J Biochem Tech (2011) 3(2):245-250
ISSN: 0974-2328

Lignocellulolytic enzymes produced by tropical white rot fungi during biopulping of *Acacia mangium* wood chips

Ahmad Husaini*, Faisalina Ahmad Fisol, Liew Chia Yun, Mohd Hasnain Hussain, Sepiah Muid, Hairul Azman Roslan

Received: 16 March 2010/ Received in revised form: 26 March 2010, Accepted: 26 November 2010, Published online: 01 July 2011,

Abstract

Pycnoporus coccineus and *Coriolus versicolor* are among the tropical white rot basidiomycetes that degrade lignin selectively. In this research work, crude enzyme produced during biopulping using both fungi were extracted, filtered and assayed using specific substrates. Both the peroxidases enzyme activities and residual lignin content were measured for the incubation period of 20, 40 and 60 days of biopulping. For both fungi, manganese peroxidase is predominant and highly expressed thus shows the highest rate compared to other ligninolytic enzymes activities in all of extract preparations. After 60 days of inoculation, manganese peroxidase activities are recorded as 270.51 U/mL and 274.36 U/mL for *C. versicolor* and *P. coccineus*, respectively. On the evaluation of the lignin content after biopulping, the lignin content showed significant decreased. Wood chip biotreated with *C. versicolor* showed higher percentage in lignin loss (9.42%) compared to with *P. coccineus* (8.10%).

Key words: *Pycnoporus coccineus, Coriolus versicolor*, lignin peroxidase, manganese peroxidase, laccase, biopulping.

Introduction

Recently, researchers from all over the world have been adapting biotechnology as tools in the pulp and paper industries. The major goal is to develop a method to improve the efficiency of the existing pulping processes in an environmentally-friendly and cost-effective way. In order to achieve this, the use of wood-rotting fungus containing ligninolytic enzymes has been explored in pretreatment of wood chips. The use of fungi can lessen the usage of energy and chemicals in the paper-making process. Biopulping is defined as the treatment of wood chips with lignin degrading fungi prior to the pulping process (Scott et al. 1998; Keller et al 2003; Singh et al. 2010).

Previous research done revealed that the white-rot basidiomycetes degrade lignin more extensively and rapidly than any other known group of organism (Cullen 1996; Singh and Chen 2000; Akhtar et al. 2000). In contrast to other fungi and bacteria, white-rot fungi are capable of completely degrades lignin to carbon dioxide and water. *P. coccineus* and *C. versicolor,* are white-rot basidiomycetes that are distributed worldwide. They have been studied in this current research for the production of ligninolytic enzymes. Laccases, peroxidases (including lignin peroxidases, manganese peroxidases, and manganese-independent peroxidases), and H_2O_2-generating oxidases are components of the lignin-degrading enzyme system (Hatakka 1994). There has been great interest in using fungal laccases and peroxidases for biotechnological processes due to their chemical and catalytic features (Messner and Srebotnik 1994; Sayadi and Ellouz 1995; Ferraz et al. 2008).

Mechanical pulping is an energy-intensive process and yields paper with lower strength compared with chemical pulping (Scott et al. 1998). Chemical pulping involved two major of processes; sulfite pulping which uses sulfurous acid (Sulfite) and sulfate pulping or alkaline (Kraft) where the chemical used is sodium sulfate. These chemical processes uses less energy and produces lower fiber yield compared to the mechanical pulping (Singh et al. 2010). However, chemical pulping gives superior paper quality as compared to mechanical pulp. But the environment consequences are very high as it produces a strong liquid effluent detrimental to the environment that needs to be treated (Ali and Sreekrisnan 2001). Investigations in the use of wood-rotting fungi utilizing their ligninolytic enzymes in biopulping processes in paper-making industries could lead to ways to reduce the energy and chemical consumption (Heinzkill et al. 1998; Scott et al. 2002). Fungi alter the lignin in the wood cells walls, which has the effect of "softening" the chips. This substantially reduces the electrical energy needed for mechanical pulping and leads to improvements in the paper strength properties.

