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COOPERATION BETWEEN LACCASE AND GLUCOSE OXIDASE IN THE OXIDATION OF LIGNIN MODEL COMPOUND

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COOPERATION BETWEEN LACCASE AND GLUCOSE OXIDASE IN THE OXIDATION OF LIGNIN MODEL COMPOUND

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Thesis submitted in partial fulfilment of the requirements
for the award of the degree of
Bachelor of Chemical Engineering

Faculty of Chemical & Natural Resources Engineering
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Dedicated to my Mother.

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ABSTRACT

Lignin is the second most abundant natural polymer. It has a significant and largely unrealized potential as a source for the sustainable production of fuels high-value chemicals. The oxidative functions of lignin, is a very promising way to go, since it holds the possibilities to yield highly functionalised products that can serve as starting materials for other processes in the chemical and pharmaceutical industries. It can replace fossil-based oil as a renewable feedstock that would bring about socio-economic and environmental benefits to our current economy. Its use and targeted functionalisation within biomass refinery processes, however, still needs to be further explored and developed. Gaining a profound knowledge about the structure of lignin, being able to analyse the most suitable enzymes, and understanding the mechanisms that guide the reactions leading to the oxidative depolymerisation of lignin samples from different renewable sources are key requirements for developing successful lignin product. Therefore, in this study lignin model compound (LMC) will be used as a preliminary study to be able to understand the interaction between the enzymes and lignin. First, the control experiment was conducted by oxidizing 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) in the presence of laccase from *trametes versicolor* (LTV). This enzyme activity was then compared with the oxidation of ABTS in the presence of LTV and glucose oxidase (GOX). The results obtained from the experiment showed the activity of the enzyme was higher when laccase was in cooperated with glucose oxidase. Finally, the oxidation of ferulic acid as a lignin model compound by the mixture of laccase and glucose oxidase (LTV-GOX) in a range of different concentration was employed. It was expected that aromatic compounds will be produced as a result of the oxidation. Therefore Vanillin was chosen to be quantified. However, none of the samples indicated the presence of vanillin due to certain factors.

ABSTRAK

Lignin adalah polimer semula jadi yang paling banyak di kedua. Ia mempunyai potensi yang besar dan sebahagian besarnya tidak nyata sebagai sumber untuk kemapanan pengeluaran bahan api bahan kimia bernilai tinggi. Fungsi oksidatif lignin, adalah cara yang sangat cerah untuk pergi, kerana ia memegang kemungkinan untuk menghasilkan produk yang sangat functionalised yang boleh dijadikan sebagai bahan permulaan untuk proses lain dalam industri kimia dan farmaseutikal. Ia boleh menggantikan minyak berasaskan fosil sebagai bahan mentah yang boleh diperbaharui yang akan membawa faedah sosio-ekonomi dan alam sekitar kepada ekonomi semasa kami. Penggunaannya dan functionalisation disasarkan dalam proses penapisan biomass, bagaimanapun, masih perlu terus diterokai dan dibangunkan. Mendapat ilmu pengetahuan yang mendalam mengenai struktur lignin, dapat menganalisis enzim paling sesuai, dan memahami mekanisme yang membimbing tindak balas yang membawa kepada penyahpolimeran oksidatif sampel lignin daripada sumber yang boleh diperbaharui yang berbeza adalah keperluan utama untuk membangunkan produk lignin berjaya. Oleh itu, dalam kajian model lignin ini kompaun (LMC) akan digunakan sebagai kajian awal untuk dapat memahami interaksi antara enzim dan lignin. Pertama, eksperimen kawalan dijalankan dengan mengoksidakan 3-ethylbenzothiazoline-6-sulphonic asid (ABTS) di hadapan laccase dari trametes versicolor (LTV). aktiviti enzim ini ketika itu berbanding dengan pengoksidaan ABTS di hadapan LTV dan oxidase glukosa (Gox). Keputusan yang diperolehi dari eksperimen menunjukkan aktiviti enzim adalah lebih tinggi apabila laccase bekerjasama dengan oxidase glukosa. Akhir sekali, pengoksidaan asid ferulic sebagai sebatian model lignin oleh campuran laccase dan glukosa oksidase (LTV-Gox) dalam pelbagai kepekatan yang berbeza telah digunakan. Ia dijangka bahawa sebatian aromatik akan dihasilkan sebagai hasil daripada pengoksidaan. Oleh itu vanillin telah dipilih untuk diukur. Walau bagaimanapun, tiada sampel menunjukkan kehadiran vanillin disebabkan faktor-faktor tertentu.

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LIST OF ABBREVIATION

LTV	Laccase
GOX	Glucose Oxidase
HPLC	High Performance Liquid Chromatography
UV-VIS	Ultraviolet-Visible Spectrophotometer
ABTS	3-ethylbenzothiazoline-6-sulphonic acid
LMC	Lignin Model Compound
LiP	Lignin peroxidase
MnP	Manganese peroxidase

CHAPTER 1

INTRODUCTION

1.1 Background of the study

The development and exploitation of renewable, non-fossil based resources has become increasingly important, since the use of fossil-based resources is no longer legitimate for practical, ecological and socio-economic reasons (Werpy and Peterson, 2004). Many technologies have been developed and successfully implemented in order to end fossil-dependent energy production and many of this technologies are based on the use of biomass (Holladay et al., 2007). Today, biorefinery processes are aiming at the degradation of lignocellulosic part of the biomass, which consist of cellulose, lignin, and hemicellulose (Argyropoulos, 2007).

Lignin, that is currently mostly obtained as waste in paper and biofuel productions is the second most abundant renewable polymer (Sakakibari, 1980). Lignin forms aromatic parts of wood composites and acts like adhesive in plant cell walls holding cellulose and hemicellulose together (Zakeski et al., 2010). Lignin is an interesting part of biomass and it is considered a biodegradable renewable source of energy and chemicals. Lignin's ability to be source of aromatic compound has the greatest potential to become an essential substance in the future, because in some ways it can be substituted for fossil fuels as source of these chemicals (Hodasova et al., 2015).

Therefore to further understand it, lignin model compounds were used not only to understand the cooperation between different type of enzymes in oxidizing lignin samples but to also gain a better understanding on the structure of lignin. Therefore in this study laccase from *trametes versicolor* (LTV) and glucose oxidase (GOX) will be used to oxidize ABTS to understand the enzyme activity and in ferulic acid; a lignin model compound with variation of LTV-GOX concentration ratio to observe the oxidative properties.

1.2 Motivation

With the increasing prices and exhaustion of source of fossil fuels, the society has started to focus on alternative source of energy and chemicals. British Petroleum recently released its Statistical Review of World Energy 2015 (BP Statistical Review, 2015) and found that consumption of fossil fuels in 2014 continued to increase (Rosillo-Calle et al., 2007). Global fuel consumption in 2014 represented 10228.3 million tonnes which is divided into oil, natural gas, coal, nuclear energy, hydroelectricity and renewables (Rosillo-Calle, et al. 2007).

World Fossil Fuel reserves-to-production ratios at end 2014 estimated on 52.53 years for oil, 55 years for natural gas and 110 years for coal (Blazej and Kosik, 1993). As known petroleum is the main source for aromatic chemicals, however the number is decreasing by time. Therefore, new alternatives using lignin is constantly be studied and experimented.

Lignin is an interesting part of biomass and it is considered a biodegradable renewable source of energy and chemicals (Gosselink, 2010). Lignin's ability to be source of aromatic compound has the greatest potential to become an essential substance in the future, because in some ways it can be substituted for fossil fuels as source of these chemicals (Smolarski, 2012). However since the structure of lignin is complex, an attempt was made to use LMC as a preliminary study to understand the interaction between LMC and LTV-GOX.

1.3 Problem Statement

To date, lignin represents the main waste stream from modern saccharification processes. As such its enhancement is necessary in order to develop sustainable biorefinery processes (Lange et al., 2013). Lignin constitutes a rich renewable source of aromatic compounds. Its complex structure offers unique routes to produce fine and bulk chemicals either by adjustment of already developed petroleum processes or by new technologies.

The variable and unpredictable structure of lignin requires improvement of systems for controlling and customizing its multi-usefulness, in particular procedures of oxidation and functionalization (Lange et al., 2013). Therefore, lignin model compound (LMC) was preferred to understand small parts of lignin that may contribute a better understanding in the lignin structure.

There are many ways to degrade lignin model compounds but the most common ways are biological and chemical. Chemical degradation uses oxidizing agents such as nitrobenzene, oxygen, and hydrogen peroxide. These chemical degradation of lignin is not a favorable option as most of the oxidizing agent are too strong and tends to over-oxidize the lignin during long reaction times (Nanayakkara et al., 2014). Besides, large portion of the product are made up of mixture of toxic derivatives, not only is it difficult to separate from the useful product, it also releases an unpleasant odor (Nanayakkara et al., 2014)

Biological methods on the other hand are divided into two ways, bacterial degradation and enzyme degradation. Using enzyme is more favorable as bacteria is a slow growing process and takes a long time to study its interactions (Brown and Chang, 2014) Enzyme degradation compared to the other methods is the most economical and favorable method as the enzymes are more selective and there are many types of enzymes. In this experiment laccase and glucose oxidase are chosen to study the oxidation of lignin model compound.

1. 4 Objectives

- To study the effect of laccase/glucose oxidase concentration in the oxidation of ferulic acid as a lignin model compound.

