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A REVIEW ON LIGNIN AND BIODELIGNIFICATION

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Abstract

Lignin is a complex phenylpropanoid polymer which present between the cell-wall of plant cells and the second largest biomass after cellulose. This structural component is important in plant as they provide physical strength imparting stiffness to the plant cells that enables the plant to transport water and solutes through the treachery elements in plant vasculature system. In pulp and paper industry, delignification is an important step to produce high quality of fiber for paper making. Nevertheless, hindrance from lignin recalcitrant has make pulping process inefficient in both chemical and mechanical methods. This has resulted in pulping process to use more chemicals, high energy consumption and releasing pollutants to the environments. A greener technology or enzyme-based processing might be one of the alternative to improve pulping process. Researchers have been studied to remove lignin using enzymes produced by isolated microorganism from the gut of wood feeding insects or soil. This paper will provide a review on lignin and previous studies about lignin degradation using enzymes such as lignin peroxidase, manganese peroxidase, versatile peroxidase, dye-decolorizing peroxidase and laccase. The discussion in this paper is focused on issues pertaining the efficiency of using enzymes to degrade lignin which also known as bio-delignification. Also, the challenges in implementing bio-based method for pulping process is mentioned in this paper.

Keywords: wood-feeding insects; lignin; ligninase; production; biodelignification and enzyme activity.

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KAJIAN SEMULA TERHADAP LIGNIN DAN LIGNIN BIO-DEGRADASI

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Abstrak

Lignin adalah polimer fenilpropanoid kompleks yang terdapat di antara dinding sel sel tumbuhan dan merupakan biomas kedua terbesar selepas selulosa. Komponen struktur ini penting dalam tumbuhan kerana ia memberikan kekuatan fizikal yang memberikan kekukuhan kepada sel-sel tumbuhan yang membolehkan tumbuhan untuk mengangkut air dan larutan nutrien melalui dalam sistem vaskular tumbuhan. Dalam industri pulpa dan kertas, penguraian lignin merupakan langkah penting untuk menghasilkan serat berkualiti tinggi untuk membuat kertas. Walau bagaimanapun, halangan dari lignin yang tahan penguraian membuatkan proses penghasilan pulpa tidak efisien dalam kedua-dua kaedah samada kaedah kimia dan kaedah mekanikal. Perkara ini menyumbang kepada penyebab proses penghasilan menggunakan lebih banyak bahan kimia, penggunaan tenaga yang tinggi dan pelepasan bahan pencemar kepada alam sekitar. Teknologi yang lebih hijau atau pemprosesan berasaskan enzim adalah salah satu alternatif untuk memperbaiki proses pulpa. Penyelidik telah mengkaji kaedah untuk penguraian lignin menggunakan enzim yang dihasilkan oleh mikroorganisma yang terpencil dari usus serangga makan atau tanah. Kertas kajian ini memberi penjelasan mengenai lignin dan kajian-kajian terdahulu mengenai penguraian lignin menggunakan enzim seperti lignin peroksidase, peroksidase mangan, peroxidase serba boleh, pewarna warna-warna peroxidase dan *laccase*. Perbincangan dalam kertas kajian ini memfokuskan isu-isu yang berkait dengan kecekapan penggunaan enzim untuk penguraian lignin yang juga dikenali sebagai bio-delignification. Juga, cabaran-cabaran dalam melaksanakan kaedah berasaskan bio untuk proses penghasilan pulpa juga dinyatakan dalam kertas kajian ini.

Kata kunci: Serangga pemakan kayu; lignin; ligninase; biodelignifikasi dan aktiviti enzim.

1.0 INTRODUCTION

Asia, North America and Europe are major consumers of paper and paperboard with an estimation about 360 million tonnes per year (Szabó *et al.,* 2009). The demand for paper and paperboard in these regions are expected to increase about 2.1% per year reaching the year 2020 (Szabó *et al.,* 2009). However, to fulfil such demand there are three major challenges that the paper-pulp industries have to overcome; the economical challenge (Karikallio *et al.,* 2011), the huge energy consumption (Fujimori and Matsuoka, 2011) and the serious environmental issues (Viikari *et al.,* 2009). The pulp-processing procedure releases substantial amount of greenhouse gases emission (GHG) and other pollutants such as chlorinated wastewater from the processing reactors (Szabó *et al.,* 2009). Reports from the Intergovernmental Panel on Climate Change (IPCC) suggests to the papermaking industries to reduce the release of GHG, toxic chemicals and energy consumptions in a way to handle global warming issues (Szabó *et al.,* 2009; Viikari *et al.,* 2009). Therefore, papermaking companies have been pressurised with these environmental issues.

The conventional method of processing the pulp is currently non-economical and harmful to the environment. This is because the current technology is inefficient in removing lignin; delignification for 30 (hardwood) to 35% (softwood) in chemical pulping and 25 to 30% in mechanical pulping (thermomechanical) (Gonzalez *et al.,* 2011, Brännvall, 2017). This is due to its resistancy against degradation which has caused a major problem not only for pulp and paper making but also in biofuel liquid production (Novaes *et al.,* 2010). For example, the pulp yield in softwood kraft pulping for bleachable paper grades is approximately about 47% and this has persisted unchanged despite improvisation on the pulping technology (Brännvall and Bäckström, 2016). Higher lignin content contributes to the yield losses (Brännvall and Bäckström, 2016). The transition of conventional lignin removal process into a modern bioprocessing by integrating with the use of enzymes is believed to improve the ability to fully utilize lignocellulosic biomass to produce high quality fibres and other potential industries (Viikari *et al.,* 2009). Therefore, a sustainable development in the lignin removal process is both economic and environmental importance.

Lignin is a complex phenylpropanoid polymer which present between the cell-wall of plant cells and the second largest biomass after cellulose. This structural component is important in plant as they provide physical strength imparting stiffness to the plant cells that enables the plant to transport water and solutes through the treachery elements in plant vasculature system (Pothiraj *et al.,* 2006). In pulp and paper industry, delignification achievement in pulping process was able to achieve for 30 (hardwood) to 35% (softwood) in chemical pulping and 25 to 30% in mechanical pulping (thermomechanical) (Gonzalez *et al.,* 2011,

Brännvall, 2017). Thus, is a major problem in pulping and paper making industry as the pulp yield is highly depending on the successfulness of lignin removal during pulping process; pulp yield: chemical: (Abdel-Hamid *et al.,* 2013; Bhalla *et al.,* 2013; Bugg *et al.,* 2011; Wang *et al.,* 2013a). While extensive research efforts have been focused on the production of ligninase from fungi, the enzymes activity from fungi is rather low. Unlike fungi, the activity of lignin degradation also carried out by gut microbes from wood feeding insect. In gut microbes, lignin degradation activity is a primary metabolism hence, it is hypothesized that the enzymes activity is faster and more efficient than the fungi. Therefore, there is a need for alternative methods to improve the delignification process using enzymes isolated from wood feeding insects' gut microbiome.

