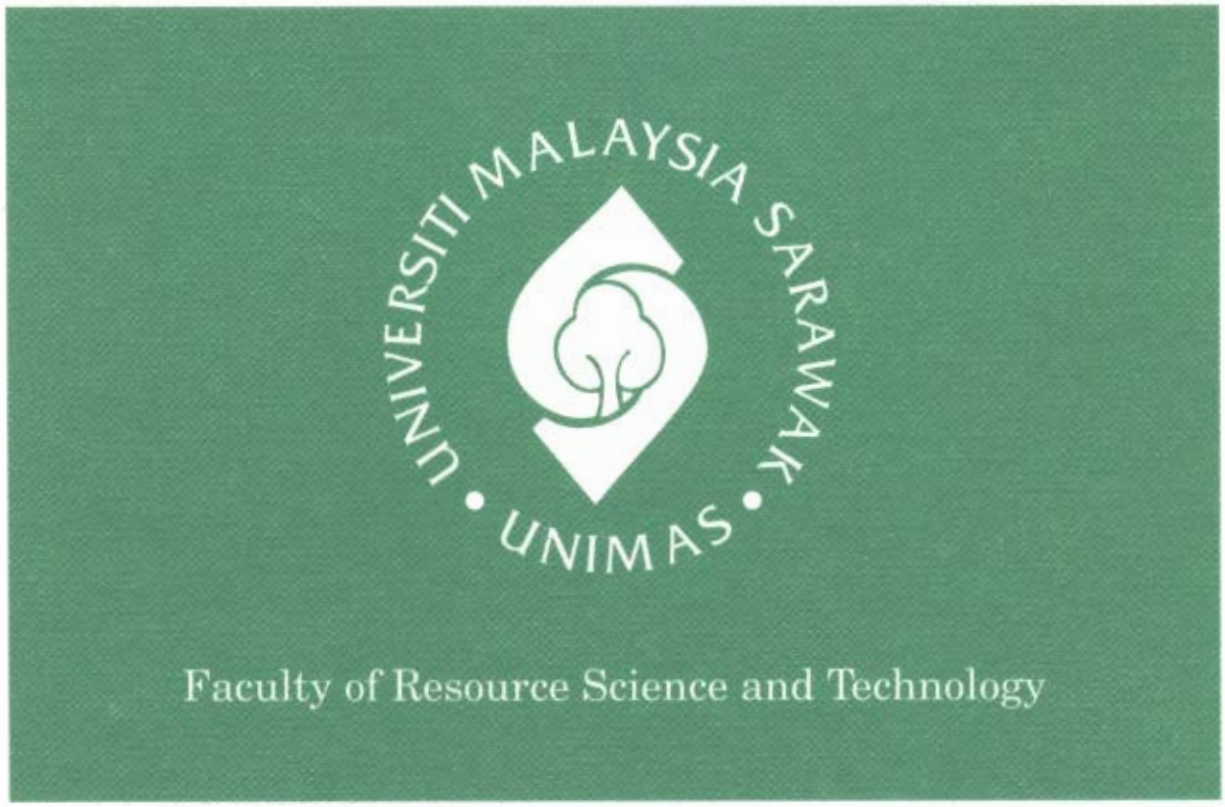

mRNA ANALYSIS OF LIGNINOLYTIC ENZYMES EXPRESSED BY *CORIOLUS VERSICOLOR* AND *PYCNOPORUS COCCCINEUS* DURING BIOPULPING OF *ACACIA MANGIUM*