1.5 Scopes of study

- To compare the activity of laccase and laccase/glucose oxidase mixture in the oxidation of ABTS.

- The activity of LTV and LTV-GOX mixture in the oxidation of ABTS will be determined by UV-Vis Spectrophotometer.

- The effect of concentration of LTV-GOX will be varied (1:20, 1:0 and 0:20) for the oxidation of ferulic acid and the product from the reaction will be identified using High Performance Liquid Chromatography (HPLC).

CHAPTER 2

LITERATURE REVIEW

2.1 Lignocellulose

Lignocellulose refers to plant dry matter (biomass), also known as lignocellulosic biomass. The term lignocellulose structure is related to the part of the plant which forms the cellular wall, composed of fibrous structures, basically constituted of polysaccharides [cellulose (40-60%) and hemicellulose (20-40%)]. These components are associated to a structure containing aromatic substances, lignin (15-25%) (Sun and Cheng, 2002). Saka and Goring, 1985 discovered that there is a large difference in the chemical composition and the lignin composition among various woods. A schematic representation of the lignocellulose complex is shown in Figure 1. It is the most abundantly available raw material on the earth for the production of bio-fuels, mainly bio-ethanol. Lignocellulosic biomass, in the form of wood fuel, has a long history as a source of energy. Since the middle of the 20th century, the interest of biomass as a precursor to liquid fuels has increased (Carol et al., 2009). To be specific, the fermentation of lignocellulosic biomass to ethanol is an attractive route to fuels that supplements the fossil fuels. Biomass is a carbon-neutral source of energy, since it comes from plants, the combustion of lignocellulosic ethanol produces no net carbon dioxide into the earth's atmosphere (Galbe and Zacchi, 2007). Aside from ethanol, many other lignocellulose-derived fuels are of potential interest. Lignocellulosic feedstocks include residues from agriculture and forestry, energy crops, and residues from biorefineries and pulp mills (Ragauskas et al., 2006).

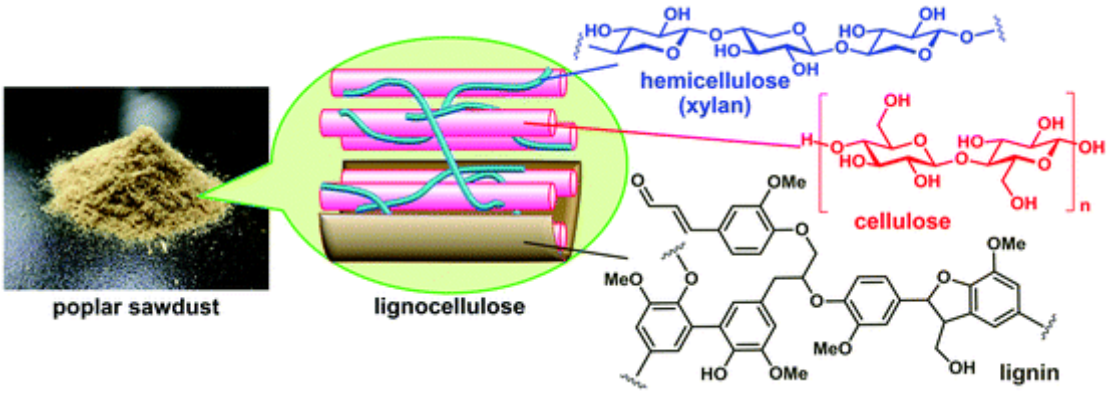

Figure 1: Scheme of the Lignocellulose Structure.

Source: Hirokazu and Atsushi, 2013

2.2 Cellulose

Cellulose is an organic compound with the formula $(C_6H_{10}O_5)_n$, a polysaccharide consisting of a linear chain of several hundred to many thousands of β1 until β4 linked D-glucose units (Crawford, 1981). Figure 2 shows the molecular structure of Cellulose. Cellulose is an important structural component of the primary cell wall of green plants, many forms of algae and the oomycetes. Some species of bacteria secrete it to form biofilms (Tony, 2018). Cellulose is the most abundant organic polymer on Earth (Klemm et at., 2005) The cellulose content of cotton fiber is 90%, that of wood is 40–50% and that of dried hemp is approximately 57%. (Piotrowski et al., 2011).

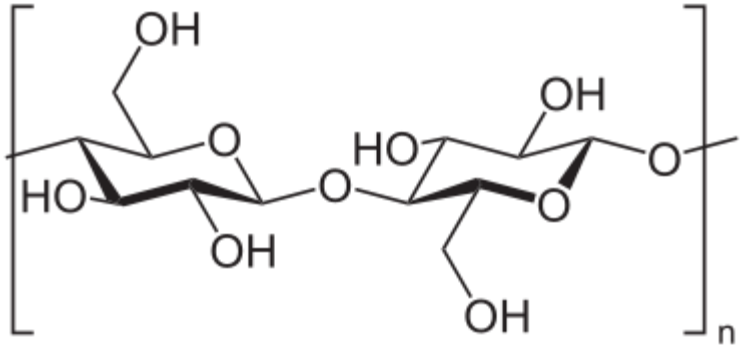

Figure 2: Structure of Cellulose

Source: (Nishiyama et al., 2002)

Cellulose is mainly used to produce paperboard and paper. Smaller quantities are converted into a wide variety of derivative products such as cellophane and rayon. Conversion of cellulose from energy crops into biofuels such as cellulosic ethanol is under research as an alternative fuel source. Cellulose for industrial use is mainly obtained from wood pulp and cotton (Klemm et at., 2005).

2.3 Hemicellulose

A hemicellulose (also known as polyose) is any of several heteropolymers (matrix polysaccharides), such as arabinoxylans, present along with cellulose in almost all plant cell walls (Scheller and Ulvskov, 2010).While cellulose is crystalline, strong, and resistant to hydrolysis, hemicellulose has a random, amorphous structure with little strength. It is easily hydrolyzed by dilute acid or base as well as myriad hemicellulase enzymes. Unlike cellulose, hemicellulose consists of shorter chains – 500–3,000 sugar units as opposed to 7,000–15,000 glucose molecules per polymer seen in cellulose (Gibson, 2013). In addition, hemicellulose is a branched polymer, while cellulose is unbranched. Structural representation of cellulose is shown in Figure 3.

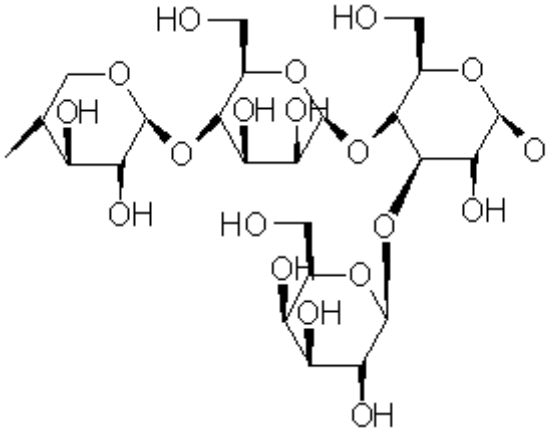

Figure 3: Structure of Hemicellulose

Source: (Scheller and Ulvskov, 2010)

2.4 Lignin

Lignin is a class of complex organic polymers that form important structural materials in the support tissues of vascular plants and some algae (Marton et al., 2009). Lignins are particularly important in the formation of cell walls, especially in wood and bark, because they lend rigidity and do not rot easily. Chemically, lignins are cross-linked phenolic polymers (Lebo et al., 2001). The composition of lignin varies from species to species. An example of composition from an aspen sample is 63.4% carbon, 5.9% hydrogen, 0.7% ash, and 30% oxygen (by difference) (Hsiang-Hui King et at., 1983) corresponding approximately to the formula $(C_{31}H_{34}O_{11})_n$. An example of the possible lignin structure is shown in Figure 4.