2.0 ABOUT LIGNIN

Lignin is the second abundant aromatic compound in plants that is wrapping around the cellulose and hemicellulose. Lignin is crucial for plants and has important function in providing physical strength, protection and transporting water through vascular system (Martínez *et al.,* 2009; Ververis *et al*., 2004). The composition of lignin varies among plant species (Zucca *et al.*, 2014). Hardwood plants have higher lignin content (>30%) when compared to softwood or herbaceous plants (Li, 2011). Lignin consists of random multiple cross-linked of phenylpropanoids compound that are derived from three major methoxylated monolignol monomers which are p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Eriksson and Bermek, 2009; Ververis *et al.,* 2004). The chemical structure of lignin polymers with monomers linkages was depicted in Figure 1. These monomers are linked together by ether bond (C=O) or carbon to carbon bonding (C=C) to form highly complex polymers (Miguel and Santos, 2014). Lignin polymer is thought to be unusual due to its heterogeneity and lack of a defined primary structure (Lange *et al.,* 2013). As the arrangement of lignin is complex and undefined, it is known to be recalcitrant for degradation against chemical treatment and also by most of organisms (Eriksson and Bermek, 2009).

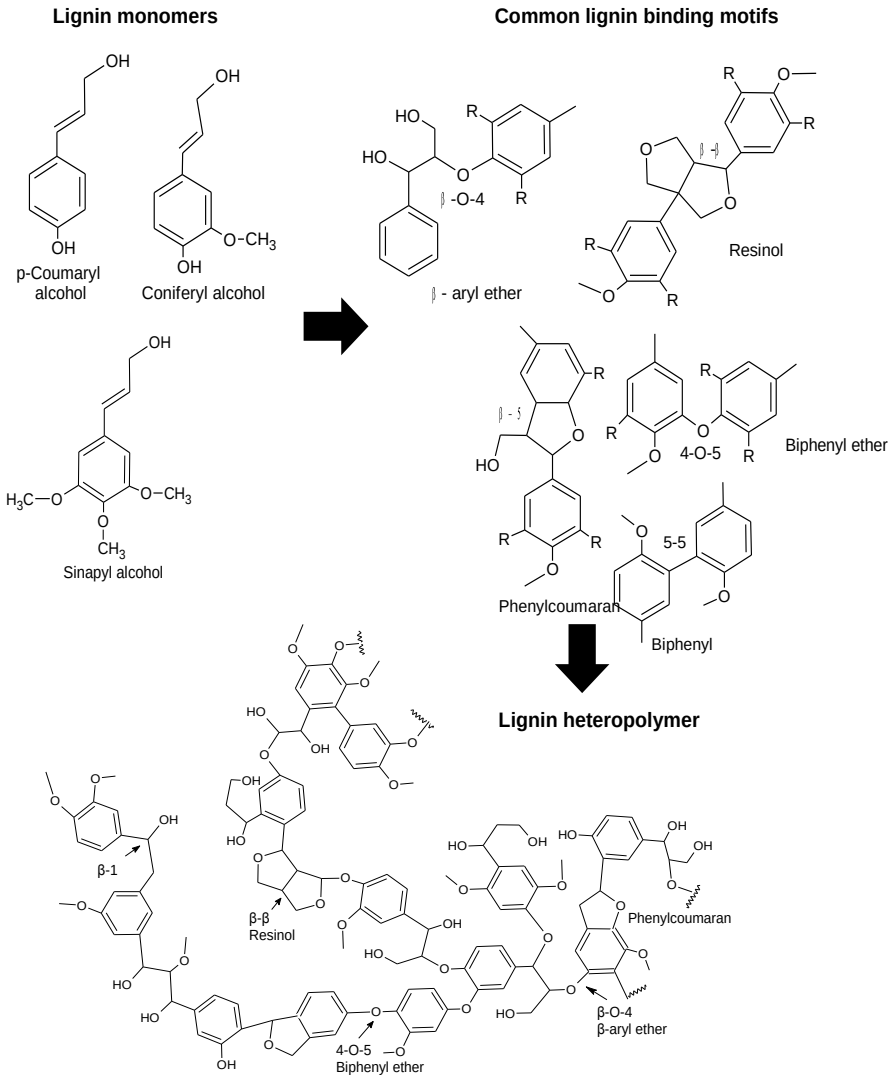

Figure 1: Chemical structure of three common lignin monomers and common lignin binding motifs that built lignin heteropolymers in plant.

2.1 Addressing lignin challenges in pulp and paper-based industry

Removing lignin from lignocellulosic materials is an essential process in pulp and paper based industry. The non-removal lignin in remaining pulp will cause the brownish effect on the paper over the time. Hence, removing most of lignin during pulping process is crucial to ensure a good quality of fibre and high fibre yield is obtained. Current technology used by pulp factory such as chemical and mechanical pulping is inefficient in removing the lignin as this compound is resistant to degradation. For an example, the inefficiency of conventional pulping process using alkaline hydrolysis, where only 66% of lignin is removed via treatment with aqueous ammonia at high temperature (Harmsen and Huijgen, 2010). Another example is through chemical agent in oxidative delignification using hydrogen peroxide to remove the lignin where only 50% of lignin is successfully removed (Harmsen and Huijgen, 2010). In fact, in industrial process, large amount of the capital cost is derived from lignin removal process with an approximate cost between 160 - 200 million dollars per year depending on types of treatment used (Harmsen and Huijgen, 2010) in order to produce high-quality fibre (Novaes *et al.,* 2010).

Table 1: Average energy used by different types of pulping processes. Adapted from Report for U.S Pulp and Paper Industry (2005)

Pulping process		Average Energy (10^{12} Btu/Ton pulp)
Chemical Pulping	Kraft Process	2.68
	Sulfite Process	5.38
Semichemical Pulping	Semichemical	3.86
Mechanical Pulping	Stone Ground Wood	5.11
	Refiner Mechanical Pulping	6.10
	Thermo-Mechanical Pulping	7.09
	Chemi-Thermo-Mechanical Pulping	7.68
	Recycled Paper Pulping	1.30

Chemical pulping process for lignin removal and bleaching process produced several major pollutants that are considered as a serious threat to the environment. These include the release of volatile organic compounds (VOCs) such as terpens, alcohol, acetone, methylethylketones (MEK), and reduced sulphur compound (TRS) as well as organo-chlorine compound (Bahar *et al.*, 2011) as depicted in Figure 2. The emissions of VOCs into the atmosphere are considered as part of hazardous air pollutants (HAPs) and acquaintance of HAPs for long period may cause adverse effect including respiratory problems and cancer on human health hence raising the public concern (Tsai and Wen-Tien, 2016). Furthermore, the

wastewater that was generated from this chemical pulping processes also consist high concentration of chemicals such as sodium sulphide, bisulfites, chlorine dioxide, calcium oxide and hydrochloric acid (Wood Product Industry: Chemical Wood Pulping, 1990). Hence, reducing the usage of chemicals involved during pulping process in early treatment is a vital in handling both environmental and economic issues.

On the other hand, in mechanical pulping, there are two types of treatment used to remove lignin which are milling (chipping or grinding) and ultrasonic pre-treatment (Andreas, 2014; Harmsen and Huijgen, 2010). The purpose of mechanical treatment is to reduce the size of particle that can easily dissolve in particular solvent to separate cellulose content with lignin (Harmsen and Huijgen, 2010). Also, the heat generated during the grinding process can soften the lignin that are binding the fibres and this mechanised forces separate the fibres from the rest part of the wood (Hänninen *et al.*, 2012). However, mechanical processing damaging the fibre which make the fibre less strong (Hänninen *et al.*, 2012) and huge consumption of electricity (Szabó *et al.*, 2009). Therefore, alternative process that can reduce the dependency on mechanical pulping is needed to improve the way fiber is treated.