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ABSTRACT

White rot fungi basidiomycete have been known for its ability in wood biodegradation. *Coriolus versicolor* and *Pycnoporus coccineus* are widely applied in biopulping for pulp and paper manufacturing. During biodegradation process, genes that encode for ligninolytic enzymes were express factors for lignin and fibers separation. Ligninolytic enzymes consist of lignin peroxidase, manganese peroxidase and laccase. Study on the level of ligninolytic enzyme expression during biodegradation period have been carried out using total ribonucleic acid (total RNA) extraction and reverse transcriptase polymerase chain reaction (RT-PCR). Their capability in producing ligninolytic enzymes are determined by inoculating *C. versicolor* and *P. coccineus* on acacia mangium wood chip for 20, 40 and 60 days. After the biodegradation period, RNA of both fungi were then isolated and RT-PCR amplification utilized to detect the lignin peroxidase, manganese peroxidase and laccases messenger RNA (mRNA) or genes. Pattern of cDNA (after agarose gel electrophoresis) was used to spot the expression pattern of ligninolytic enzymes. However, no RT-PCR products were obtained.

Keywords: White rot fungi, Basidiomycete, Biopulping, ligninolytic enzymes.

ABSTRAK

Kulat bassidiomycete, white rot telah diketahui kebolehannya dalam proses biodegradasi. Coriolus versicolor dan Pycnoporus coccineus adalah kulat white rot yang sering digunakan dalam proses biopulping untuk menghasilkan kertas. Semasa proses bidegradation, gen yang mengkodkan enzim-enzym ligninolitik diekspreskan untuk memisahkan lignin dan fiber. Ligninolitik enzim terdiri dari lignin peroksida, mangan peroksida dan laccas Kajian ke atas tahap pengekpresan enzim ligninolitik semasa tempoh biodegradasi telah dijalankan melalui teknik pengestrakkan keseluruhan ribonucleic acid (total RNA) dan polymerase chain reaction transkripsi terbalik. (RT-PCR). Kebolehan fungi ini menghasilkan enzim ligninolitik dikenalpasti dengan cara meginokulasikan C. versicolor dan P. coccineus ke atas kepingan kayu acacia mangium selama 20, 40 dan 60 hari. Selepas tempoh biodegradasi, RNA kedua-dua kulat tersebut kemudian dipencilkan dan amplifikasi RT-PCR digunakan untuk mengenalpasti gen atau mesej RNA (mRNA) untuk lignin peroksida, mangan peroksida dan juga laccas. Paten cDNA (selepas melalui gel elctrophoresis) digunakan untuk mengetahui corak ekspresi enzim ligninolitik. Namun begitu, tidak ada RT-PCR produk yang diperolehi.

***Kata kunci**: Kulat White Rot, Basidiomycete, Biopulping, enzim ligninolitik.*

CHAPTER I

INTRODUCTION

For decades, manufacturing of pulps and papers has become one of the most important industries throughout the world. Approximately 125 million metric tones of papers were produced around the globe annually. This numbers will continue to rises for next few decades, as requirement for both papers and paper products are still very demanding. Paper and paper products can and have been used for a wide variety of purposes including writing, printing, drawing and packaging.

The major component inside the wood are usually fibers; including cellulose, hemicellulose and lignin. In paper industries, these fibers will be separated to increase the flexibility of pulps. In the past, the lignin inside the woods was separated from other components by means of mechanical or chemical actions. However, these conventional lignin removal methods had cause many unwanted environmental conflicts and these lead to the introduction of new and more environmental friendly method. It had been proven that biopulping can become a very effective alternative for replacing the conventional pulping methods. This is due to the reasons that biopulping does not required any harmful chemicals such as chlorine with only little amount of energy consumption. In addition, biopulping will also improve the papers strength. As a result, biopulping will provide a better source of paper production with lower manufacturing costs but higher products quality.

According to Priscila *et al.*, (2004), biopulping can be defined as a solid-state fermentation process in which the enzymes produced by the fungus remain adsorbed on the wood chips. Biopulping that use a natural wood decay or degradation organisms has big potential in pulp and paper industry. Fungi alter the lignin of wood cell walls using their

extracellular enzymes, thus 'softening' the wood chips. Application of fungi in biopulping also helps to reduce a pitch problem in paper making and lowered the effluent toxicity. Since the fungal pretreatment is a natural process, therefore no adverse environmental consequences are foreseen (Kirk *et al.,* 1993 and 1994; Akhtar *et al.,* 1996 and 1997; Syke 1994).