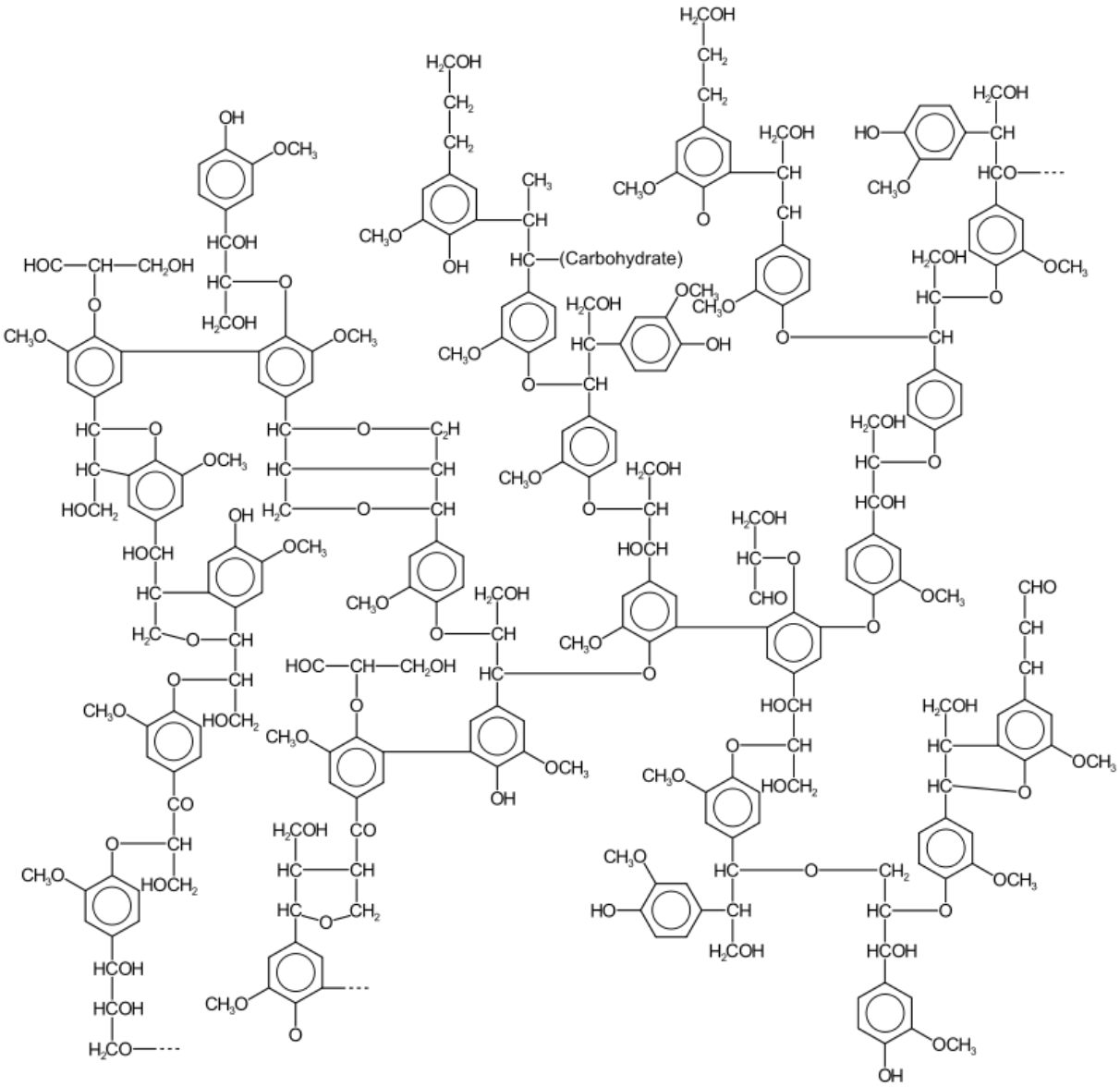

Figure 4: An example of a possible lignin structure

Source: (Hsiang-Hui King et at., 1983)

As a biopolymer, lignin is unusual because of its heterogeneity and lack of a defined primary structure. Its most common function is the support through strengthening of wood (xylem cells) in trees (Wardrop, 1969). Lignin was first mentioned in 1819 by a Swiss botanist, AP. DE Canidolle, who described it as fibrous, tasteless material, insoluble in water and alcohol. Lignin constitutes 20-35% of dry mass wood (Boerjan et al., 2003). It is commonly known that lignin is a possible source of low molar mass compound. As fossil source of these compounds are running out and also their price is rising, focus of science is on the renewable source of chemicals (Laurichesse and Averous, 2014). In order to develop successful products from lignin, we need a profound understanding on the mechanism that guide the reaction that leads to oxidative degradation of lignin. Therefore, lignin

samples are constantly being used to further understand the oxidation properties of lignin. These lignin samples mainly refers to Lignin Model Compund (LMC) which can be found in various types for example used in this experiment is ferulic acid. Due to the complex cross-linked three-dimensional network structure, lignin is highly rigid and difficult to degradation (Ruiz-Dueñas and Martínez, 2009).

The industry started first using lignin in the 1880's, when lignosulfonates were used in leather tanning and dye baths. From that time onwards, lignin has found applications in food products, serving as emulsifiers in animal feed and as raw material in the production of vanillin, as component in the pharmaceutics product formulation and also as fragrance in the perfume industry. The derivative product applications of lignin will expand literally, creating impacts in a lot of industrial segments (Van Dam et al., 2006). Although hundreds of applications for lignin can be pointed out, its main use in the pulp and paper industry is as bio-fuel to replace fossil fuels in heat or power generation, and the lignin-depleted black liquor can be reused in the cooking operation. (Van Dam et al., 2006). Figure 5 shows the products from lignin.

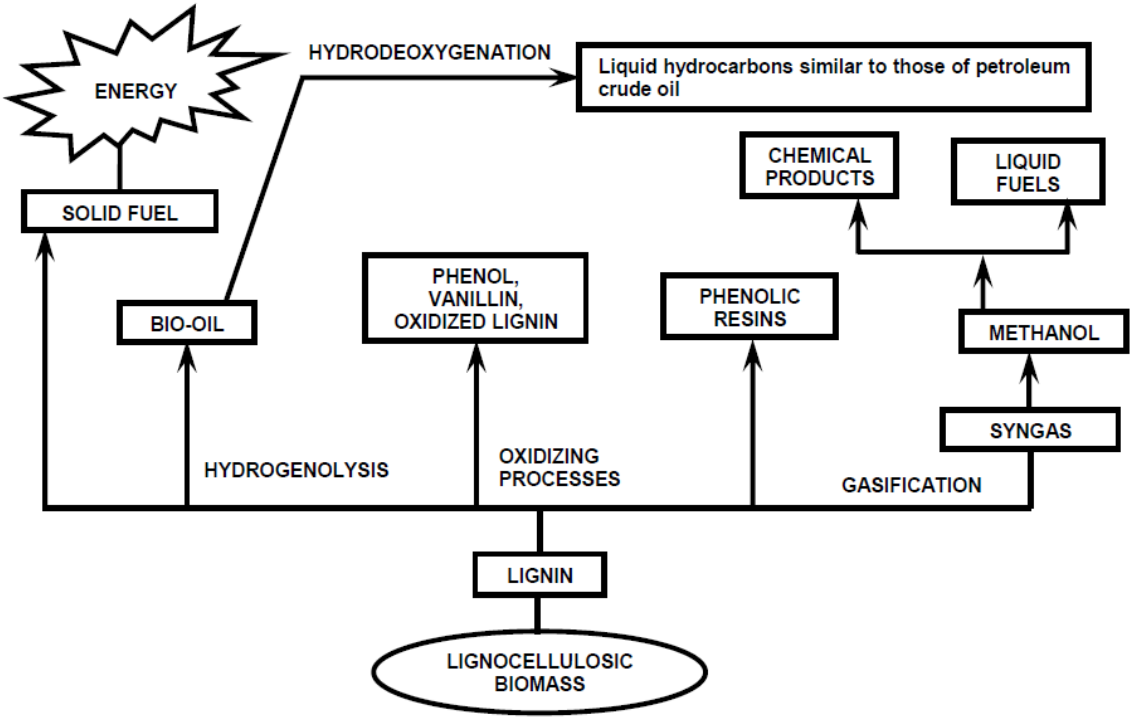

Figure 5: Lignin Products

Source: (Nei Pereira et al., 2008)

2.5 Lignin Model Compound

The lignin model compound used for this experiment is ferulic acid, Ferulic acid is a hydroxycinnamic acid, a type of organic compound. It is an abundant phenolic phytochemical found in plant cell wall components such as arabinoxylans as covalent side chains. It is related to trans-cinnamic acid (Saulnier et al., 1999). As a component of lignin, ferulic acid is a precursor in the manufacture of other aromatic compounds. Molecular structure of ferulic acid is shown in Figure 6.

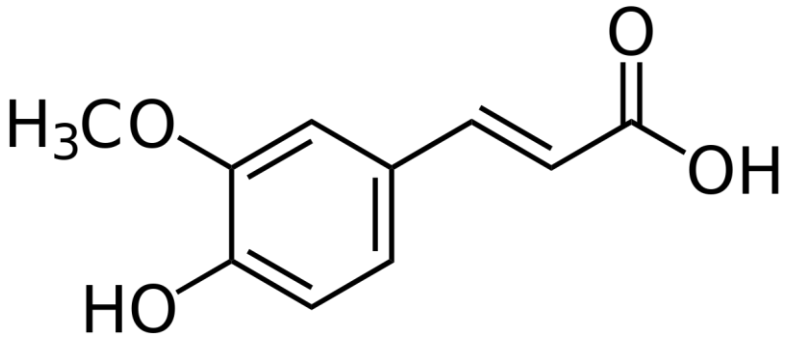

Figure 6: Structural representation of Ferulic Acid

Source: (Saulnier et al., 1999).

Ferulic acid, being highly abundant, may be useful as a precursor in the manufacturing of vanillin, a synthetic flavoring agent often used in place of natural vanilla extract (Kawsar et al., 2008). However, biotechnological processes may be the most efficient method to use ferulic acid as a precursor, and as such, research is still ongoing (Anson et al., 2009). There are a few possible products from the oxidation of ferulic acid, as shown in Figure 7, The product that will be quantified is Vanilin.