Table 2: Summary of environmental aspect of pulping processes. Adapted from Report for U.S Pulp and Paper Industry (2005)

Pulping rocess	**Air emission**	**Process effluents**	**Wastes, residuals, or byproducts**
Chemical Pulping - Kraft Process	Noncondensibles (TRS[a], VOC[b]) from blow and vent gases	Digester condensates containing VOC, TRS. Spent liquor and byproduct spills containing BOD[c], COD[d], AOX[e], TSS[f], color[g] Water Flow: >30,000 gallons/ton of pulp BOD: 23 lb/ton pulp TSS: 12 lb/ton pulp	Turpentine, methanol
Chemical Pulping - Sulfite Process	Noncondensibles (VOCb) from blow and vent gases; SO_2	Digester condensates containing VOC, TRS. Spent liquor and byproduct spills containing BOD, TSS	Lignosulfonates, sugars, organic acids for use as binders in brickette and pellet manufacturing and in other applications

Semichemical Pulping	Not available	White water from pulp refining and spent liquor and byproduct spills containing BOD, TSS	No significant wastes, residuals, or byproducts
Mechanical Pulping	No significant air emissions	White water from pulp refining, containing BOD, TSS Water Flow: 5,000-7,000 gallons/ton of pulp	No significant wastes, residuals, or byproducts

a-Total reduced sulfur (TRS) emissions include hydrogen sulfide, methyl mercaptan, dimethyl sulfide, anddimethyl disulfide. **b**-Volatile organic compounds. **c**-Biological oxygen demand (BOD) is the amount of oxygen required by aerobic microorganisms to decompose organic matter in a sample of water. BOD5 measures the oxygen consumed in a 5-day testing period. **d**-Chemical oxygen demand (COD) measures the amount of oxygen required to oxidize organic matter in the sample. **e**-Adsorbable organically bound halogen (AOX) can include chlorinated organic compounds such as dioxins, furans, and chloroform. **f**-Total suspended solids (TSS) is a measure of the solids in water that can be trapped by a filter. **g**-Color is measured in platinum-cobalt (Pt-Co) units.

Sustainable developments in the pulp processing especially the alternatives for lignin removal are both economic and environmental importance. Continuous disapproval and pressure by a number of environmental protection agencies such as EPA (Environmental Protection Agency) to reduce the release of VOCs and chlorine-based pollutants from pulp and paper industries has accelerated the research efforts at global scale to urge for alternatives in replacing chemical pulp bleaching technology with a more environmental friendly, greener technology or enzyme-based processing. Discoveries of lignin degradation enzymes produced by microorganism are one of the ideas to propose as green technology for pulping process. Therefore, transitioning from the conventional pulping processing into a modern bioprocessing by integrating the use of enzymes are considered to improve the ability to fully utilize all components of substrates to produce high quality fibres and will give rise to other potential industries.

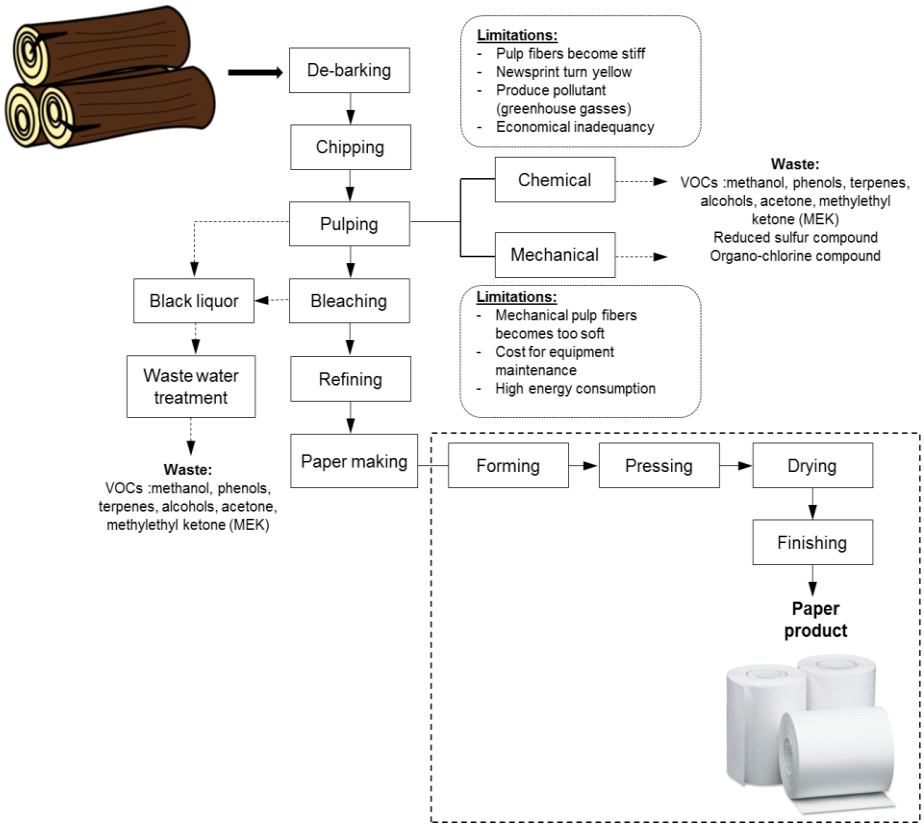

Figure 2: Process flow diagram in pulp and paper industry. The figure also describes waste generated during pulping and bleaching.

2.2 Improving lignin removal via enzymatic process

Enzymatic pulping involved the use of enzymes as pre-treatment to replace or reduced the amount of chemical used during pulping process. An enzyme is a protein that acts as a biological catalyst to carry out specific metabolic chemical reactions in living organisms (Bajpai, 2009). Currently, a lot of studies have been conducted to integrate the use of enzymes as a pre-treatment before subjected to either chemical (bio-chemical) or mechanical (bio-mechanical) pulping (Abdel-Hamid *et al.*, 2013; Bholay *et al.*, 2012; Waung and Chemistry, 2010). In pulping process, the raw material was first treated with enzymes such as laccases or peroxidases to remove lignin and enhanced the efficiency of later pulping process (Abdel-Hamid *et al.*, 2013; Zucca *et al.*, 2013). Incorporating enzymatic pre-treatment has shown to reduce the amount of chemical usage in chemical pulping (Gavrilescu and Chisti, 2005; Miguel and Santos, 2014; Wang *et al.*, 2013a). This is because less chemical is required to degrade lignin, allowing more access to cellulose and hemicellulose (Gavrilescu and Chisti, 2005; Miguel and Santos, 2014; Wang *et al.*, 2013a). Also, the treatment reduces the chemical for bleaching

to remove the brownish effect that is due to presence of lignin (Eriksson and Bermek, 2009; Wang *et al.,* 2013a). Furthermore, biodelignification has mild reaction conditions, higher product yields, few side reactions, less energy demand and less reactor requirements to resist pressure and corrosion (Bajpai, 2009; Miguel and Santos, 2014; Yu *et al.,* 2011). These criteria make the enzymatic pulping favourable compared to chemical and mechanical pulping. Table 3 summarises the comparison of conventional pulping (chemical and mechanical pulping) and alternative pulping using enzyme known as bio-pulping.