Fungi used in the degradation are known as wood rotting fungi (Eaton *et al.,* 1993). These wood rotting fungi are responsible for decay in timber while those fungi that feed on the cell content, causing the stains. Wood rooting fungi usually will grow on the precious timbers, and consuming cell wall constituents such as lignin and other fibers leading to the disintegration of wood tissue, thus reducing the values of timbers.

White rots are a form of wood decaying process that resulted in bleaching of the wood (Eaton et al., 1993) and very often-applied in biopulping. White rot decreases the lignin steadily. Cellulose is attacked only in the later stages of decay. This simultaneous rot is distinct micromorphologically and where the lignin and cellulose are attacked together, cell wall becoming thinner (Haque, 1997). Sometimes, wood-bleaching process can cause the formation of dark 'zone lines' due to the removal or modification of chromogenic material in the wood.

Understanding the aspects that influence the gene expression of white rot fungi is important and useful for future application of these organisms in biopulping. Determination of manganese peroxidase, laccases and lignin peroxidase (ligninolytic enzymes) produced by white rot basidiomycete species during biopulping have been studied by Priscila *et al.,* (2004). However, they are still doubts regarding the ability of white rot basidiomycetes, such as *Coriolus versicolor* and *Pycnoporus coccineus* to express ligninolytic enzymes at optimum level during biopulping process. Therefore, molecular biology methods such as ribonucleic acids (RNA) extraction and reverse transcriptase polymerase chain reaction (or RT-PCR)

were carried out in order to detect the expression level of lignolytic enzymes by these fungi under optimal condition.

The main objective of this study is to identify the expression pattern of ligninolytic enzymes (manganese peroxidase, lignin peroxidase, and laccase) by analyzing the messenger RNA (or mRNA) expression level of *Coriolus versicolor* and *Pycnoporus coccineus*, and to study the relationship between inoculation period and enzyme expression. In addition, this study will also help in generating cDNA library for the manganese peroxidase, lignin peroxidase, and laccase encoding genes for future expression purposes.

CHAPTER II

LITERATURE REVIEW

2.1 White rot fungi Basidiomycetes

White rot basiodiomycetes are important to the forest ecosystem because they are the only fungi that capable of degrading all cell wall components (cellulose, lignin, hemicelluloses) of woods. Some white rot fungi are found to remove all cell wall components simultaneously while others remove lignin and hemicelluloses by leaving the cellulose behind. Some researchers have categorized these fungi as white rot and corrosive rot. In addition, some authors also categorized the previous fungi as simultaneous rot and the latter as white rot instead of corrosive rot (Otjen and Blanchette, 1986).

Blanchette *et al.*, (1988) has discovered the existence of white rot basiodiomycetes that cause wood decay, with 'selective delignification' characteristic. These fungi are suitable for biopulping requirements because that can remove the entire lignin in the secondary wall and middle lamella but leaving the large quantities of cellulose in the S_2 layer of the cell wall. White rot of the simultaneous type is characterized by the removal of both cellulose and lignin, leaving cells either riddled with bore holes and erosion troughs, or with extensively thinned secondary wall (Otjen *et al.*, 1987). 'Selective delignification' of wood by white rot fungi has attracted interest because the associated lignolytic (lignin-degrading) fungi or their enzymes have industrial potential in biological pulping processes, biobleaching of pulps, lignin removal for release of polysaccharides, and production of ruminant animal feeds (Blanchette *et al.*, 1988). White rot Basidiomycetes species, *Pycnoporus coccinues* and *Coriolus versicolor* are two of lignin-degrading fungi that mostly applied in biopulping. The

lignin-degradation system of white rot fungi consists of non-specific oxidative reactions that are catalyzed by multiple isoenzyme of phenoloxidase (Tien *et al.*, 1983; Kirk *et al.*, 1987; Morohoshi *et al.*, 1987; Kawai *et al.*, 1988; Gold *et al.*, 1989; Wariishi *et al.*, 1991; Limura *et al.*, 1995). Because they have a system for the degradation of lignin, it has proposed that these fungi also useful for bioremediation. (Eriksson *et al.*, 1985 and Bumpus *et al.*, 1987).