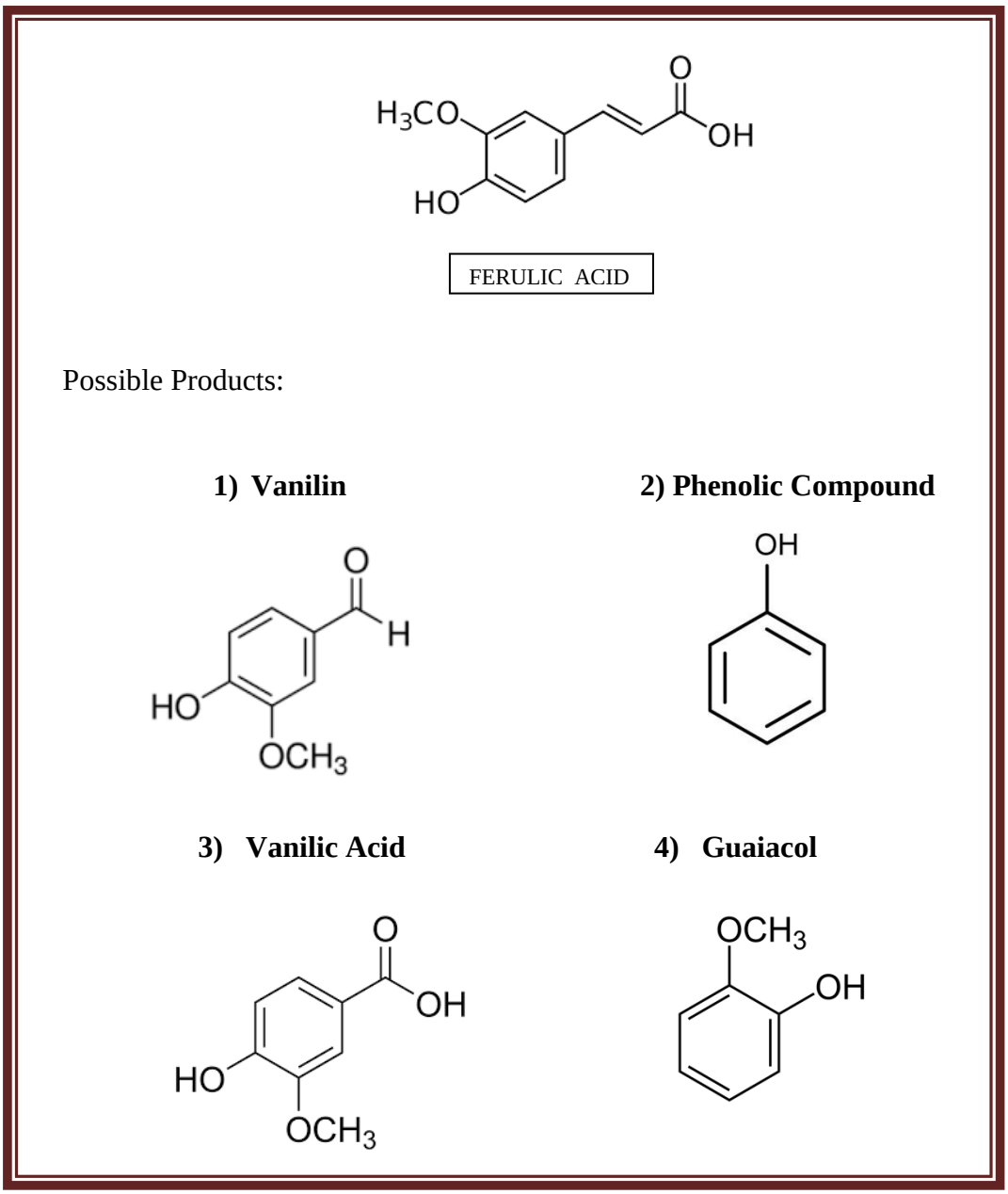

Figure 7: Possible products from the oxidation of Ferulic acid.

2.6 Chemical Oxidation

There are two alternative paths for the breakdown of the lignin polymer, chemical and biological. Chemical degradation of lignin model compound simply means using oxidizing agents such as nitrobenzene, oxygen with or without the presence of copper sulphate pentahydrate as catalyst, and hydrogen peroxide. Various chemical oxidations of lignin have been investigated over many years.

The use of strong oxidizing agents has shown the breakup of the aromatic ring whereas milder oxidizing agents caused changes in the side-chain whilst keeping the ring intact. Oxidation reactions were initially used to identify the structure of lignin polymers and the mode of linkage between the precursors (Morohoshi and Glasser, 1979). Nitrobenzene was one of the earliest chemical oxidants used and it produced reasonable quantities of syringaldehyde and vanillin depending on the source of lignin. These reaction products were extracted with acid and identified using GC-MS and LC-MS techniques (Moodley et al., 2011). These chemical degradation of lignin is not a favourable option as most of the oxidizing agent are too strong and tends to over-oxidize the lignin during long reaction times (Nanayakkara et al., 2014). Besides, large portion of the product are made up of mixture of toxic derivatives, not only is it difficult to separate from the useful product, it also releases an unpleasant odor (Nanayakkara et al., 2014) Besides that, the chemicals used are not stable in high pressure and temperatures (Montane and Farriol, 2014).

2.7 Biological Oxidation

Biological method on the other hand are divided into two ways, bacterial degradation and enzyme degradation. Using enzyme is more favorable as bacteria is a slow growing process and takes a long time to study its interactions (Brown and Chang, 2014). Enzyme degradation simply means using suitable enzymes and mediator to oxidize LMC. Enzyme degradation compared to the other methods is the most economical and favorable method as the enzymes are more selective and there are many types of enzymes. Enzymes used to oxidize lignin are called ligninolytic enzyme, such as laccase, lignin peroxidase (LiP) and and manganese peroxidase (MnP). In nature, different organisms degrade lignin; however, the most effective ones are the fungi belonging to the white rot fungi class (Wan and Li Y, 2011). These microorganisms possess an enzymatic system to effectively degrade lignin. This system contains three principal enzymes laccase, manganese peroxidase (MnP), and lignin peroxidase (LiP) (Nigam et al., 2009). These microorganisms have been used to remove lignin from different substrates such paper, animal feed, and biofuels (Lu C et al., 2010).In biofuel production, the ligninolytic enzymes have two principal purposes, delignification and detoxification. Delignification methods apply

ligninolytic enzymes to reduce the lignin content in several feedstocks. Detoxification utilizes the ligninolytic enzymes to reduce the toxic compounds present in the biomass hydrolysates after chemical pretreatments (Lu C et al., 2010). In this experiment laccase and glucose oxidase are chosen to study the oxidation of lignin model compound.

2.8 Ligninolytic Enzymes

Ligninolytic enzymes are enzymes used to oxidize lignin, Those enzymes are laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP).

2.8.1 Laccase

Laccases is also known as benzenediol. Yoshida, at the end of the nineteenth century, was the first to extract laccases from exudates of the Japanese tree Rhus vernicifera (Sergio R, 2006). Laccases are widely distributed in microorganisms, insects, and plants, showing a specific function in each of them.. From this group, white rot fungi are the most studied laccases. These fungi use their enzymes to break the plant's lignocellulosic wall and obtain the host's nutrients. The structural shape of laccase is shown in Figure 8.

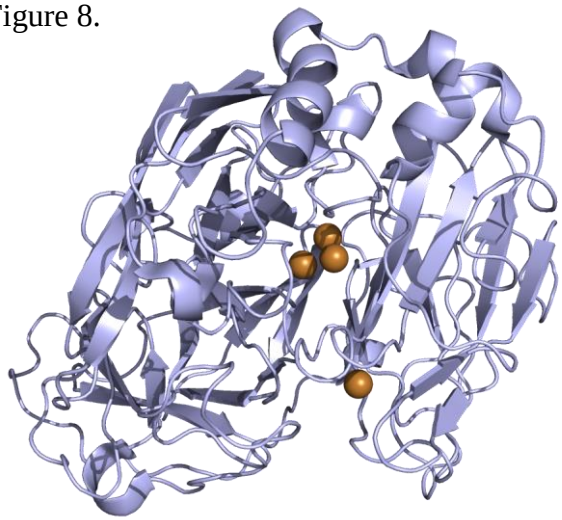

Figure 8: Structural Representation of Laccase

Source: (Sergio R, 2006)

Besides their catalytic characteristics, these enzymes are attractive for biotechnological process, because they are extracellular and inducible, do not need a cofactor, and have low specificity (Couto and, Toca-Herrera, 2006). Laccase production is increased by the addition of molecules as copper, dyes, or other recalcitrant compounds (Minussi et al., 2007). Laccase employs oxygen as an oxidizing agent and cofactor instead of other expensive cofactors such as pantothenic acid, thiamine, or biotin (Matera et al., 2008). Laccases have low substrate specificity, this characteristic allows the degradation of several compounds with a phenolic structure (Zouari-Mechichi et al., 2006). Therefore, laccases have been employed in several areas such as bioremediation of aromatic recalcitrant compounds, treatment of effluents polluted with lignin, chemical synthesis, degradation of a wide number of textile dyes, and biomass pretreatment for biofuel production (Zouari-Mechichi et al., 2006). The broad characteristic of laccases and their ability to utilize atmospheric oxygen as electron donor instead of other expensive cofactor used by peroxidases makes these enzymes a promising candidate for diverse industrial applications (Couto and Herrera, 2007; Medhavi and Lele, 2009). For this experiment, laccase from *Trametes Versicolor* will be used as shown is Figure 9.

Figure 9: *Trametes Versicolor*; Source of Laccase

2.8.2 Lignin Peroxidase (LiP)

LiPs were originally discovered in nitrogen- and carbon-limited cultures of Phanerochaete chrysosporium (Dias et al., 2007). LiP possess high redox potential (700 to 1,400 mV), low optimum pH 3 to 4.5, and the ability to catalyze the degradation of a wide number of aromatic structures such veratryl alcohol (3,4-dimethoxybenzyl) and methoxybenzenes (Piontek et al., 1993). Figure 10 shows the structural representation of LiP. LiP oxidizes aromatic rings moderately activated by electron donating substitutes (Khindaria et al., 1996).