Table 3: Summary and comparison of three different types of pulping; i) Mechanical Pulping ii) Chemical Pulping and iii) Enzymatic pulping.

Description	**Mechanical Pulping**	**Chemical Pulping**	**Bio - Pulping**
Process	The raw material (wood) is converted into small chips via grinding. This grinding process generates heat which can soften the lignin binding the fibres and the mechanised forces detached the fibres from the rest part of the wood.	The raw material (wood chips) is cooked with chemicals (acidic or alkaline) under high pressure. Lignin is dissolved and separates the wood into cellulose fibres.	The raw material (wood) is pre-treated with enzyme before undergone chemical or mechanical pulping. Selectively degrade or modify lignin.
Energy Consumptions	1000 KW/tonne of pulp	Self sufficient	Less energy required compare to chemical and mechanical pulping
Fibre Yield	90-94%[a]	40-65% [a]	80 – 90% [b]
Fibre Strength	Varies size of fibre fragments	Long fibre	Long fibre
Paper Strength	Low	High	High
Production Cost	Lower than chemical pulping	Higher than mechanical pulping	Not yet implement in industrial scale
Delignification	Thermomechanical: 25-30%[c]	Kraft pulping: Hardwood – 30%[d] Softwood – 35%[d]	Fungi: 20-45%[e] Bacteria: NA
This table is adapted and modified with additional information from Miguel and Santos (2014).**a**- US Department of Energy, (2005), **b**-Kirk, (1992), **c**-Gonzalez *et al*., (2011), **d**-Brännvall, (2017), **e**- Risdianto *et al*., (2012).			

3.0 CLASSES OF LIGNIN DEGRADATION ENZYMES: PEROXIDASE AND LACCASE

Lignin degrading enzyme (ligninase) is an extracellular enzyme that has been classified into two large groups; peroxidases and laccases (Abdel-Hamid *et al.,* 2013). Both, peroxidases and the laccases use different mechanisms in degrading lignin. Unlike many other enzymes, these enzymes degrade lignin through oxidative (electron transfer) instead of hydrolytic (Wong, 2009). In most of lignin degrading organisms, both enzymes are present during lignocellulosic degradation. Therefore, it is suggested that cocktails of lignin-degradation enzymes like peroxidases and laccase are necessary in enhancing the degradation of lignocellulosic material. These extracellular enzymes peroxidase and laccases are the keys to the delignification of lignin. Figure 3 depicts the three dimensional structure of peroxidases enzymes and laccase isolated and characterized from *P. chryosporium* (fungi), *P. eryngii* (fungi), *R.jostii* RHA1 (bacteria) and *B. subtilis* (bacteria).

3.1 Peroxidases

Peroxidases are an extracellular class II Peroxidases secreted by majority of basidiomycete species. In peroxidases family, there are four types of peroxidases enzymes that are known to have the ability to degrade lignin. This includes lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP) (Abdel-Hamid *et al.,* 2013) and dye-decolorizing peroxidases (DyP) (Brown and Chang, 2014a). These peroxidases are heme-containing enzymes with catalytic cycles that involve the activation by hydrogen peroxidase (H_2O_2) and substrate reduction (Abdel-Hamid *et al.,* 2013; Brown and Chang, 2014a). LiP, MnP and VP are expressed by many basidiomycete species (fungi) and not many bacterial species possess these types of peroxidase. In contrast, DyP are largely expressed by bacteria and small portion of basidiomycete species. All these four types of peroxidases consist three conserved binding domains; i) heme-binding site, ii) Ca^{2+} binding site and iii) H_2O_2 binding site. Although all these four types of enzymes are categorized under the same peroxidases group, but the enzyme mechanisms are different by targeting different part of lignin substrate compound.

Table 4: Summary on the isolated bacteria with lignin degrading ability based on previous studies.

Species	**Applications**	**Enzymes related to lignin degradation**	**References**
K. pneumoniae (GU193983)	Decolorization and lignin removal of paper mill effluent	Laccase	Paliwal *et al.* (2012)
Klebsiella sp. strain BRL6-2	Lignin decomposer in tropical soil	Four putative peroxidases	Woo *et al.* (2014)
Klebsiella aerogene	Biopulping of sugarcane bagasse	MnP	Jha and Patil, (2011)
Klebsiella sp. strain C2A	Degrade high concentrations of tannic acid and polyphenols	Tannase and peroxidase	Pepi *et al.* (2013)
Klebsiella sp. PRW-1	Bioconversion of lignocellulosic biomass	endoglucanase, exoglucanase, β-glucosidase, filter paperase (FPU), xylanase and glucoamylase	Waghmare *et al.* (2014)
Enterobacter lignolyticus SCF1	Lignin degradation for biofuel production	catalase/peroxidase enzymes	DeAngelis *et al.* (2013)
Enterobacter sp.	Bioremediation of pulp and paper mill effluent by	Tannase and LiP	Singh *et al.* (2011)
Enterobacter sakazakii (IITRM16, FJ581031).	decolourisation of synthetic melanoidins from industrial wastewater	MnP	Yadav *et al.* (2011)
Serratia marcescens (GU193982)	Decolorization and delignification of paper mill effluent	Laccase	Paliwal *et al.* (2012)
Serratia marcescens (MTCC 4822)	Biotechnological applications	Laccase	Kaira *et al.* (2015b)
Pseudomonas aeruginosa PKE117	NA	Dyp-type peroxidase (DyPPa)	Li *et al.* (2012)
Pseudomonas putida MET94	biocatalytis for environmental or industrial biotechnology.	Dyp-type peroxidase (PpDyP)	Santos *et al.* (2014)
Pseudomonas sp. Strain YS-1p	Plant biomass conversion technologies.	Laccase, peroxidase and enzymes for aromatic ring-oxidation and ring-cleavage	Prabhakaran, *et al.* (2015)

3.2 Lignin Peroxidase (LiP)

Lignin peroxidase (LiP) was originally discovered and characterized in *Phanerochaete chrysosporium*, white rot fungi by scientists in 1984 with an average protein size 374 amino acid (Brown and Chang, 2014a; Tien and Kirk, 1984). LiP oxidizes aromatic compounds with high redox potentials by single electron abstraction from H_2O_2 and produces the radical cation (Oscar Sánchez, 2011; Wong, 2009). This radical cation then undergoes rearrangements and non-enzymatic degradations which in turn lead to lignin degradation (Figure 4) (Oscar Sánchez, 2011; Wong, 2009). This enzyme is able to degrade both, the phenolic and non-phenolic compounds. LiP works best under condition of low pH optimum (acidic conditions) with the presence of H_2O_2. Uniquely, in the case of non-phenolic substrates LiP does not require the involvement of mediators during oxidation reaction. Thus, LiP is a stronger oxidant than MnP and laccases.