2.1.1 *Coriolus versicolor*

White rot basidiomycetes species, *Coriolus versicolor* is a fungi that degrading lignin selectively and very often used in biopulping (Appendix 1a). *C.versicolor* known with a various name including *Trametes versicolor, Boletus versicolor, Polyporus versicolor, Polytictus versicolor* and "Turkey tail" (Jeffrey, n.d). It is also known as "kawarate" among Japanese people, means mushroom by the riverbank, whereas the. Chinese culture called it "yun-zhi" as look as cloud fungus and grows best in the rain (Jeffrey, n.d). Furthermore, Jeffrey (n.d) also stated that, "*C. versicolor* of North America, Asia and Europe has fan-shaped fruiting bodies which grow in overlapping clusters on dead trees with color in brown, white grey or blue shades and sport hairy bands".

2.1.2 *Pycnoporus coccineus*

Pycnoporus species is a bright reddish-orange brackets fungi which widespread on dead wood (Appendix 1b). *P. coccenius* and *P. sanguineus* are two species of *Pycnoporus* that can be found in Australia (Heino, 2003). Besides applied in biopulping, P, coccineus also has been widely used in medical field. *P. coccineus* provide a good treatment for mouth by sucking to cure sore mouths, rubbed inside the mouths of babies with oral thrush, and rubbed on sore lips (Heino, 2003). Consequently, it also can be used as a teething ring.

2.2 Enzymes

Based on James (1989), enzymes are proteins that accelerate the rates of the wide variety of chemical sections, which occur in biological system under thermodynamically unfavorable condition. A catalyst is a substance that participates in a chemical reaction to enhance its rate without destruction or irreversible modification during the reaction. Basically, enzymes are globular protein and exist in form of tertiary or quaternary structure. This three-dimensional conformation play important role in orders to decide the activation of enzyme and guided by the bonding arrangement that fold the protein in specific manner. The specific region of enzyme suit for substrate binding is known as active site. Active sites are formed by a number of amino acids R group for attracting and orientating the substrates and responsible for the specificity of the enzyme. Substrates are bound to the activation site by the chemical and physical properties of amino acid. Enzymatic reactions are highly specific due to the specific three-dimensional shape of the active site that are different in each enzyme.

2.2.1 Lignin-degrading enzyme system

Laccasses, peroxidases (manganese peroxidase, lignin peroxidase and manganese-independent peroxidase) and hydrogen peroxide-generating (H_2O_2) oxidases are major components in the lignin-degrading enzyme system (Hatakka, 1994) for white rot fungi. The existence of this system in many white rot fungi has increased the possibility for applying the white root fungi for bioremediation purpose.

2.2.2 Laccases

Laccases (EC 1.10.3.2; benzenediol: oxygen oxidureductases with molecular weights between 60,000 and 80,000) are glycosylated polyphenol oxidases which contain four copper ions per molecule (Reinhammar et al., 1984). They catalyze the reduction of one dioxigen molecule to two molecules of water, stimulataneously oxidizing their aromatic substrates (Thurston et al., 1994). Laccases are produced by the majority of white rot fungi described to date as well as by other types of fungi and plants. In white rot fungi, they are produced as a number of isoenzymes (Bollag et al., 1984; Collins et al., 1996; Von Hunolstein et al, 1986) encoded by gene families (Kojima et al., 1990; Yaver et al., 1996). Laccases genes from a number of ligninolytic fungi, including *C.versicolor*, have previously been cloned and characterized (Coll et al., 1993; Giardina et al., 1995; Jonsonn et al., 1995; Kojima et al., 1990; Saloheimo et al., 1991; Yaver et al., 1996), and it has been suggested that gene encoding variouis isoenzymes are differently regulated, with some being constitutively expressed and others being inducible (Bollag et al., 1984 and Yaver et al., 1996).