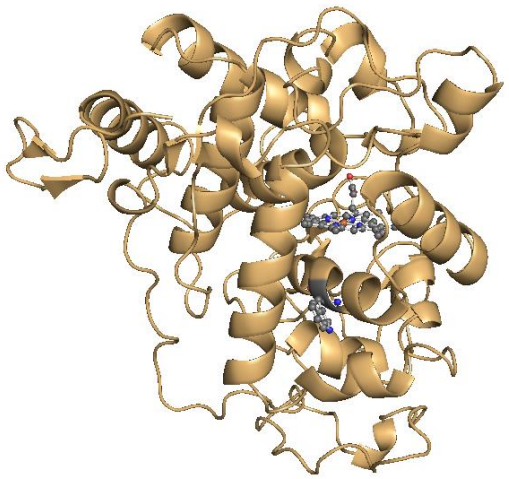

Figure 10: Structure of LiP

Source: (Piontek et al., 1993)

2.8.3 Manganese Peroxidase (MnP)

Kuwahara in 1984 found the first MnP in batch cultures of Phanerochaete chrysosporium (Dias et al., 2007). They are glycoproteins with a molecular weight between 38 and 62.5 kDa (Martin H, 2002). MnP structure has two domains with the heminic group in the middle, ten major helixes, a minor helix, and five disulfide bridges. One of those bridges participates in the manganese (Mn) bonding site. The structure of MnP is shown in Figure 11. This site is a characteristic that distinguishes MnP from other peroxidases (Sundaramoorthy et al., 1994)

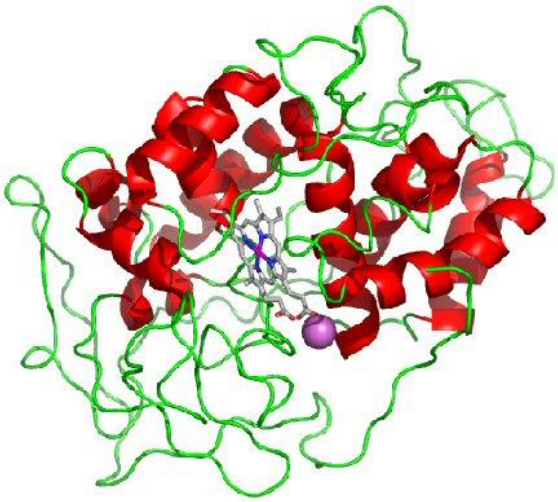

Figure 11: Structure of MnP

Source: (Sundaramoorthy et al., 1994)

CHAPTER 3

METHODOLOGY

3.1 Materials

3-ethylbenzothiazoline-6-sulphonic acid (ABTS), sodium acetate, lacasse from *trametes versicolor* (LTV), glucose oxidase (GOX), ferulic acid.

3.2 Enzyme Activity

3.2.1 Laccase

The device used in determining the activity of Laccase is Ultraviolet-Visible Spectrophotometer (UV-VIS Spectrophotometer). Laccase is prepared by diluting 0.008 g of lacasse from *trametes versicolor* into 100 ml of distilled/ purified water. (Bourbonnais and R.Paice, 1990). Detailed calculations of laccase solution preparation are shown in Appendix.

3.2.1.1 Buffer solution

Acetate buffers are prepared by mixing equimolar solutions of acidic and basic forms of acetate in order to achieve the desired pH. The acidic form is acetic acid (HOAc, or HAc), and the basic form is the sodium salt.

Sodium acetate buffer, 0.1 M

Solution A: 14.8 ml glacial acetic acid/liter (0.2 M)
Solution B: 35.3 ml sodium acetate ($NaC_2H_3O_2$ $3H_2O$)/liter (0.2 M)

Referring to the table below for desired pH, the indicated volumes of solutions A and B will be mixed, then diluted with H_2O to 100 ml

Preparation of 0.1 M Sodium and
Potassium Acetate Buffers

Desired pH	Solution A (ml)	Solution B (ml)
3.6	46.3	3.7
3.8	44.0	6.0
4.0	41.0	9.0
4.2	36.8	13.2
4.4	30.5	19.5
4.6	25.5	24.5
4.8	20.0	30.0
5.0	14.8	35.2
5.2	10.5	39.5
5.4	8.8	41.2
5.6	4.8	45.2

3.2.1.2 Sample Preparation

Laccase activity was determined by oxidation of 3-ethylbenzothiazoline-6-sulphonic acid (ABTS). The reaction mixture (sample) contained various concentrations of ABTS, 0.2mM, 0.4mM, 0.6mM, 0.8mM and 1.0mM, 0.1M sodium acetate buffer, pH 5.0, and a suitable amount of enzyme. (Bourbonnais et al.,1995). Detailed calculations of ABTS solution preparation are shown in Appendix.

3.2.1.3 Ultraviolet-Visible Spectrophotometer (UV-VIS Spectrophotometer).

The activity of the enzyme was determined by using UV-VIS Spectrophotometer at 420 nm at a reaction time of 10 minutes.

3.2.2 Laccase and Glucose Oxidase

The activity of LTV-GOX was determined by the oxidation of 3-ethylbenzothiazoline-6-sulphonic acid (ABTS). The reaction mixture contained various concentrations of ABTS, 0.2mM, 0.4mM, 0.6mM, 0.8mM and 1.0mM, 0.1 sodium acetate buffer, pH 5.0 with LTV-GOX concentration ratio of 1:20. (Szklarz and Leonowicz, 1986). Detailed calculations of GOX solution preparation are shown in Appendix.

3.3 Oxidation of ferulic acid

For this experiment, ferulic acid was used as a lignin model compound. The reaction mixture contains 0.5 M ferulic acid, 0.1 M sodium acetate buffer, pH 5.0, 1.0mM of ABTS and different variation of LTV-GOX concentrations. The concentration ratio of LTV-GOX will be varied at 1:0, 0:20 and 1:20 respectively. (Adelakun et al., 2012). Next the product from ferulic acid oxidation was identified using HPLC. Detailed calculations of ferulic acid solution preparation are shown in Appendix.

3.4 High Performance Liquid Chromatography (HPLC)

Acetonitrile gasifier was used as a carrier for the samples by using ratio 1:10. The product of oxidation was analysed by reverse-phase HPLC using a Microbondapak C-18 column by setting the temperature at 37 ˚C and the retention time of 30 min. (William, 2004)

3.5 Preparation Standard Solution of Vanillin

The standard solution of vanillin with concentration of 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM, 0.7 mM, 0.8 mM, 0.9 mM and 1.0 mM was prepared by dissolving vanillin powder of mass 0.0015 g, 0.003g, 0.0046 g, 0.0061 g, 0.0076 g, 0.0092 g, 0.011g, 0.0112 g, 0.0137 g, and 0.0152 g in 10 mL of distilled water respectively. The standard curve for vanillin concentration was determined from the product analysis of vanillin concentration. (Booth, 2006).

CHAPTER 4

RESULT AND DISCUSSION

4.1 Laccase Activity

The figure shown below shows the enzymatic activity of LTV over time in the oxidation of ABTS. The graph shows the activity of LTV at different concentration of ABTS ranging from 0.2 until 1.0 Mm.

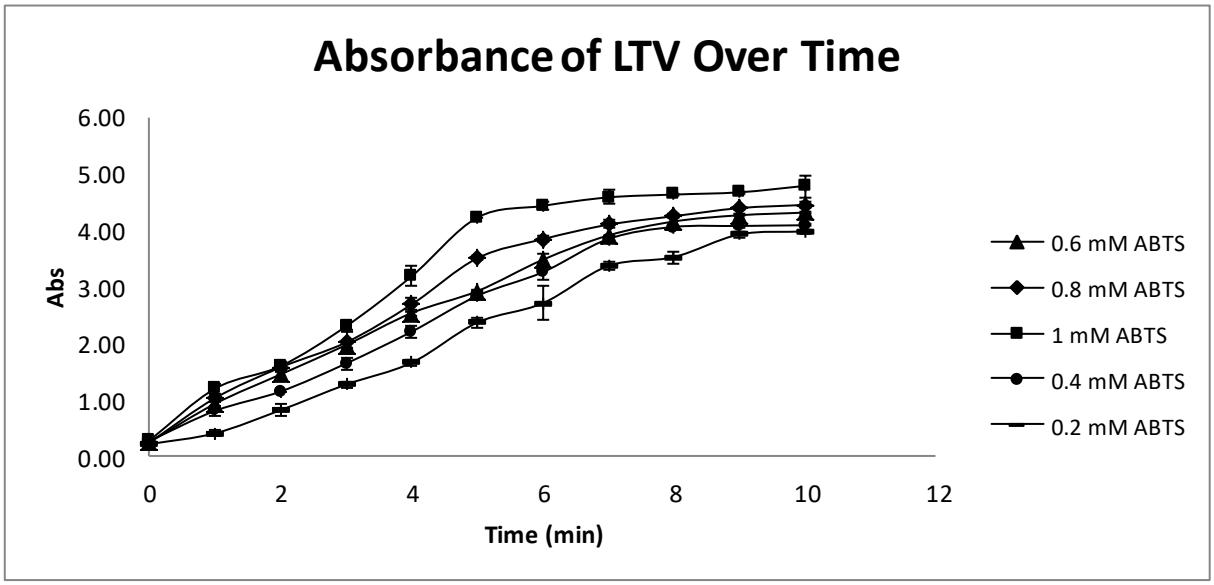

Figure 12: Enzymatic Activity of LTV at Different Concentrations of ABTS

Laccases are widely distributed in nature, and their involvement in both lignin synthesis (Dean & Eriksson, 1994) and lignin degradation (Thurston, 1994) has been recently reviewed. Laccase has the ability to catalyze one-electron oxidations resulting in the formation of radicals which undergo numerous spontaneous reactions. These, in turn, lead to various bond cleavages including aromatic ring fission (Shoemaker and Leisola 1990). In the past, the laccase substrate range was thought to be limited to these classes of compounds and the enzyme was considered to be inactive with non-