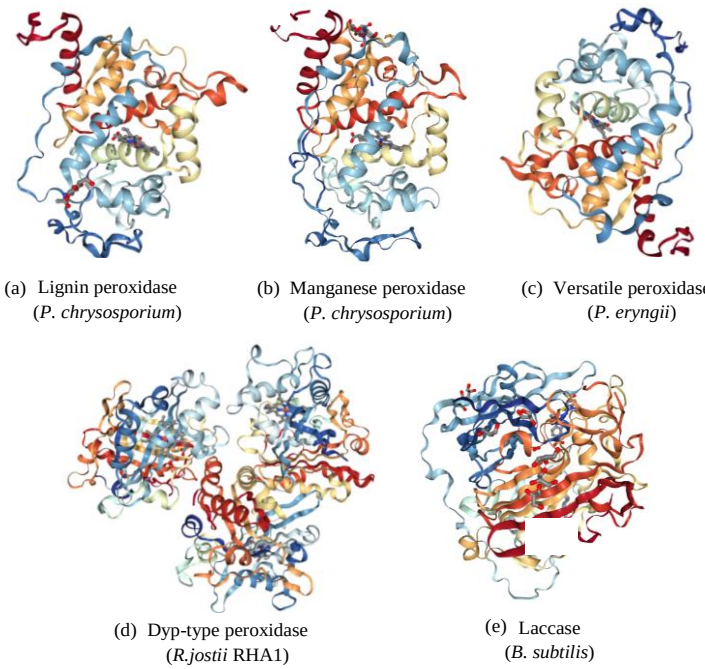

Figure 3: Three-dimensional structure of lignin-degrading enzymes retrieved from protein databank PDB (https://www.rcsb.org/ Retrieved on 16 July (2017)). From the left (a) Lignin peroxidase (LiP) from *P. chrysosporium*; ID: 1LGA (Poulos *et al.*, 1993) (b) Manganese peroxidase (MnP) from *P. chrysosporium*; ID: 1MNP (Sundaramoorthy *et al.*, 1994) (c) Versatile peroxidase from *P. eryngii*; ID: 3FM4 (Ruiz-Dueñas *et al.*, 2007). (d) Dye-decolorizing type peroxidase (Dyp) from *R. jostii*; ID: 3VEC (Singh *et al.*, 2012). (e)Laccase from T. versicolor; PDB ID: 3ZDW (Enguita *et al.*, 2004).

About 77 protein sequences have been recorded in Peroxibase Databank, an online database with collections of peroxidases protein (Peroxibase Databank, 2017). The majority of this LiP is identified from nine white rot fungi of basidiomycete species; *Bjerkandera adusta, Phlebia brevispora, Phlebia radiate, Phlebiopsis gigantean, Phanerochaete carnosa, Phanerochaete chrysosporium, Cerena unicolor* and *Trametes versicolor* (Peroxibase Databank, 2017). Nevertheless, not many LiP proteins from soil bacteria or wood feeding insects have been sequenced and characterized. Hence, the exploration of LiP from bacteria either from soil or wood feeding insects will unveil new knowledge to this field.

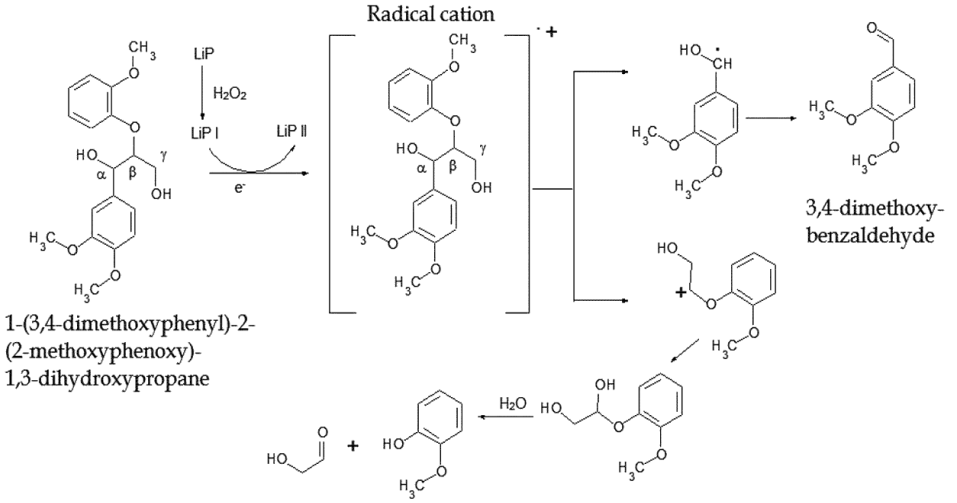

Figure 4: Mechanism of lignin (model compound) degradation by lignin peroxidase (LiP). Adapted from Oscar Sánchez (2011).

3.3 Manganese Peroxidase (MnP)

Lignin degradation by Manganese peroxidase (MnP) is dependent on Mn^{2+} ion. MnP exists in several types of isoforms and the protein sizes ranging between 378 and 382 amino acids, with an average length of 380 amino acids (Morgenstern *et al.*, 2008). Similarly, MnP are able to oxidise both phenolic and non-phenolic compounds like LiP (Wong, 2009). Unlike LiP, there is an addition of interaction sites of Mn^{2+} ion with H_2O_2 which converts Mn^{2+} to Mn^{3+} upon the redox reactions (Wong, 2009). During MnP catalysis, the Mn^{3+} ion oxidizes the phenolic substrates in a second-order reaction result in phenoxy-radical cation (Wong, 2009). These phenoxy-radical cations disrupt the lignin structure (lignin model compound) and led to a series of cascade lignin degradation (Figure 5).

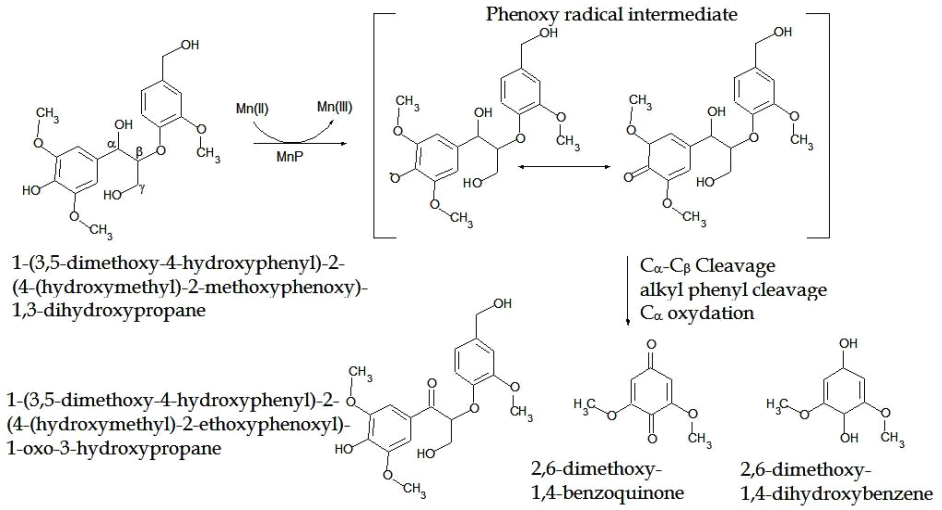

Figure 5: Mechanism of lignin degradation by manganese peroxidase (MnP). Adapted from Oscar Sánchez (2011).