Ahmad Husaini*, Faisalina Ahmad Fisol, Liew Chia Yun, Mohd Hasnain Hussain, Hairul Azman Roslan

Department of Molecular Biology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak 94300 Kota Samarahan, Sarawak, Malaysia

Sepiah Muid

Department of Plant Science and Environmental Ecology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak 94300 Kota Samarahan, Sarawak, Malaysia

*Tel.: 0060 82582960, Fax: 0060 82583160
Email: haahmad@frst.unimas.my

The fungal pretreatment is a natural process; therefore, no adverse environmental consequences are foreseen. Through the use of the proper lignin-degrading fungi, at least 30% electrical energy can be saved in mechanical pulping and paper strength properties are improved (Kirk et al. 1993; Kirk et al. 1994; Akhtar et al. 1996; Akhtar et al. 1997; Akhtar et al. 1998; Akhtar 2000). In addition, the fungal pretreatment for mechanical pulping has less environmental impact than chemical pretreatments (Sykes 1994; Singh et al. 2010).

The objective of this research work is to investigate the ligninolytic enzymes; peroxidase, manganese peroxidase, laccase, and cellulase production patterns during lignin degradation by *P. coccineus* and *C. versicolor* used and applied in the biopulping of *Acacia mangium* wood chips.

Material and methods

Fungus, inoculums preparation and wood biodegradation

P. coccineus and *C. versicolor* pure cultures were kindly provided by Associate Prof Dr. Sepiah Muid (UNIMAS Fungal Collection, Universiti Malaysia Sarawak). The cultures were maintained on 20 g/L malt-extract agar (MEA) plates at 4ºC. MEA was prepared by dissolving 20 g/L of malt-extract (Oxoid) in distilled water, which resulted in a culture broth with pH 5.6. Before sterilization, 2% agar was added to this broth to prepare the solid MEA. 200 mL of liquid medium containing potato extract broth (24 g/L) (DIFCO, USA)and yeast extract (7 g/L) (Oxoid) was inoculated with 20 discs (8 mm in diameter) of *P. coccineus* and *C. versicolor* pre-cultured solid medium. This liquid culture was maintained unshaken for 10 days at 27ºC. The grown mycelium mat was filtered and washed with 300 mL of sterile water. Mycelium that was obtained from several cultures was blended with 100 mL of sterile water in three cycles of 15 s. The mycelium suspension was used to inoculate the wood chips in 1 L conical flask.

A. mangium wood chips (2.5cm x 1.5cm x 0.2cm) were cut from a 6-year-old tree. Before the biodegradation experiments, the wood chips were immersed in water for 16 h. The surplus water was drained and the wood chips were sterilized inside of a 1 L conical flask at 111ºC for 15min. This procedure was repeated one more time after 24h. Each conical flask was loaded with 100 g (dry basis) of wood chips and 10 mg (dry basis) of blended mycelium. After inoculation, the conical flask was shaken, mixed well and incubated at 27ºC for the duration of 20, 40, and 60 days. The conical flask with only wood chip was used as the control in the experiments.

The enzymes were extracted with 50mM sodium acetate buffer (pH 5.5) supplemented with Tween 80 (0.1 g/L). Each 1L conical flask containing cultured wood chips was added with 500 mL of extraction buffer. Extractions were performed with agitation at 120 rpm for overnight at 10 ± 1ºC (de Souza-Cruz et al. 2003). The crude extract preparations were recovered by filtration through fine filter paper. In all experiments, 500 mL of enzymatic extract was treated with 2.5 g of PVP (BDH Laboratory supplies, England) by shaking the mixture at 120 rpm and 27ºC for 1 h. After treatment, the enzyme extract was recovered by filtration through filter paper Whatman No. 1.