phenolics. In recent years, however, a number of synthetic compounds have been identified (Bourbonnais & R, Paice, 1990) which allow the oxidation of nonphenolic substrates by laccases. In the presence of ABTS, the most extensively studied of these synthetic compounds, laccase can oxidize lignin model dimers (Bourbonnais & R, Paice, 1990), polymeric lignin (Bourbonnais et al., 1995), and other nonphenolic aromatics such as veratryl alcohol and PAHs (Collins et al., 1996). These compounds have been proposed to function as electron transfer mediators between the enzyme and its substrate. However, no direct evidence for such a mediated process exists. In contrast, a number of findings suggest that the role of ABTS is not that of a redox mediator. Bourbonnais and Paice have reported that although the laccase/ABTS couple oxidizes veratryl alcohol, $ABTS^{+}$ itself cannot. Similarly, it was found that laccase/ABTS oxidizes the PAH anthracene (Collins et al., 1996) but that $ABTS^{+}$ in the absence of the enzyme has no effect. Data from experiments investigating the oxidation of ABTS by laccase has led these workers to conclude that ABTS functions to transfer one electron to the enzyme, thus initiating the ability of the enzyme to accomplish electron transfer from the substrate to dioxygen in a two-electron transfer process.

In this way, ABTS would act as a co-oxidant which activates the enzyme rather than as an electron mediator of the substrate. Based on Figure 12, it shows that when the concentration of ABTS increases, absorbance of the enzyme also increases. This is due to the fact that in enzymatic reaction, the rate of product formation varies with the substrate concentration (Hamidi, 2013). The absorbance was at its highest when ABTS of concentration 1mM was used.

4.2 Laccase and Glucose Oxidase Activity

The figure shown below shows the enzymatic activity of LTV-GOX over time in the oxidation of ABTS. The graph shows the activity of LTV-GOX at different concentration of ABTS ranging from 0.2 until 1.0 Mm.

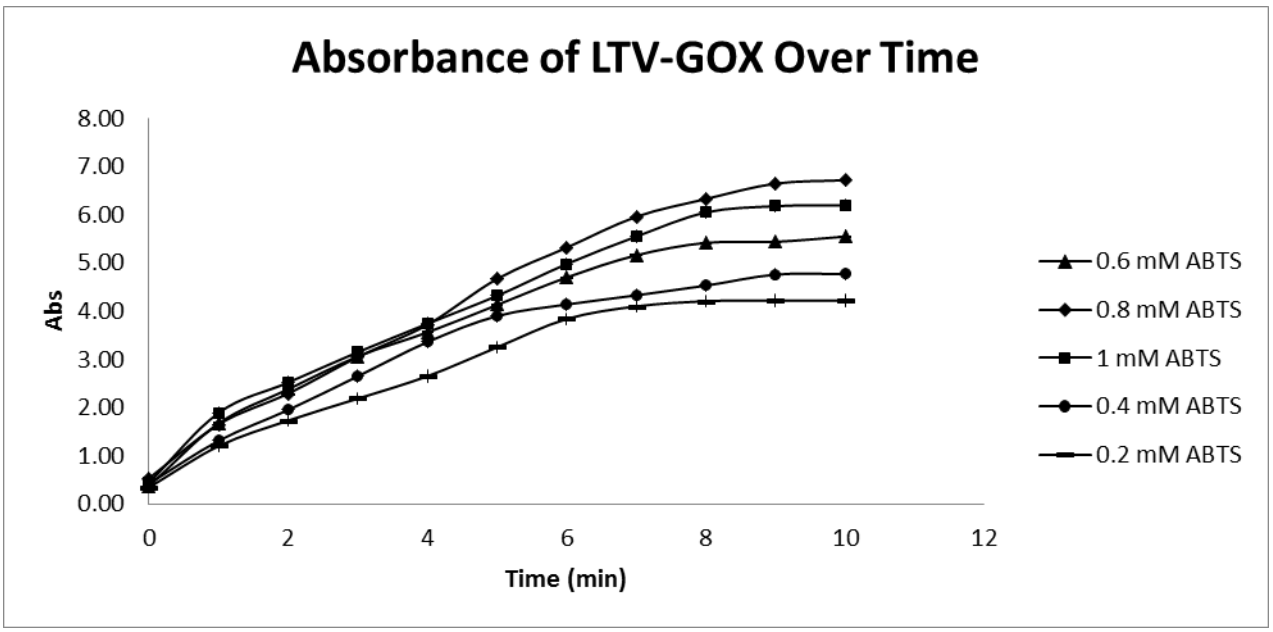

Figure 13: Enzymatic Activity of LTV-GOX at Different Concentrations of ABTS

Laccase oxidizes preferentially phenolic lignin structures to phenoxy radicals which subsequently form quinones. This spontaneous rearrangement can also lead to the fission of carbon-carbon or carbon-oxygen bonds inside the lignin phenyl-propane subunits resulting either in the degradation of both side chains (Fukuzumi 1960) and aromatic rings (Kawai et al., 1988), or in demethylation processes (Harkin and Obst, 1974). In this respect, laccase can co-operate with various FAD-containing oxidases like glucose oxidase (Szklarz and Leonowicz, 1986), veratryl alcohol oxidase (Marzullo et al., 1995) and quinone oxidoreductase (Westermark and Eriksson 1975). Although the actual role of laccase in lignin biodegradation is still under discussion and not completely understood, several authors have reported that laccase acts on the lignin polymer in the ways different from ligninolytic peroxidases. Therefore, In order to present the role of laccase more clearly the experiment of oxidation of ABTS was done with laccase and in-cooperating it with glucose oxidase. Based on Figure 13, it shows that when the concentration of ABTS increases until 0.8 mM, absorbance of the enzyme also increases but when a concentration of 1mM of ABTS was used. The result shows a slightly lower absorbance than 0.8 mM of ABTS. Therefore, the absorbance was at its highest when ABTS of concentration 0.8 mM was used.

4.3 Initial Rate of Reaction of LTV-GOX

The graph below shows the initial rate of reaction of LTV-GOX for the oxidation of ABTS at concentrations of 0.2 mM, 0.4mM, 0.6mM, 0.8mM and 1.0mM.

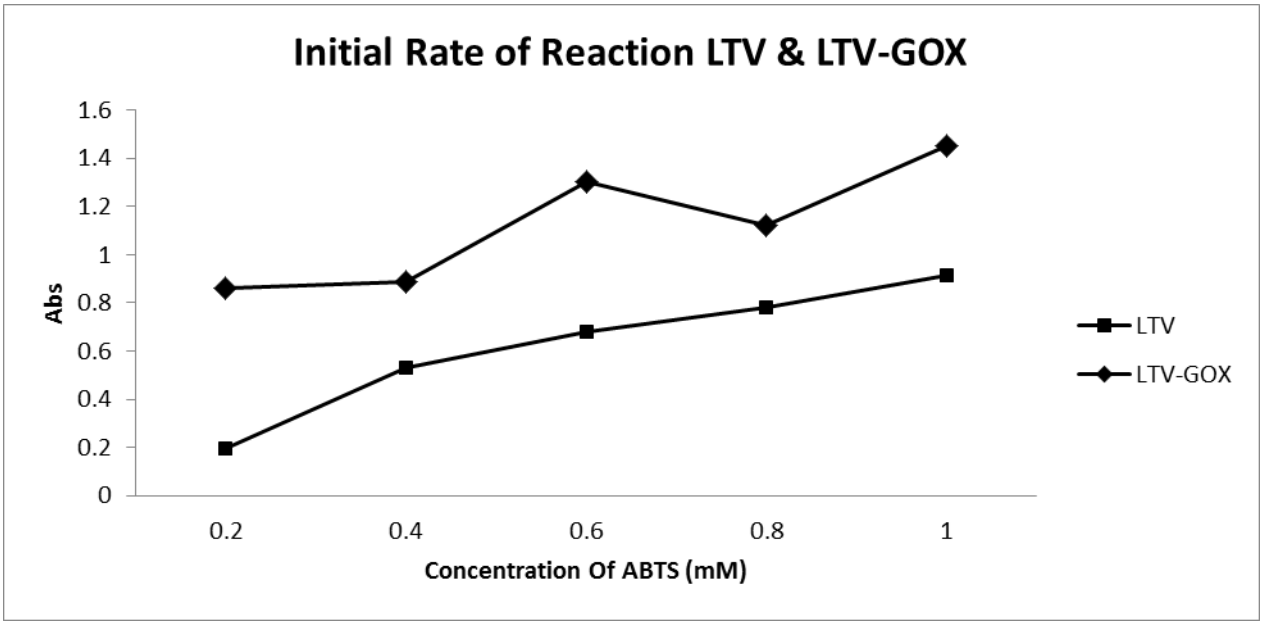

Figure 14: Initial Rate of Reaction of LTV and LTV-GOX.

