjukkan pilihan yang paling berpotensi dalam menguraikan serpihan lignin, yang pada masa yang sama boleh menghasilkan produk kimia yang bernilai. Oleh itu, penyelidikan ini tertumpu pada biopenukaran alkali lignin (AL) bagi tujuan penyahpolimeran lignin dengan menggunakan beberapa enzim daripada beberapa bakteria yang telah melalui proses pemencilan. Dua bakteria, *Streptomyces* sp. strain S6 dan *Bacillus subtilis* strain S11y, telah dipilih sebagai strain yang berpotensi untuk mendapatkan sumber enzim yang bertanggunjawab bagi penyahpolimeran lignin. Penjujukan genomik DNA strain ini berjaya mendedahkan empat calon gen yang berkemampuan dalam penyahpolimeran lignin, di mana dua gen dikenal pasti sebagai penyahwarna pewarna peroksidase (DyP2, ~41 kDa) dan oksidase multikuprum (CuO1, ~44 kDa) dari *Streptomyces* sp. strain S6, serta 2 gen yang dikenal pasti sebagai Cu / Zn jenis-superoksida dismutase (SOD2, ~22 kDa) dan katalase monofungsi yang mengandung hem (Kat2, ~55 kDa) dari *Bacillus subtilis* strain S11y. Gen-gen ini berjaya dinyatakan sebagai enzim rekombinan dan diperakui mempunyai kemampuan untuk mendegradasi polimer AL. Bezaan spektrum ultraungu tampak (Δε-spektrum) bagi AL yang dirawat dengan enzim menunjukkan peningkatan keresapan pada ~295 nm dan 350 nm selepas rawatan, menunjukkan peningkatan struktur fenol bebas dan berkonjugat kesan daripada penyahpolimeran. Enzim ini juga menunjukkan aktiviti untuk pengoksidaan AL, dengan pengurangan ~100-240 Da pecahan berat molekul tinggi AL dalam tempoh 24 jam rawatan. Tindak balas komponen bagi setiap jenis enzim dengan AL dianalisis dengan menggunakan kromatografi cecair tekanan ultra-tinggi-spektrometri jisim dan kromatografi gas-spektrometri jisim menunjukkan beberapa produk berat molekul rendah terjana dari pelbagai kumpulan kimia, seperti alkohol *vanillyl*, vanillin, asid dihidro-ferulit, asid salisilik, asid benzoik, 2,4-dimetil-benzaldehid dan asid oksalik. Berdasarkan produk penyahpolimeran, mekanisma tindak balas yang dilakukan oleh setiap enzim berjaya dijelaskan, yang melibatkan beberapa jenis tindak balas, termasuk pemecahan ikatan β−O−4, Cα-Cβ, Cβ-Cγ, Aryl-Cα, pendemetilan-O, polimerisasi, dekarboksilasi, pengoksidaan benzilik dan belahan oksidatif gelang aromatik. Setiap enzim dilihat boleh menjana radikal yang terbentuk pada permukaan lignin, menyebabkan beberapa belahan ikatan dan pengubahsuaian struktur dalam AL selepas rawatan enzim membuktikan keupayaan mereka untuk menyahpolimer lignin polimer. Keberjayaan penilaian enzim penyahpolimeran lignin menunjukkan kebolehgunaan enzim sebagai proses pra-rawatan lignin dalam penghasilan tenaga hijau serta penjanaan bahan kimia bernilai di loji penapis biologi.