2.2.3 Manganese Peroxidase

Manganese peroxidase (MnP) is the most common enzyme produced by ligninolytic white rot basidiomycete that is useful for lignin degradation. Mnp (EC1.11.1.13, molecular weights, 43,000 to 49,000) is a heme-containing glycoprotein that requires hydrogen peroxide (H_2O_2) as an oxidant (Glenn *et al.,* 1985; Kuwara *et al.,* 1984). *C. versicolor* produces multiple of MnP and lignin peroxidase (LiP) isoenzymes, which are encoded by families of structurally related gene. (Johansson *et al.,* 1993 and 1996). Catalysis reaction of manganese peroxidase is similar to lignin and laccases. MnP oxidize Mn(II) to Mn(III), which then oxidizes phenolic rings to phenoxy radicals via 1 electron oxidation, and finally to the

composition of compounds. (Glenn *et al,* 1986; Tuor *et al.,* 1992). Besides that, system of MnP have been reported to catalyze cleavage of nonphenolic lignin model compounds (D'Annibale *et al.,* 1996; Hammel *et al.,* 1989; Jensen *et al.,* 1996; Maltseva *et al.,* 1991). Because of nonspecificity of Mn(III), the MnP system is also able to oxidize a variety of organic pollutants and xenobiotic compounds. This function of the enzyme is environmentally important because lignin which is the second most abundant biopolymer on earth could be converted into useful chemical and fuels through controlled degradation.

2.2.4 Lignin peroxidase

Lignin peroxidase (LiP) (EC 1.11.1.14; diarylpropane: oxygen, hydrogen peroxide oxidoreductases with a molecular weights, 38,000 to 43,000) is similar with manganese peroxidase where it is also an extracellular haem enzyme that involved in the degradation of lignin (Heinzkill *et. al.,* 1998). The first step in lignin degradation is depolymerisation, catalized by the LiP. LiP will be secreted along with hydrogen peroxidase (H_2O_2), by white rot fungi under condition of nutrient limitation. This enzyme oxidize aromatic compounds by one-electron to the corresponding radical cation (Schoemaker *et al.,* 1990; Kersten *et al.,* 1990; and Khindaria *et al.,* 1995) which may undergo various non-enzymic reactions such as addition in water (Schoemaker *et al.,* 1990 and Joshi *et al.,* 1996) or splitting off a proton to form a radical. Like manganese peroxidase, lignin peroxidase also potentially valuable in chemical waste disposal since it is able to degrade environmental pollutant.

2.3 Reverse transcription polymerase chain reaction (RT-PCR)

Polymerase chain reaction (PCR) can be defined as an *in vitro* technique that increase (amplify) the amount of small, specific target DNA sequence into billions of molecules using enzyme within a short period of time. The PCR imitates the natural process of DNA duplication that occurs in most of organisms.

The main features that make PCR method chosen is that it provide greater sensitivity of detection and specificity, with the auxiliary benefit of rapidity compared to other molecular biology procedures (Old *et al.*, 1989.). PCR is valuable to researchers because it allows them to multiply unique regions of DNA so they can be detected in large genomes.

Besides that, PCR also has been applied in cDNA amplification process. The version of standard PCR reaction that responsible for synthesizing cDNA sequence is known as reverse transcription-PCR (RT-PCR). RT-PCR is a highly sensitive and specific method for the detection of rare transcripts (Erlich, 1989 and Carding *et al.*, 1992). During RT-PCR amplification, mRNA isolated from sources such as mycelium of fungi, bacterial cells and blood cells of human or animal is converted into cDNA using reverse transcriptase enzyme, and the cDNA is then used as a template for PCR amplification.