Figure 14 shows the initial rates of reaction which was determined by varying the substrate concentration with LTV and LTV-GOX. Based on Figure 14, LTV shows a slower activity compared to LTV-GOX. This is due to the fact that LTV often have difficulty in reaching the substrate (Homaei et at., 2013). The higher activity of LTV-GOX proves the review shows that glucose oxidase co-operates effectively with laccase in lignin degradation. Thus, the results of Ramasamy et al. on glucose oxidase as an agent involved in lignin degradation were proven (Srinivasan et al., 1995).

4.4 Oxidation Product Identification Using HPLC.

The graph below shows the peak graph results obtained from HPLC for the oxidation of Ferulic acid using different concentration ratios of LTV-GOX after 24 hour incubation.

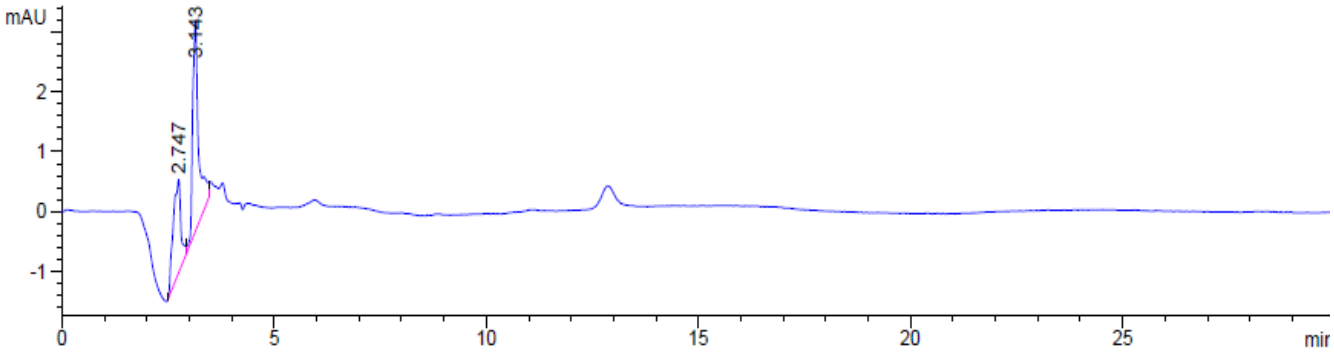

Figure 15: Peak Graph for LTV-GOX ratio of 1:20

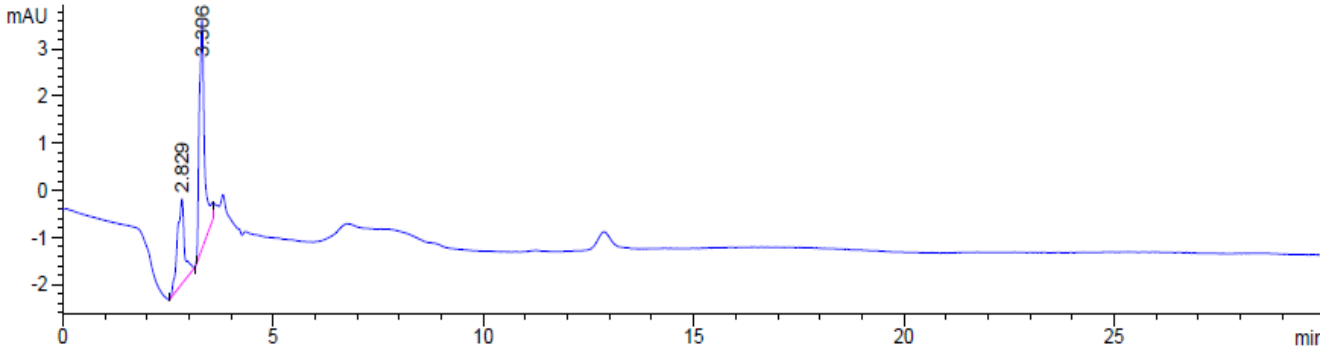

Figure 16: Peak Graph for LTV-GOX ratio of 1:0

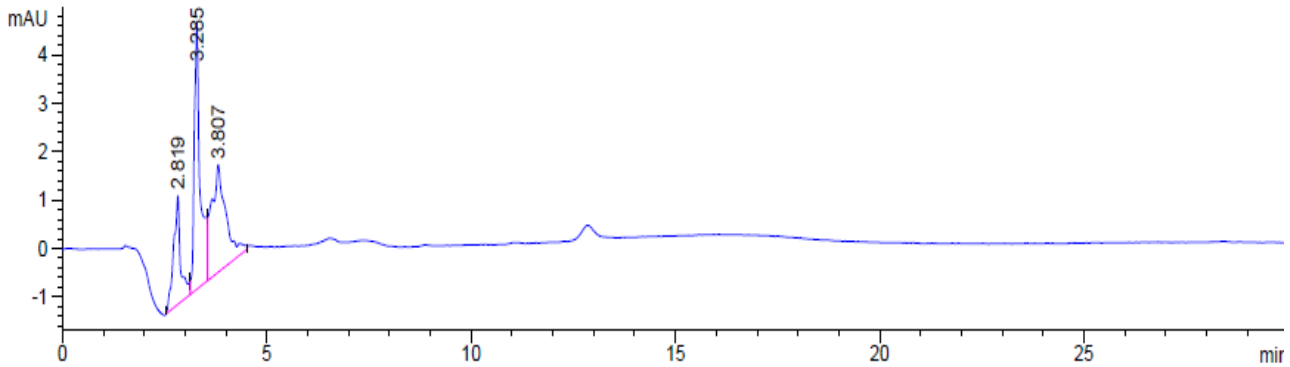

Figure 17: Peak Graph for LTV-GOX ratio of 0:20

The second part of this experiment was to determine whether vanillin will be produced as a result of the oxidation of a lignin model compound, Ferulic Acid. Three sample of the model compound was incubated for 24 hours at temperature of 50^{o}C in concentrations of LTV- GOX of 1:0, 0:20 and 1:20. Figure 15, Figure 16 and Figure 17 shows the peak graph of the products formed upon oxidation of ferulic acid. Few standard solution of vanillin was also prepared and ran through HPLC to compare the peak value with the value of the sample. From Figure 18, 19 and 20 shows the peak area of vanillin for concentration ranging from 0.1M to 1.0M was between 27.08 mAU to 27.751 mAU. Therefore comparing this value with Figure 1.4, 1.5 and 1.6, vanillin (4-Hydroxy-3-methoxybenzaldehyde; 121-33-5), which is the benchmark of product from this research, was not detected. The absence of vanillin might be caused by the over oxidation of Vanillin. Vanillin is a reactive aldehyde which is easily oxidized to give carboxylic acids (Kumar et al., 2012). Besides that, sample degradation might also be one of the reason vanillin was not detected. Sample degradation is often caused by exposure of sample to certain conditions, environment, or time. Hence, sample degradation might have contributed in the absence of vanillin in the product. Even though vanillin was not detected, there were several other product that were formed but due to time and cost restrain, those peaks could not be identified as only vanillin standards was available.

4.5 Vanillin Standard Peak

The graph below shows the peak graph results obtained from HPLC for vanillin standard.