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LIST OF ABBREVIATIONS

AL	-	Alkali Lignin
AAO	-	Aryl Alcohol Oxidases
ABTS	-	2,2′-Azino-bis(3-Ethylthiazoline-6- Sulfonate)
APS	-	Ammonium Persulfate
BLAST	-	Basic Local Alignment Search Tool
° C	-	Degrees Celsius
Da	-	Dalton
DDVA	-	2,2' Dihydroxy-3,3′-Dimethoxy-5,5′-Dicarboxybiphenyl
DMSO	-	Dimethyl Sulfoxide
DNA	-	Deoxyribonucleic Acid
dNTPs	-	Deoxynucleotide Triphosphates
DyP	-	Dye-Decolorizing Peroxidases
E. coli	-	Escherichia Coli
EC	-	Enzyme Commission
EDTA	-	Ethylenediaminetetraacetic Acid
ESI	-	Electrospray Ionization
G	-	Guacyl Lignin Subunit
GC/MS	-	Gas Chromatography Mass Spectrometry
GGE	-	Guaiacylglycerol-Beta-Guaiacyl
GPC	-	Gel-Permeation Chromatography
H	-	p-Hydroxyphenyl Lignin Subunit
H_2O_2	-	Hydrogen Peroxide
HMW	-	High Molecular Weight
IPTG	-	Isopropyl-B-D-1-Thiogalactopyranoside
Kat	-	Catalase
KatG	-	Catalase-Peroxidase
L	-	Litre
LAC	-	Laccase
LIP	-	Lignin Peroxidase

LMCO	-	Laccase-Like Multicopper Oxidase
LMW	-	Low Molecular Weight
m/z	-	Mass To Charge Ratio
mL	-	Millilitre
MNP	-	Manganese Peroxidase
MSM	-	Minimal Salt Medium
Mw	-	Average Molecular Weight
NaOH	-	Sodium Hydroxide
NCBI	-	National Center for Biotechnology Information
NGS	-	Next Generation Sequencing
NIST	-	National Institute of Standards and Technology
$O_2^{\cdot -}$	-	Superoxide Anion Radical
OD600	-	Optical Density at 600 nm
OH·	-	Hydroxyl Free Radical
OPEFB	-	Oil Palm Empty Fruit Bunch
ORF	-	Open Reading Frame
PCR	-	Polymerase Chain Reaction
POME	-	Palm Oil Mill Effluent
PSI-BLASTP	-	Position-Specific Iterated Blast
RAST	-	Rapid Annotation Using Subsystem Technology
RB5	-	Reactive Black 5
ROS	-	Reactive Oxygen Species
S	-	Syringyl Lignin Subunit
SDS-PAGE	-	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SOC	-	Super-Optimal-Broth
SOD	-	Superoxide Dismutase
TEMED	-	Tetramethylethylenediamine
UHPLC/MS	-	Ultra- High-Pressure Liquid Chromatography Mass Spectrometry
UV-vis	-	Ultraviolet Visible
VP	-	Versatile Peroxidase

LIST OF SYMBOLS

ε	-	Extinction coefficient
λ	-	Wavelength

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CHAPTER 1

INTRODUCTION

1.1 Research Background

The industrial production of fuels, chemicals, and synthetic polymers depends on non-renewable fossil resources, which are depleting and can pose serious environmental problems, such as waste generation and global warming. Developing sustainable products from renewable natural resources is in demand to minimize this issue. Lignocellulosic biomass is the most abundant renewable organic carbon source on earth. Utilization of this renewable source is expected to be the most assuring alternative feedstock to petroleum-based resources as it is widely available in nature. Besides, lignocellulosic feedstocks are inedible compared to food-based crops and will not compete with food supplies. As a tropical country with enormous biomass resources, Malaysia generated at least 168 million tons of biomass annually, ranging from palm oil, rubber, wood, and rice husks (Agensi Inovasi Malaysia, 2013). The palm oil sector contributes to the most significant biomass generation in the country, with about 85.5 % generation. From the palm oil biomass, only approximately 10% can be converted into crude palm oil, while the remaining 90% are generated as biomass (Ng et al., 2012), which are still seen as low-value by-products. This residual biomass can be re-utilized and converted into downstream value-added products, including but not limited to bioethanol, biobased chemicals, and biopolymers, which can generate revenue for the country.