In addition, RT-PCR can also be use for providing rough quantitation of expression of particular gene, which can be use in the case of all cell types or tissues that are not easy to access in great quantity (Daniels *et al.*, 1995). Furthermore, Pyket *et al.*, (1994) reported that "RT-PCR can be useful for identifying and studying the different isoforms of an RNA transcript. For example, different mRNA isoforms may be produced by alternative spilicing

and can be identified when exon specific primers identify extra amplification products in addition to the expected products".

mRNA differential display is a form of RT-PCR in which reverse transcriptase catalyzes cDNA synthesis by using a modified oligo (dT) primer that binds to the poly(A) tail of subset of mRNA (Liang *et al.,* 1993). The resulting amplification patterns are designed in order to produce a bands ladder when size-fractionated in polyacrylamide gel. The most usefulness of mRNA differential display is the comparison of gene expression in cells at different physiological or developmental stages (Strachan *et. al.,* 1996).

CHAPTER III

MATERIALS AND METHODS

3.1 Organisms

Basidiomycetes culture which producing ligninolytic enzymes are *Coriolus versicolor* and *Pycnoporus coccineus*. Both pure cultures were obtained from UNIMAS Fungal Collection.

3.2 Inoculation preparation

Approximately $8mm^2$ of fungus cubes that cut from agar plates were cultured on solid, potato dextrose agar (PDA media) for 5 to 7 days in an incubation temperature of 27°C. After that, 20 discs (8mm in diameter) of fungus from that solid media were inoculated in 200ml of liquid media containing malt extract (ME) broth (24g/l) (DIFCO, USA). All liquid cultures were maintained unshaken for 14 to 17 days at 27°C depending on the mycelium growth. The grown mycelium was then filtered and washed with 300ml of sterile distilled water, and then blended with 100ml of sterile distilled water in three cycles for 5 minutes. The blended mycelium suspension was used to degrade the wood chips in 1L conical flask.

3.3 Wood biodegradation

Wood chips (2.5cm X 1.5cm X 0.2cm) were taken from *Acacia mangium* tree. 200mg of the wood chips were filled into 1L conical flask and sterilized at 121°C for 15 minutes. Sterilization of these wood chips will then be repeated after 24h. In this study, six conical flasks each containing 200g (dry basis) of sterilized wood chips were prepared and loaded with 20mg (dry basis) or 100ml (v/v) of blended mycelium suspension. After that, the conical flasks were shaken by hand and incubated at 27°C for 20, 40 and 60 days. All conical flasks were covered with an aluminum foil to prevent contaminations.

3.4 Mycelium collection

After 20, 40 and 60 days of biodegradation period, all growing mycelium were separated from the surface of the wood chip media (in 1L conical flask) by filtration process. Mycelium of the fungus grow on the wood chips will be harvested according to the 20 days, 40 days and 60 days of biodegradation period by means of separation from the wood chips.

Mycelium were extracted with extraction solution containing 50mM sodium acetate buffer (pH5.5) supplemented with Tween 80 (0.01%). 500ml of the extraction solution were added to the entire content of conical flask and was shaken at 120rpm for overnight at 20±1°C. After overnight culture, the mixture then was filtered in order to collect all the mycelium from the wood chips.

3.5 Preparation of total RNA from fungi.

The RNA extraction method used in this study was performed using hot phenol protocol, a method introduced by Govender (1998) and reviewed by Nel (2001). Due to optimization purpose, this method was modified to suit local experimental requirements.

Growing mycelium that were scraped off from the wood chip media were ground to a fine powder in liquid nitrogen with pestle and mortar in order to break down the cell walls. Then, mycelium powders were transferred into 2 ml Eppendorf tube containing 500µl of phenol (pre-heated at 65°C), 500µl of STE buffer (100mM NaCl, 10mM EDTA, 250mM Tris HCl, pH7.2) and 10µl of 10%SDS (sodium dodecyl sulphate). This mixture was vortexed before shaken for 4 minutes at 65°C and left on ice for 5 minutes.