Enzyme assays

Oxidative enzymes were assayed with a number of substrates with and without the addition of $MnSO_4$ and H_2O_2. The enzyme activities were expressed as units per milliliter, where 1 U was defined as 1 millimole of substrate oxidized per min or otherwise stated.

Laccase was assayed using 2, 6-dimethoxyphenol (Fluka, Swizerland) as substrate. Reactions were carried out in 3 mL cuvette containing 0.6 mL of 100mM citric acid buffer at pH 5.0, 0.2 mL water, 1.0 mL of enzyme extract and 0.2 mL of 1.0mM substrate. Reactions with 2, 6-dimethoxyphenol (DMP) were monitored at 468 nm. Manganese peroxidase activity was measured by the oxidation of $MnSO_4$ (Sigma). The reactions were conducted in 3 mL cuvette containing 2.5 mL of 20mM sodium tartrate buffer at pH 4.5, 1.0 mL of 1.0mM $MnSO_4$, 1.0 mL of enzyme extract and 0.5 mL of 2.0mM H_2O_2. The absorbance was measured at 238 nm. Lignin peroxidase was assayed using veratryl alcohol (Fluka, Swizerland) as a substrate. Reactions were carried out in 3 mL cuvette containing 1.25 mL of 50mM sodium tartrate buffer at pH 2.5, 0.5 mL of enzyme extract, 0.25 mL of 2.0mM veratryl alcohol and 0.5 mL of 500µM H_2O_2. The reactions were monitored at 310 nm.

Total cellulolytic activity of the enzymatic extracts was determined using dinitrosalicyclic acid (DNS) assay method which was originally described by Bernfeld (1955) with some modifications. Glucose was used as standard. The reaction mixture consists of 0.5 mL of enzyme extract with addition of 0.5 mL of 1% (w/v) carboxylmethylcellulose (CMC). The suspension was incubated for 3 minutes at 25ºC. Five hundred microliter of sterile ddH_2O was used as blank. The reaction was stopped by addition of 1.0 mL of DNS color reagent and subsequently incubated in boiling water for 5 minutes. The mixture was cooled down to room temperature and 10 mL of sterile ddH_2O was added. The mixture was vortexed before measuring the absorbance at 540 nm. The cellulose activity was defined as the activity that produced 1 millimole of glucose equivalents per minute under the optimum assay conditions.

The standard Bradford method was used to measure protein activity with bovine serum albumin (BSA) as the standard. In all the experiments, 1 mL of the crude enzyme preparation was mixed with 1 mL of the Bradford reagent (Amresco, USA). Protein concentration was measured with absorbance at 595 nm.

Determination of residual lignin (TAPPI Standard T222)

The determination of biodegraded lignin is measured according to Smook (1989). One gram of air-dried extractive-free, grounded sample was weighed out accurately in a weighing bottle into a 50 mL beaker. Ten milliliters of 72% (v/v) sulphuric acid was carefully added with a pipette and with a small glass rod, the mixture was stirred. Subsequently the mixture was left with irregular stirring at room temperature for 2 hours. The mixture was next transferred to 500 mL conical flask, and dilute with water until the final volume was 300 mL and covered with reversed smaller conical flask. The mixture was heated on the hotplate for an hour. This process was continued for an extra 2 hours. A crucible (fine or medium porosity) was oven dried for an hour at 105ºC, then allowed to cool in a desiccator and weighed accurately. The insoluble lignin was recovered when the heating was completed, by filtration through the crucible. The lignin was washed with 250 mL of hot distilled water. The crucible containing the lignin was dried at 105ºC for a day, cooled in desiccator and weighed. The lignin content was reported as percentage by weighed of the dry sample.

Results and discussion

Determination of lignocellulolytic activities in wood samples biotreated with fungi.