Despite the abundance and potential of biomass, the main challenge for its commercial application is the technology for converting lignocellulosic biomass into sugars for bioethanol/biochemicals and aromatic-based chemicals. There is still technology uncertainty in the initial pre-treatment process of breaking down the lignin fraction of the biomass structure before converting it into individual sugars that can later be fermented into bioethanol or bio-based chemicals. Lignocellulosic biomass is

the main component of plant structure, which mainly consists of two carbohydrate polymers, cellulose and hemicellulose, and an aromatic polymer, lignin, organized and interlinked together. Cellulose and hemicellulose are chains of polysaccharides that can be easily degraded by microbial enzymes or chemical hydrolysis and are considered the primary candidates for bioethanol and biochemical production. Meanwhile, lignin, the most complex among them, is a three-dimensional heterogeneous cross-linked macromolecule comprising numerous aromatic phenylpropanoid monomeric units identified as guaiacyl (G), p-hydroxyphenyl (H), and syringyl (S) units (Guerriero *et al.*, 2016). In the plant cell wall, lignin acts as a cellular glue between cellulose fibers and is covalently bonded with hemicellulose, thus enhancing its strength and rigidity. In addition, being aromatic and relatively hydrophobic, lignin acts as an antimicrobial and waterproofing agent and provides carbohydrates protection from hydrolysis by microbial enzymes (Hatti-kaul and Ibrahim, 2013; Olajuyigbe *et al.*, 2018). However, due to these stable aromatics and complex structures, lignin is highly resistant to degradation. Thus, the crosslinked networks prevent enzymatic access to cellulose and hemicellulose, which hinders the downstream saccharification and fermentation process for bioethanol conversion.

Several pre-treatment approaches include mechanical, chemical, physicochemical, and biological methods to depolymerize the recalcitrant lignin fraction of lignocellulosic biomass. Biological pre-treatment using microbial enzymes appears to be the most promising alternative, as it offers a more environmental-friendly treatment with lower energy requirements (Olajuyigbe *et al.*, 2018; Xu *et al.*, 2018). Besides, since lignin is the only available natural source for aromatic chemicals production, biological pre-treatment could also offer advantages to support the generation of lignin-derived compounds alongside the saccharification of carbohydrates from cellulose and hemicellulose. Through biological pre-treatment, microbial degradation can be controlled to achieve desired, valuable aromatic and phenolic by-products, such as vanillin, catechol, styrene, as well as polyphenols (Bugg and Rahmanpour, 2015; Fang and L. Smith, 2016).

It is reported that some white-rot and brown-rot fungi have the ability to degrade lignin, and many studies have been reported on the production of lignin-

degrading enzymes from fungi (Salvachúa *et al.*, 2013; Sharma and Aggarwal, 2017). According to Vicuña (1988), lignin degradation occurs in nature mainly the result of two processes. First is the depolymerization of native polymeric lignin to yield low molecular weight aromatic compounds, followed by mineralization of those resultant aromatics. Depolymerization of native lignin is driven by oxidoreductases enzymes, such as lignin peroxidase (LIP), manganese peroxidase (MNP), versatile peroxidase (VP), and laccase (LAC). White- and brown-rot fungi can generate numerous oxidoreductases and are expected to be the main contributors to the lignin depolymerization stage. Meanwhile, bacteria are thought to have a lesser amount of these powerful ligninolytic enzymes. Due to this, bacteria are generally predicted to take a major part in the second stage, which is the mineralization of lignin-derived aromatic compounds. Bacterial enzymes involved in the depolymerization of lignin are still poorly understood until now.

However, most of the studies on fungal ligninolytic enzymes have not turned into a commercial process for lignin degradation, and the lignin degradation problem is still becoming an issue. There are limitations in producing a large-scale growth due to their slow reproduction rate and maintaining the enzyme activities' stability at broad reaction conditions (Taylor, 2013). Besides, fungi also have a relatively complex genetic and protein expression characteristics (Ahmad *et al.*, 2011), making it challenging to perform genetic manipulation.

1.2 Problem Statements

It is proposed that bacterial systems can be suitable for generating lignin-oxidizing enzymes for lignin depolymerization. Although less well-identified and characterized, especially in their depolymerization ability, bacteria are well understood for their biochemical versatility, rapid growth, and good environmental adaptability. Their lignin metabolism can also be further explored (Bandounas *et al.*, 2011; Taylor, 2013). Performing molecular genetics and protein expression from bacteria is also more convenient than fungi, especially on a large scale (Lee *et al.*, 2019). Various gram-positive and gram-negative bacteria have been reported to have the ability to