3.4 Versatile Peroxidase (VP)

Versatile peroxidase (VP) was first isolated and discovered from *P. energii*, a white rot fungus. VP represents a unique family of fungal peroxidases that combine both the catalytic properties of LiP and MnP (Morales *et al.,* 2012). Since VP possess both catalytic sites, these enzymes are able to oxidize both phenolic and non-phenolic compounds as well as other aromatic compounds (Martínez, 2002; Morales *et al.,* 2012). Like many other previous peroxidases, the catalytic cycles of VP are similar to the LiP and MnP.

3.5 Dye Decolorizing Peroxidase (DyP-type)

Dye-decolorizing peroxidase (DyP) is largely expressed by bacteria including Gammaproteobacteria species, Actinobacteria species, α-proteobacteria species, β-proteobacteria species and many others (Torres and Ayala, 2010). The main role of DyP is to oxidise and decompose dyes and other aromatic xenobiotics (Brown and Chang, 2014a). DyPs are catogerized into four different clades; DyP-A, DyP-B, DyP-C and DyP-D depending on enzymes reactivity (Abdel-Hamid *et al.*, 2013; Brown and Chang, 2014a; Fernández-Fueyo *et al.*, 2014). It is known that DyP from clade A and B are less active and small compared to DyP-D (Brown and Chang, 2014a). Whereas, DyP-C is largely known to came from prokaryotic origin (Brown and Chang, 2014a).

3.6 Laccases (Lac)

Laccase is a glycosylated multicopper enzyme that is widely distributed in nature and can be found in many organisms including *basidiomycete* and *ascomycete* (fungi), bacteria and insects (Table 5). Unlike peroxidases family, Lac utilises oxygen instead of the H_2O_2 as a substrate mediator (Christopher *et al.*, 2014). Due to the large size of laccase and the high complexity of lignin structure, in most cases Lac do not degrade lignin directly, instead laccase needs the presence of intermediate substrates or chemical mediators which is oxidized radicals (Christopher *et al.*, 2014). The oxidized radicals are able to induce the degradation of lignin complex substrates (model lignin compound) via three different mechanisms; cleavage of C=C bond, alkyl aryl production and carbon alpha oxidation depicted as in Figure 6. The cellular localization of Lac depends on the organism, with plant and fungal laccase is excreted as extracellular enzymes, while an intracellular localization is observed in most of the bacterial laccase.

Table 5: Total number of laccases enzymes that have been identified and characterized in fungi and insects. These records are according to the laccase and multicopper oxidase engineering database (https://lcced.biocatnet.de). Retrieved on 17 July, (2017).

Super family	**No. of Laccase identified**	**Structure characterized**
A. Basidiomycete Fungi (7 Homologous Family)	733 proteins	29
B. Ascomycete Fungi (10 Homologous Family)	570 proteins	12
C. Insect Laccase (8 Homologous Family)	258 proteins	None
D. Bacteria MCO	1400 proteins	1
E. Bacteria CueO	1374 proteins	47
F. Bacteria CopA	1269 proteins	None

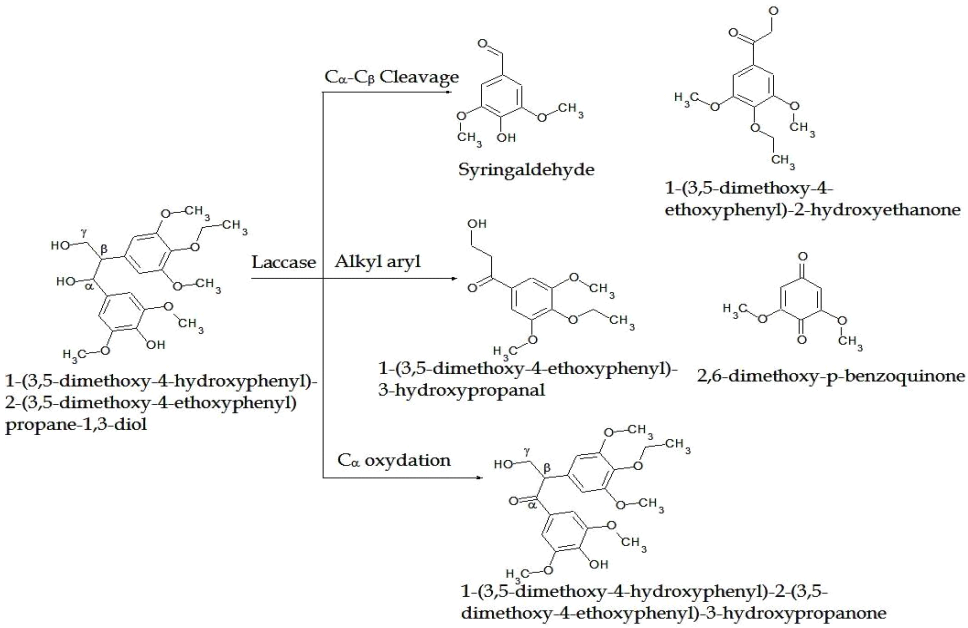

Figure 6: Mechanism of lignin degradation by laccase. Adapted from Oscar Sánchez (2011).

4.0 A STUDY ON POTENTIAL LIGNIN-DEPOLYMERISING ENZYMES BY BACTERIA

A study by Yadav and Chandra, (2015) on syntrophic (symbiotic relationship where one species lives off the products of another species) co-culture of *B. subtilis* and *K. pneumonia* demonstrated the ability of *K. pneumoniae* to degrade kraft lignin from the discharged of pulp industry. Confirmation with GC-MS and HPLC analysis showed almost 60% of kraft lignin was degraded and decolourisation of black liquor were observed. It was detected that there was high laccase activity presence during the treatment process (Yadav and Chandra, 2015). Furthermore, a genomic analysis on *Klebsiella sp.* strain BRL6-2 revealed that its ability to degrade lignin was likely due to the possession of genes related lignin to degradation (Woo *et al.*, 2014). There were six putative peroxidase genes, two putative lactate dehydrogenase genes and two putative catalase genes were discovered in omics analysis likely to express peroxidase and laccase enzymes (Woo *et al.*, 2014). Another interesting characteristic of *Klebsiella sp.* (strain C2A) was the ability to not only degrade polyphenols (lignin) but also tannic acid within 35 hours. During the degradation process, *Klebsiella sp*. strain C2A was producing gallic acid as part of the by product (Pepi *et al.*, 2013). The capability of *Klebsiella sp.* strain C2A to adapt to the toxicity of tannic acid characterized in this study could be used in bioremediation processes of wastes from the industrial activity (Pepi *et al.*, 2013). *Klebsiella sp.* PRW-1 demonstrated utilisation of pure

cellulosic substrates such as avicel and carboxymethylcellulose (CMC). Not only that, *Klebsiella sp.* PRW-1 has versatility to use different agricultural wastes like sugarcane bagasse, sugarcane barbojo, sorghum husks, grass powder, corn straw and paddy straw by producing a large amount of lignin depolymerising enzymes (laccase and peroxidase), endoglucanase, exoglucanase, β-glucosidase, xylanase and glucoamylase (Waghmare, *et al.,* 2014).