After centrifugation for 10,000 rpm at 15 minutes, the upper phase was collected and extracted twice with an equal volume of phenol/ chlorophorm/ isoamyl alcohol (25:24:1 [v/v/v]). This was followed by precipitation with 1/10 volume of 3M sodium acetate (NaOAc) and 2 volume of cold absolute isopropanol for overnight at -20°C.

After overnight precipitation, the mixture was pelleted by centrifugation for 15 minutes at 10,000 rpm. The supernatant was discarded and total RNA pellet (which not always visible) dried approximately for 5 minutes. The total RNA pellet was re-suspended in 5µl of 10X reaction buffer with $MgCl_2$, 18 µl of double distilled water (DEPC treated water) and 5 µl of RNase-free Deoxyrinuclease I (Fermentas). The mixture then was shaken at 90 rpm at 37°C for 30 minutes. Finally, 1µl of 25mM EDTA added, and incubated again in water bath at 65°C for 10 minutes before being chilled on ice for 4 minutes and stored at -20°C or -70°C. The total RNA prepared was subjected to agarose gel electrophoresis analysis and absorbance spectrophotometer assay for quantification, prior to being used as a template for reverse transcription polymerase chain reaction (RT-PCR) method.

3.6 Quantitation of total RNA

The concentration and purity of RNA was determined by measuring the absorbance with wavelength of 260nm (A_{260}) and 280nm (A_{280}) in a spectrophotometer. Absorbance readings at 260nm (A_{260}) will measure RNA concentration and ratio between the absorbance values at 260 and 280 nm gives an estimated value of RNA purity. For determination of concentration and purity of total RNA, 5µl of RNA diluted with 495µl DEPC treated water using cuvette.

3.7 Electrophoresis and staining

Agarose gel electrophoresis of total RNA was carried out in a 1.0% agarose gel with 1 X TAE running buffer solution. Buffer solution consist of 0.8 M Tris base, 0.4 Acetic acid glacial, 20mM EDTA solution and formaldehyde loading buffer (loading dye). Electrophoresis was carried out at 80V for approximately 1 hour. Staining was then carried out using ethidium bromide for approximately 15 minutes and visualized under ultraviolet light (UV). Documentation of gel was done using a polaroid camera containing polaroid film.

3.8 Reverse transcriptase Polymerase Chain Reaction (RT-PCR) Amplification

First-strand cDNA generation and PCR product amplification were performed using the standard Access RT-PCR kit protocol (Promega). Total RNA extracted from 20 days, 40 days and 60 days culture of *C. versicolor* and *P. coccineus* were used as the template in the RT-PCR amplification. Approximately 2 µl of total RNA template were added to the RT-PCR mixture containing 12.5 µl of 2X AccessQuick ^{TM} Master Mix (Promega);1 µl of forward and reverse primers; each at concentration of 10 µM; 0.5 µl (5U) AMV reverse transcriptase (Promega); and nuclease-free water to a final volume of 25 µl. Taq DNA polymerase, dNTPs, magnesium chloride ($MgCl_2$) and RT-PCR buffer were concluded in 2X AccessQuick ^{TM} kit. RT-PCRs were done in a thermal cycler GeneAmp PCR System Model 9700 (Perkin-Elmer co. Ltd.).

Three different sets of primers were applied in this study to identify the size of ligninolytic enzymes (manganese peroxidase, lignin peroxidase and laccases) genes in *Coriolus versicolor* and *Pycnoporus coccineus*. Name and sequences of each forward and reverse primer were listed in **Table 1**.

A transcription of mRNA corresponding to glyceraldehyde-3-phosphate (*gpd*), will be also examined as in housekeeping gene. Templates for *gpd* sequences were construct in the same manner as manganese peroxidase, lignin peroxidase and laccases genes. The sequences of the primer that responsible for those housekeeping gene also listed in **Table1.**