break down lignin, such as *Sphingomonas paucimobilis* SYK-6), *Pseudomonas putida* mt-2, and *Rhodococcus jostii* RHA1 (Timothy Bugg *et al.*, 2011). However, since bacteria are predicted to involve in the mineralization of lignin-derived aromatic compounds, many of the previously reported bacterial lignin degradation utilized low molecular weight lignin-derived compounds, such as dimers (β-Aryl ether, biphenyl) and monomers as the lignin model (Santos *et al.*, 2014; Min *et al.*, 2015; Zhou *et al.*, 2017; Granja-Travez *et al.*, 2018; Haq *et al.*, 2018). These results will be inaccurate to represent the actual depolymerization ability of the organisms as the native lignin is in polymeric form. Besides, rather than focusing on exploring specific lignin depolymerizing enzymes, more of the previous studies on bacterial lignin degradation pays attention to finding the enzymes involved in the catabolic pathways of the lignin-derived aromatics (Sato *et al.*, 2009; Abdelaziz *et al.*, 2016; Rhee, 2016). Looking closer at these studies, most of the enzymes were only directed to break down the ether linkages. Reports on the actual degradation of polymeric lignin are still limited.

Exploration of lignin depolymerizing enzymes secreted by bacteria that are evaluated directly on polymer lignin substrate is needed to understand the actual mechanism of lignin depolymerization and attain suitable enzymes for this purpose. Numerous studies have attempted to utilize polymeric lignin, such as Kraft lignin, Alkali Lignin (AL), and Organosolv lignin model that can closely mimic the natural lignin to evaluate lignin degradation by bacteria (Y. H. Chen *et al.*, 2012; Kumar *et al.*, 2015; C.-X. Yang *et al.*, 2017; Jackson *et al.*, 2017). However, in all these reports, lignin-depolymerizing enzymes by the bacteria were determined through enzymatic assays using the basis of fungal ligninolytic enzymes (LIP, MNP, and LAC detection). Knowledge about bacterial lignin degradation was based mainly on empirical studies on producing the enzymatic activities of those peroxidase enzymes. Nevertheless, the actual enzymatic system utilized by bacteria responsible for lignin depolymerization is still not fully explored. Some bacterial enzymes were reported to contribute to depolymerization of lignin, such as dye-decolorizing peroxidase (DYP) from *Rhodococcus jostii* RHA1 (Ahmad *et al.*, 2011), superoxide dismutase (SOD) from *Sphingobacterium sp. T2* (Rashid *et al.*, 2015) and multi-copper oxidase from *Ochrobactrum* sp. (Granja-Travez and Bugg, 2018). These enzymes are thought to have broad substrate specificity, allowing the degradation of lignin polymeric

compounds. However, understanding bacterial enzymes for lignin depolymerization is limited to these few strains. Since different bacterial strains would generate diverse sets of lignin-degrading enzymes, further exploration of the responsible enzymes is necessary. Besides, due to their limited studies, it is still uncertain whether or not bacterial enzymes are good candidates for lignin depolymerization, which requires further evaluation.

1.3 Objectives of Study

This study aimed to perform bioconversion of AL polymer using several candidate enzymes for lignin depolymerization from several bacteria isolates. The objectives of the study are as follows:

(a) To obtain the potential bacterial strains and candidate enzymes with lignin depolymerizing ability.

(b) To construct recombinant enzymes for lignin depolymerization using heterologous expression in *Escherichia coli.*

(c) To evaluate the ability of candidate enzymes for the depolymerization of lignin structure using AL as substrate.

1.4 Scopes of Study

This study involves the selection of lignin-degrading bacteria with better performance from the previously reported strains, isolated from palm oil wastes. The bacterial strains include: four mesophilic bacterial strains from the genus of *Agrobacterium* sp. S2, *Streptomyces* sp. S6, *Aureimonas* sp. S26, and *Rhodococcus* sp. S58, that were previously isolated from decayed oil palm empty fruit bunch (OPEFB), and two thermophilic bacteria from the genus of *Stenotrophomonas* sp. S2N and