The facultative anaerobe *E. lignolyticus* SCF1 was isolated from forest soils in Puerto Rico growth on lignin as sole carbon source. The forest soils decompose litter by soil bacteria using oxygen-independent enzymes such peroxidase and multi-copper oxidase (laccase) (DeAngelis *et al.,* 2013). *E. oryzaes* isolated from soil was used for bioremediation of lignin from discharged of pulp and paper mill effluents by Singh *et al.* (2011). It had been recorded that there was a reduction in lignin by 73% and decolourisation of black colour in liquor by 82% (Singh *et al.,* 2011). *E. sakazakii* (IITRM16, FJ581031) showed a maximum decolourisation of industrial wastewater containing high concentration of melanoidins up to 60 % (Yadav *et al.,* 2011). It is known that *E. sakazakii* expressed potential MnP enzymes that are responsible for the decolourisation activity (Yadav *et al.,* 2011).

A psychrotolerant strain of *Serratia marcescens* (MTCC 4822) could produces laccase at wide range of temperature between 4 to 45 °C with an optimal temperature at 25 °C and pH between 3 to 14 with an optimal pH of 5 (Kaira *et al.,* 2015b). Meanwhile, a study on the lignin degradation of paper mill effluent by *Serratia mercescens* (GU193982) was described by Paliwal *et al.* (2012). Co-cultured of three strains *Serratia marcescens* (GU193982), *Klebsiella pneumoniae* (GU193983) and *Citrobacter sp.* (HQ873619) have showed a maximum reduction in lignin (53%) and decolourisation (67%) in 192 hours (8 days) of incubation (Paliwal *et al.,* 2012). The extracellular LiP enzymes and laccase both show activities and were responsible in breaking down lignin and discharging low molecular weight phenolic products (Paliwal *et al.,* 2012).

A new DyP-type peroxidase, DyP-type Peroxidase A (DyPPa) from *P. aeruginosa* PKE117 was identified and characterized to exhibit high decolorizing activity. DyPPa has range of substrate specificity and a novel peroxidase from DyP type family (Li *et al.,* 2012). Another peroxidase from DyP-type family produced by *P. putida* MET94 also showed to have high redox potential for degradation of aromatic compounds such as synthetic dyes, phenolic and nonphenolic lignin (Santos *et al.,* 2014). The similarity between peroxidase produced by *P. aeruginosa* PKE117 and *P. putida* MET94 is the optimal pH of both enzymes which falls into acidic condition (pH 4 to pH 5) and optimal temperature between 20 and 30 °C (Li *et al*., 2012; Santos *et al*., 2014). Other *Pseudomonas sp.* such as *Pseudomonas sp*. strain YS-1p is capable of degrading lignin and lignin-like compounds as the sole source of carbon (Prabhakaran *et al.,* 2015). Genomic analysis on *Pseudomonas sp.* strain YS-1p revealed there are

existence of genetic codes for enzymes that involve in lignin degradation pathways such as laccase, peroxidase, β-etherase, vanillate-O-demethylase, carboxyl esterase and chloroperoxidase (Prabhakaran *et al.*, 2015). Also, genes encode for aromatic ring degradation including phenol-2-monooxygenase, 4-hydroxybenzoate-3-monooxygenase, catechol 2,3-dioxygenase and protocatechuate 3,4-dioxygenase were discovered (Prabhakaran *et al.*, 2015). Table 4 summarises the isolated bacteria with lignin degrading ability and its application based on previous researches.

4.1 Production of ligninase by microorganisms

Ligninase enzymes are categorised into two large groups known as peroxidase and laccase. Both peroxidase and laccase are extracellular enzymes that are related to lignin degradation (Nandal *et al.*, 2013; Rai *et al.*, 2016). The biggest advantage of using enzymes as biocatalysts is that it causes no impairment on the ground water reservoirs and hazard of air and soil pollution (Singh *et al.* 2007; Virk *et al*. 2012). Also, the potential of enzymatic treatments prior to their subsequent bleaching with chlorine, chlorine dioxide and hydrogen peroxide would substantially reduce in the usage of bleaching chemicals (Viikari *et al.*1986). Hence, the use of microbe as lignin removal agent could be the green alternative to the current conventional pulping process.

4.2 Organisms with Lignin Degrading Capacity

Naturally, lignin is degraded by ligninase produced by compost organism such as white rot fungi and soil bacteria (Janusz *et al.*, 2013). These organisms have the ability to modify and degrade lignin by expressing different types of lignin degrading enzymes as seen in Table 6 (Eriksson and Bermek, 2009). Although, both fungi and bacteria have the ability to degrade lignin, the reactivity shown in both organisms are different (Brown and Chang, 2014a). For fungi, lignin degradation is known to be in secondary metabolic process as lignin is not essential for fungi survival (Kirk, 1988; Martínez, 2002; Paliwall *et al.*, 2012). However, in bacteria lignin degradation is known as primary metabolic process and hence are thought to be more robust in term of its expression and activity (Brown and Chang, 2014; Li *et al.*, 2012). Lignin degradation has allowed the bacteria to get access to cellulose and breakdown the cellulose into glucose as the carbon source to be utilised for survival activities (Li *et al.*, 2012). Compare to fungi, the degradation of lignin by bacteria is incomplete leaving behind aromatic chemical compounds (Brown and Chang, 2014a). Large lignin polymers are degraded and decomposed by bacteria into smaller counter part of aromatics which can be imported into cell for further aromatic catabolism (Brown and Chang, 2014a). Whereas for fungi, complete lignin degradation is carried out by cleaving C-C aromatic bonds that linked between the two lignin monomers

(Janusz *et al.*, 2013b). These findings suggested that these two organisms utilised different biochemical pathways in lignin modification and degradation.

Apart from fungi and soil bacteria, it is known that the wood-feeding insects such as termites and longhorn Asian beetle are also having the ability to degrade lignin (Geib *et al.*, 2008; Scully *et al.*, 2013). It is likely that, the majority of wood-feeding insects have the capacity to degrade lignin as they consume lignin-rich lignocellulose materials as part of their dietary. Researches in termites showed that lignin degrading enzymes were highly expressed inside the midgut and the salivary by the simbiont microorganisms (Geib *et al.*, 2008; Nakashima *et al.*, 2002; Ni and Tokuda, 2013; Scharf *et al.*, 2011). Extensive study on meta-genomics and meta-transcriptomic level of termite revealed the potential of termites in degrading wood material by expressing enzymes that degrade phenol aromatic compound (Scully *et al.*, 2013; Tartar *et al.*, 2009). Hence, there are possibilities that there is other organism than fungi that could degrade lignin with more appealing processing features.

Table 6: List of organisms that have the ability to degrade lignin and their related enzymes. Three groups of organisms that are known to involve in lignin degradation; i) fungi ii) soil bacteria and iii) bacterial insects' gut.