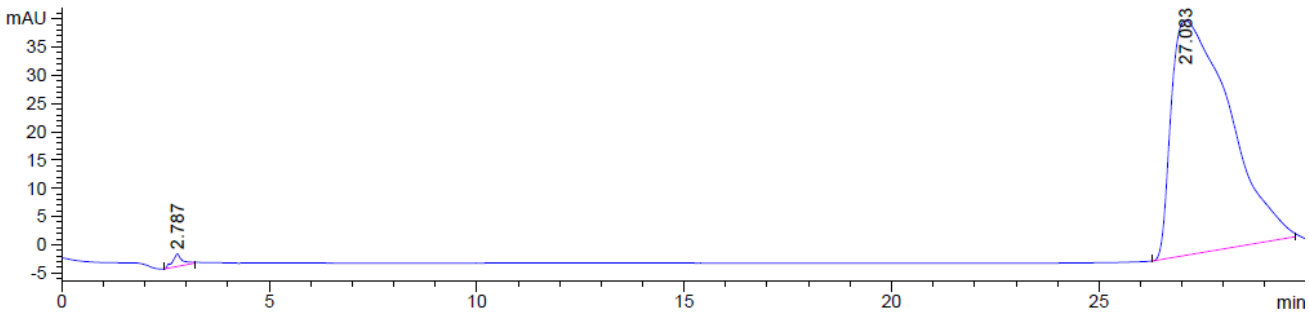

Figure 18: Peak Graph for Vanillin Concentration of 0.1 M

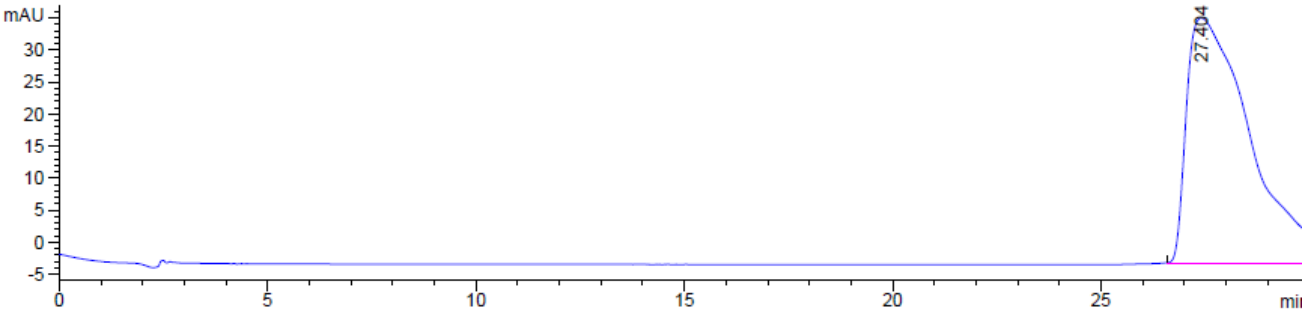

Figure 19: Peak Graph for Vanillin Concentration of 0.5 M

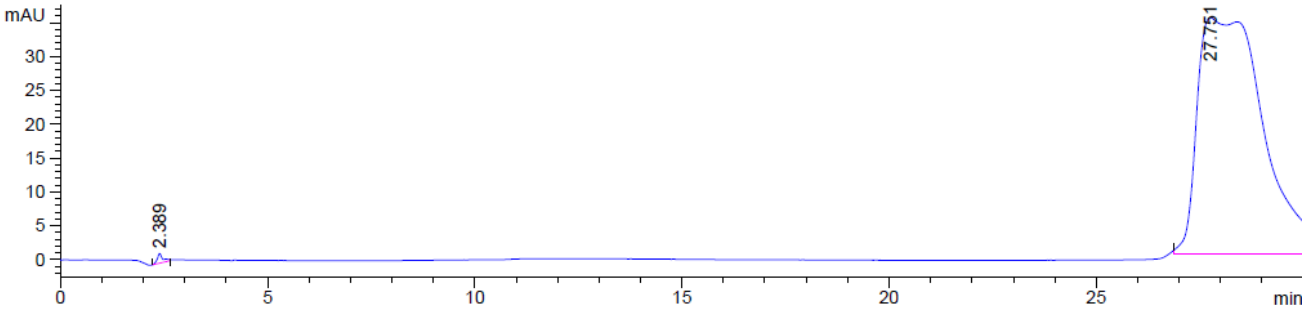

Figure 20: Peak Graph for Vanillin Concentration of 1.0 M

CONCLUSION

Laccase may have many of advantages, but using laccase alone as an oxidizing agent proves to have low rate of reaction due to its difficulty in accessing the substrate. This is caused by internal diffusion of the enzyme. Therefore, by cooperating laccase and glucose oxidase increases the rate of reaction of the enzyme. The second objective was to analyze the product of depolymerization and vanillin was chosen to be quantified as the expected product. However, none of the samples indicated the presence of vanillin due to presence of impurities, delay in sample submission due to HPLC availability and sample degradation due to over immobilization of the sample. Although vanillin was not detected in the reaction, the results from HLPC shows several peak which means there are few other products from the oxidation of ferulic acid. But due to time and money constrain, those products could not be identified as standards solutions could not be prepared.

For recommendation, product analysis should always be done immediately after the separation to minimize sample degradation thus avoid failed analysis. If the delay is inevitable, samples should be preserved and stored at the most suitable condition to minimize sample degradation. Nevertheless, the delay should be shortened as possible.

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APPENDIX

Laccase Calculation

The detailed calculation of laccase solution preparation is shown below:

Molecular weight of LTV is 97kDa

No. of Mole, n = $\frac{MV}{1000}$

= $\frac{(1mM)(100ml)}{1000}$

= 1×10^{-4}

Mass = Molecular Weight x n

= 97kDa x 1×10^{-4}

= 9.7×10^{-3}

= **<u>0.0097g</u>**

The Dalton (or atomic mass unit (amu) is a unit of mass defined as 1/12 weight of carbon-12 atom in ground state.
1 Da = 1/12 m (12C)
The number of atoms in 1 mole is Avogadro's number (6.023 x 10^23) so weight of 1 carbon atom is = (12/ 6.023 x 10^23) g
This means 1Da (1 amu) = 1/12 x (12/ 6.023 x 10^23) g = 1/ 6.023 x 10^23 g which is g/mole
(1 MOLE = 6.023 x 10^23)

=> **1 Da = 1g/mole**

Now molar mass is molecular weight expressed in grams so 1 mole of C-12 is exactly 12 grams. This means 12gm/mole = 12 daltons

The simple answer is, to convert from g/mol to Dalton is to, **Multiply by one.**

Glucose Oxidase Calculation

Molecular weight of LTV is 80kDa

No. of Mole, n = $\frac{MV}{1000}$

= $\frac{(1mM)(100ml)}{1000}$

= 1×10^{-4}

Mass = Molecular Weight x n

= 80kDa x 1×10^{-4}

= 9.7×10^{-3}

= **<u>0.008g</u>**

ABTS Calculation

Molecular weight of ABTS is <u>548.7 g/mol</u>

No. of Mole, n = $\frac{MV}{1000}$ = $\frac{(0.2mM)(10ml)}{1000}$ = 2 x 10^{-6} Mass = Molecular Weight x n = 548.7 g/mol x 2 x 10^{-6} = 1.0974 x 10^{-3} = **<u>0.001g</u>**	No. of Mole, n = $\frac{MV}{1000}$ = $\frac{(0.4mM)(10ml)}{1000}$ = 4 x 10^{-6} Mass = Molecular Weight x n = 548.7 g/mol x 4 x 10^{-6} = 2.1948 x 10^{-3} = **<u>0.002g</u>**
No. of Mole, n = $\frac{MV}{1000}$ = $\frac{(0.6mM)(10ml)}{1000}$ = 6 x 10^{-6} Mass = Molecular Weight x n = 548.7 g/mol x 6 x 10^{-6} = 3.2922 x 10^{-3} = **<u>0.003g</u>**	No. of Mole, n = $\frac{MV}{1000}$ = $\frac{(0.8mM)(10ml)}{1000}$ = 8 x 10^{-6} Mass = Molecular Weight x n = 548.7 g/mol x 8 x 10^{-6} = 4.3896 x 10^{-3} = **<u>0.004g</u>**
No. of Mole, n = $\frac{MV}{1000}$ = $\frac{(1mM)(10ml)}{1000}$ = 1 x 10^{-5} Mass = Molecular Weight x n = 548.7 g/mol x 1 x 10^{-5} = 3.2922 x 10^{-3} = **<u>0.005g</u>**	

Vanillin Calculation

Molecular weight of Vanillin is 152.15 g/mol

No. of Mole, n = $\frac{MV}{1000}$ = $\frac{(0.1mM)(100ml)}{1000}$ = 1 x 10^{-5} Mass = Molecular Weight x n = 152.15 g/mol x 1 x 10^{-5} = 152.15 x 10^{-3} = **0.0015g**	No. of Mole, n = $\frac{MV}{1000}$ = $\frac{(0.2mM)(100ml)}{1000}$ = 2 x 10^{-5} Mass = Molecular Weight x n = 152.15 g/mol x 2 x 10^{-5} = 3.043 x 10^{-3} = **0.003g**
No. of Mole, n = $\frac{MV}{1000}$ = $\frac{(0.3mM)(100ml)}{1000}$ = 3 x 10^{-5} Mass = Molecular Weight x n = 152.15 g/mol x 3 x 10^{-5} = 4.5645 x 10^{-3} = **0.0046g**	No. of Mole, n = $\frac{MV}{1000}$ = $\frac{(0.4mM)(100ml)}{1000}$ = 4 x 10^{-5} Mass = Molecular Weight x n = 152.15 g/mol x 4 x 10^{-5} = 6.086 x 10^{-3} = **0.0061g**
No. of Mole, n = $\frac{MV}{1000}$ = $\frac{(0.5mM)(100ml)}{1000}$ = 5 x 10^{-5} Mass = Molecular Weight x n = 152.15 g/mol x 5 x 10^{-5} = 7.6075 x 10^{-3} = **0.0076g**	No. of Mole, n = $\frac{MV}{1000}$ = $\frac{(0.6mM)(100ml)}{1000}$ = 6 x 10^{-5} Mass = Molecular Weight x n = 152.15 g/mol x 6 x 10^{-5} = 9.129 x 10^{-3} = **0.0091g**

No. of Mole, n = $\frac{MV}{1000}$ = $\frac{(0.7mM)(100ml)}{1000}$ = 7 x 10^{-5} Mass = Molecular Weight x n = 152.15 g/mol x 7 x 10^{-5} = **<u>0.0107g</u>**	No. of Mole, n = $\frac{MV}{1000}$ = $\frac{(0.8mM)(100ml)}{1000}$ = 8 x 10^{-5} Mass = Molecular Weight x n = 152.15 g/mol x 8 x 10^{-5} = **<u>0.0122g</u>**
No. of Mole, n = $\frac{MV}{1000}$ = $\frac{(0.9mM)(100ml)}{1000}$ = 9 x 10^{-5} Mass = Molecular Weight x n = 152.15 g/mol x 9 x 10^{-5} = **<u>0.0137g</u>**	No. of Mole, n = $\frac{MV}{1000}$ = $\frac{(1mM)(100ml)}{1000}$ = 1 x 10^{-4} Mass = Molecular Weight x n = 152.15 g/mol x 1 x 10^{-4} = **<u>0.0152g</u>**