Bacillus subtilis S11y that were isolated from matured OPEFB and palm oil mill effluent (POME) compost. Initial selection was performed by evaluating the growth of isolated bacteria in a medium containing AL as sole carbon source. AL is used as a polymeric lignin model compound, that can sufficiently mimic the native lignin. Since there is limited study on ligninolytic enzymes from bacteria, the known ligninolytic enzyme assays that were previously detected and reported in fungi and some bacteria, were used to further select the potential strains. The enzymes include, LIP, MNP, DYP, LAC and auxiliary enzyme, AAO. Bacterial strains with better overall growth and enzymatic activities were selected as the potential strains. Draft genome sequences of the potential strains were extracted and analyzed to confirm the strain species and to search for homology sequences that encode for the known ligninolytic enzymes being tested. Since bacteria showed low homology similarities with the reference genes encoding for the known ligninolytic enzymes, the draft genome sequences were also used to evaluate and identify the presence of other candidate genes that are responsible for the lignin depolymerization in bacteria. All the potential gene of interest were TA cloned using pGEM-T Easy vector in *E. coli* JM109 host. Successful recombinant plasmids were heterologously expressed in *E. coli* BL21(DE3) using IPTG induction. Their enzymatic properties on polymeric lignin were study to confirm the catalytic activities of the enzymes for lignin depolymerization. To do this, polymeric AL was treated with the overexpressed whole-cell recombinant enzymes. Direct structural characterization on the lignin was performed to evaluate the enzymatic performance. The cell harboring the blank vector pGEM-T Easy (*E. coli* BL21(DE3)/pGEM) was used as negative control. The control and enzymatic treated lignin samples were then analyzed by ultraviolet–visible spectroscopy (UV-vis). Successfully characterized lignin depolymerizing enzymes were further analyzed to evaluate the extent of depolymerization performed by the enzymes, by using several analytical instruments including gel-permeation chromatography (GPC), ultra- high-pressure liquid chromatography mass spectrometry (UHPLC/MS), and gas chromatography mass spectrometry (GC/MS). Due to limited understandings on bacterial ability to depolymerize lignin and its enzymology, the degradation study by the recombinant-enzymes were performed using whole-cell enzymes rather than free enzymes. The used of whole-cell biocatalysts were also targeted to minimize the time-consumption and material-intensive from enzyme purification process. Besides, due to the recalcitrant structure of lignin structure, the enzymatic treatment was performed on

polymer lignin-mimicking model (Alkali Lignin), and not on dimers or directly on the native lignocellulose medium. In this study, the bacterial lignin-degrading enzymes are only limited to those that are secreted by the isolated bacterial strains. Moreover, biochemical characterizations and enzyme kinetics were not performed as the activity against the test substrates under the specific reaction conditions might have different outcome on the degradation of actual polymeric lignin substrate. Thus, the catalytic activities of the enzymes were directly assessed using lignin as substrates and evaluated the structural changes using analytical instruments (UV-vis spectroscopy, GPC, UHPLC/MS, and GC/MS).

1.5 Significance of study

This research provides the engineering of whole-cell enzymes for green chemistry applications. Uncovering bacterial genes and enzymes in its lignocellulolytic system is important for improvement in the industrial applications. This is because, analyzing the genome sequences of the bacteria strains, could reveal many potential genes with unique properties and contributes to information on lignin depolymerization mechanism by bacteria. In addition, the research on exploring bacterial enzymes for lignin depolymerization will also contribute to additional knowledge about lignin-depolymerizing enzymes and could be applied by industrial enzyme for lignin depolymerization process. A commercial lignin depolymerizing pre-treatment process could also subsequently provide a global solution associated to the underutilized lignocellulosic biomass generation and aid in the development of biomass conversion to value-added product, such as bioethanol and bio-based chemicals production. Maximizing the potential of lignocellulose biomass in biorefinery can subsequently improve profitability of the biorefinery and provide circular economy to the biorefinery.

1.6 Thesis Organization

This thesis consists of Five Chapters with appendices. Chapter One is the background of study, problem statements, and objectives related to the studies, the research scopes, as well as significance of study. Chapter Two discussed about lignocellulose biomass, specifically on lignin, including its structures, its isolation as well as its potential as renewable feedstocks. Several pre-treatment processes for degradation of lignin were covered, mainly on biological pre-treatment in the form of ligninolytic enzymes. Short reviews on microbial enzymes, especially from bacteria responsible for lignin degradation were also written to understand the limitation in the related-reported studies, and to highlight the potential of bacterial enzymes in depolymerizing lignin. Some discussion on the DNA recombinant technology, and protein expression for production of recombinant enzymes for lignin depolymerization were also covered. Some analytical methods to study and characterize the structure of lignin and lignin-derived breakdown products were also included. Chapter Three explained all the materials and methodologies required to complete the entire objectives of the research. Chapter Four presented the experimental results and thorough discussion of the research findings. Chapter Five concludes the overall research studies with some recommendation for future related research.