Organism	Enzyme degrading lignin	References
Fungi		
Phanerochaete chrysosporium	LiP, MnP and Lac	Kirk *et al.*(1986); Ntwampe *et al.* (2010)
Ganoderma lucidum	LiP, MnP and Lac	Hariharan and Nambisan, (2013a)
Trametes versicolor	LiP, MnP and Lac	Iqbal *et al.* (2011)
Lentinula edodes	MnP	Silva *et al.* (2008)
Pleurotus ostreatus	VP	Moreira *et al.* (2007)
Pleurotus eryngii	VP	Moreira *et al.* (2007)
Pleurotus pulmonarius	VP	Moreira *et al.* (2007)
Bjerkandera adusta	VP	Moreira *et al.* (2007)
Ceriporiopsis subvermispora	MnP	Tello *et al.* (2000)
Phanerochaete sordida	MnP	Hirai *et al.* (2013)
Fusarium Solani	LiP, MnP and Lac	Obruca *et al.* (2012)
Trichophyton rubrum	MnP	Bermek *et al.* (2004)
Soil Bacteria		
Rhodoccocus jostii	DyPs	Ahmad *et al.* (2011)
Aneurinibacillus aneurinilyticus	DyP	Raj *et al.* (2006)
Insects' Gut Bacteria		

Coptotermes Formosanus (Termite)	MnP, DyP and Lac Phenol acid esterases	Li *et al.* (2012)
Coptotermus curvignathus (Termites)	Ligninase	Bakar *et al.* (2013)
Reticulitermes flavipes (Termite)	Phenol-oxidizing Laccase (LacA)	Scharf *et al.* (2011a)
Anoplophora glabripennis (Asian longhorned beetle)	Lac, DyPs and β-estherases	Scully *et al.* (2013)

4.3 Mechanism of lignin degradation by ligninase: Fungi versus bacteria

Generally, there are two mechanisms utilised by microorganism to degrade lignin polymer. One of the mechanisms is through non-selective oxidation of lignin inter-monomer side-chain linkages and another is demethylation of linkage that connected lignin with hemicellulose (Knežević *et al.*, 2013). These enzymes are H_2O_2-heme dependant peroxidases which requires the H_2O_2 reaction to oxidize the co-factor; Mn (II) in the case of manganese peroxidase and convert this co–factor into a Mn (III) reactive ion species (Lange *et al.*, 2013). These reactive species are diffusible and able to penetrate in between the structure of lignin polymer thus attacking the methyl and methylene groups that are close to the phenolic group. The result of this phenomenon generates radical phenolic cation that further cascading lignin degradation reaction (Lange *et al.*, 2013). Extensive research efforts have been focused on the production of ligninase enzymes from fungi such as *P. chrysosporium* (Leisola *et al.*, 1985), *P. sordida* (Sugiura *et al.*, 2009), *C. subvermispora* (Babič *et al.*, 2012) and *T. versicolor* (Iqbal *et al.*, 2011) for industrial applications. However, enzymes activity from fungi is rather low as lignin modification is a secondary metabolism in this organism by which the enzyme will only be expressed under particular favourable conditions to the organism.

Other than fungi, symbiotic microorganisms within the gut of wood feeding insects also have the ability to modify lignin. Insects such as termites able to efficiently modify lignin at ambient temperature and pressure within the termite gut system (Ni and Tokuda, 2013; Scharf *et al.*, 2011a). A study done by Ni and Tokuda (2013) observed lignin removal in the midgut of termite digestive tract. The process for lignin removal occurs in the midgut of termites involved three forms of reactions; demethylation, hydroxylation and propyl side-chain modification (Brown and Chang, 2014a; Ni and Tokuda, 2013). Delignification of lignin in bacteria is a very complex mechanism involving series of steps including cleavage inter-monomeric linkages, demethylation, hydroxylation, side chain modifications, aromatic ring fission and dissimilation aliphatic metabolite (Paliwal *et al.*, 2012). Since the degradation of lignin is such a complex event, the complete removal of this compound in industrial scale is almost impossible. Unlike fungi, the activity of modifying lignin by this consortium is conducted as a

primary metabolism hence, it is hypothesized that the enzymes activity are faster and more efficient in removing lignin from cellulose and hemicellulose contents.

Both fungi and bacteria can be used as host to harvest invaluable enzymes. Nonetheless, the process using fungi proved to be difficult to regulate due to its sensitivity to the shear forces presence in the bioreactor and the agitation during the fermentation inhibit ligninase production and activity (Asgher *et al.*, 2006). Furthermore, researches on ligninase are not only useful in degrading lignin but are also useful for degrading phenolic pollutants, bioremediation of soil, industrial waters (Brown and Chang, 2014a) and biofuel production (Lu and Ralph, 2010). Until today, the biodelignification using ligninase enzymes in fermentation is not yet ready to be commercialised due to these limitations. More studies have focused on finding the alternatives for effective lignin removal techniques. Utilising lignin-degrading bacteria would be the best alternatives to replace the conventional method.

4.4 Limitation of ligninase production by fungi

Natural lignin degrader such as fungi is well studied for its ability to produce both enzymes (Knežević *et al.*, 2013). However, fungi are difficult to regulate due to its sensitivity to the shear forces that are presence in the bioreactor and the agitation during the process which inhibit ligninase production and activity (Asgher *et al.*, 2006). Also, the enzyme activity in fungi is low and requires long reaction time to achieve a maximum enzyme activity (Janusz *et al.*, 2013). For example, study in the white rot fungi for ligninase production by Górska *et al.* (2014) have taken 30 days of incubation to achieve maximal ligninase production. Other fungal species such as *Schyzophyllum commune* and *Ganoderma lucidum* studied by Asgher *et al.* (2013) had obtained optimal crude ligninolytic enzymes using agro substrate in SSF only after 10 days of culture. Time constrain is one of the challenges to apply enzymatic treatment in pulping process at large scale, therefore less practical for industrial scale purposes.

4.5 Advantage of ligninase production by bacteria

On the other hand, bacteria from the soil and the wood feeding insects are seen as one of the highly sought potential to identify new novel ligninase enzymes with high enzymatic activity (Brown and Chang, 2014a). These organisms act as decomposer in soil (Burns *et al.*, 2013) and symbiont within the gut of the wood-feeding insects (Krishnan *et al.*, 2014). Lignin-depolymerising bacteria have the ability to modify and solubilise lignin by expressing different types of ligninase (Eriksson and Bermek, 2009). The uniqueness of the red palm weevil (*R. ferrugineus)* larva showed to be suitable candidates for the discovery of novel potential lignin-depolymerising species. *R. ferrugineus* which is known as a major pest in many palm species in Malaysia has this ability as they consume massive

plant tissues (Vatanparast *et al.*, 2014; Yin *et al.*, 2013). Lignin degradation has allowed bacteria to access to cellulose and breakdown the cellulose into glucose to be utilised for survival activities (Li *et al.*, 2012). Due to this essential activity, lignin degradation is known as primary metabolic process in bacteria (Brown and Chang, 2014a) hence, higher activity with better production which is suitable to the industrial activity. Therefore, a study on production of ligninase from bacteria will provide convenient way to utilise ligninase for industrial scale applications.

5.0 CONCLUSION

Lignin recalcitrant against degradation is a major challenge in industrial processing including pulp and paper-based, and biofuel production. Using biological process to remove lignin through the exploitation of lignin degrading microbes propose an ideal process. Biological process offers an efficient and an environmentally friendly processing. This is due to their ability to catalyse specific biochemical reactions that enable an efficient process to be achieved as well as the generation of chemical compound that have significant commodities value. Bacterial gut from wood feeding insect is seen as one of the potential to identify new strains with the ability to produce enzymes related to lignin biodegradation (ligninase) and high enzymatic activity. However, understanding the mechanism of lignin degradation by microbes and utilising microbes in pulping process were still vague.

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