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LIST OF PUBLICATIONS

Journal with Impact Factor

1. **Riyadi, F.A.,** Tahir, A.A., Yusof, N., Sabri, N.S.A., Noor, M.J.M.M., Akhir, F.N.M., Zakaria, Z. and Hara, H., (2020). Enzymatic and Genetic Characterization of Lignin Depolymerization by *Streptomyces* sp. S6 Isolated from A Tropical Environment. *Scientific Report* 10, 7813 (2020). https://doi.org/10.1038/s41598-020-64817-4 **(Q1, 4.379)**
2. Faisal, U. H., Sabri, N., Yusof, N., Tahir, A. A., Said, N., **Riyadi, F. A**., Akhir, F., Othman, N., & Hara, H. (2021). Draft Genome Sequence of Lignin-Degrading *Agrobacterium* sp. Strain S2, Isolated from a Decaying Oil Palm Empty Fruit Bunch. *Microbiology resource announcements*, *10*(19), e00259-21. https://doi.org/10.1128/MRA.00259-21 **(Q4, 0.88)**
3. Rakhmania, Kamyab, H., Yuzir, M. A., Al-Qaim, F. F., Purba, L., & **Riyadi, F. A.** (2021). Application of Box-Behnken design to mineralization and color removal of palm oil mill effluent by electrocoagulation process. *Environmental science and pollution research international*, 10.1007/s11356-021-16197-z. Advance online publication. https://doi.org/10.1007/s11356-021-16197-z **(Q2, 3.056)**
4. Yusof, N., Abdullah Tahir, A., **Riyadi, F. A**., Ahmad Sabri, N. S., Md Akhir, F. N., Othman, N., Chin, K. L., Tseng, K. Y., Chin, S. T., Zakaria, Z., Lai, C. Y., & Hara, H. (2020). Complete Genome Sequence of Lignin-Degrading *Streptomyces* sp. Strain S6, Isolated from an Oil Palm Plantation in Malaysia. *Microbiology resource announcements*, *9*(5), e01332-19. https://doi.org/10.1128/MRA.01332-19 **(Q4, 0.88)**

Non-indexed Journal

1. Razali, M. S., Hara, H., **Riyadi, F. A**., Akhir, F. N., Othman, N., Ali, M. and Yuzir, M. (2021) 'Isolation and Characterisation of Copper Leaching Microbes from Sanitary Landfill for E-waste Bioleaching', *Journal of Advanced Research in Applied Sciences and Engineering Technology*, Penerbit Akademia Baru (M) Sdn. Bhd, 1(1), pp. 56–66, https://doi.org/10.37934/araset.23.1.5666.

Conference Proceedings

1. **Riyadi, F. A**, Akhir, F. N. M., Othman, N., Zakaria, Z., and Hara, H (2019). "Identification of Lignin- Depolymerizing Enzymes by Bacteria Isolated from Palm Oil Waste." Poster Presented at 2nd Hyper-Interdisciplinary Conference 2020, Kuala Lumpur.
2. **Riyadi, F. A**, Akhir, F. N. M., Othman, N., Zakaria, Z., and Hara, H (2019). "Characterization and Draft Genome Sequence of Lignin Degradation Bacteria from Tropical Environment." Poster Presented at 1st Hyper-Interdisciplinary Conference 2019, Kuala Lumpur.
3. **Riyadi, F. A**, Azman, N. F., Yusof, N., Othman, N., Hara, H (2018). "Ligninolytic Enzyme Activity of Thermophilic Lignin-Degrading Bacteria Isolated from Palm Oil Empty Fruit Bunch." Paper presented at 5th Malaysia-Japan Joint International Conference (MJJIC 2018) (pp. 75 – 76). Kuala Lumpur.