A. mangium wood chips were biotreated by *C. versicolor* and *P. coccineus* under solid-state fermentation simulating the biopulping process. Development of appropriate experimental procedures for

recovery and determination of enzymes produced by *C. versicolor* and *P. coccineus* under these conditions were necessary to evaluate the actual role of each enzyme in biopulping. Fungal growth was homogeneous (de Souza-Cruz et al. 2003), a mycelial net was easily visible on the wood chips from the 20th day of biopulping with continuous incubation, the mycelial net became dense and after 60 days of biopulping, the wood chips were almost completely covered with mycelium mat.

Biotreated wood samples were extracted with a buffer solution to recover the extracellular enzymes produced during the fungal growth. As previously described, enzyme production was highly dependent on the cultivation conditions of an organism (de Souza-Cruz et al. 2003). Most white rot fungi started lignin degradation when nitrogen, carbon, or sulfur became limited (Gold and Alic 1993). In this study the lignocellulolytic enzymes expression pattern during lignin degradation of *P. coccineus* and *C. versicolor* during biopulping of hardwood chips *A. mangium* were analyzed.

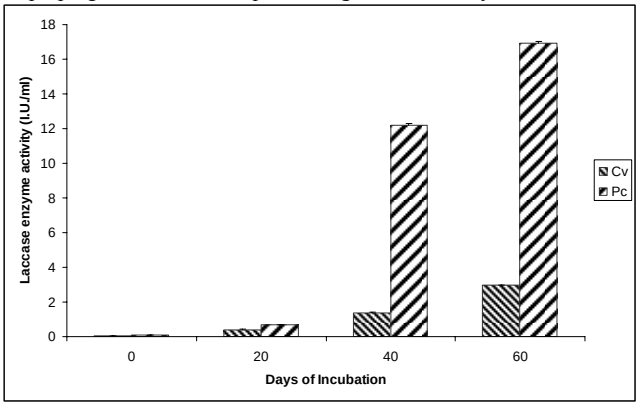

Fig. 1. Laccase activities over days of incubation with *C. versicolor* (Cv) and *P. coccineus* (Pc). Results were means of six independent samples with three enzymes assays per sample and standard deviation. The laccase activity for *C. versicolor* at 0, 20, 40 and 60 days of incubation was determined to be 0.05±0.006, 0.410±0.008, 1.364±0.056 and 2.974±0.012. Meanwhile for *P. coccineus* was 0.078±0.013, 0.667±0.033, 12.205±0.084 and 16.950±0.058.

Laccase activities

Laccase enzyme was assayed using 2, 6-DMP as a substrate. As illustrated in Fig. 1, laccase activities increased steadily over days of incubation after biotreated with both *C. versicolor* and *P. coccineus*. In the present study, it was noted that *P. coccineus* expressed higher rate of laccase activities which were 0.667 U/mL, 12.205 U/mL and 16.950 U/mL compared to *C. versicolor* (0.410 U/mL, 1.364 U/mL and 2.974 U/mL) for each 20, 40 and 60 days of treatment, respectively.

Production of laccases by *C. versicolor* and *P. coccineus* depended on the presence of carbon and nitrogen sources to the culture medium. Laccase production by *P. chrysosporium* was not detected in low nitrogen or high nitrogen medium with glucose as the carbon source but was readily demonstrable when the organism was grown in low nitrogen or high nitrogen media with cellulose (Srinivasan et al. 1995) or with glucose and 0.4 mM $CuSO_4$ (Dittmer et al. 1997). This study showed that laccase production was not inhibited in nitrogen rich conditions with glucose as the carbon source. The study also demonstrated high laccase production in both fungi species in nitrogen rich conditions with glucose. The presence of glucose also contributed to low cellulase activities (Fig. 4). In this respect, the present study is in agreement with earlier findings reporting high levels of laccase production under nitrogen rich

The high rate of laccase activities than other ligninolytic enzymes in this study for extract from *P. coccineus* was due to the high content of copper in *A. mangium* wood (de Souza-Cruz et al. 2003). Copper had been reported to be a strong laccase inducer in the fungal species such as *Trametes versicolor* and *P. chrysosporium* (Collins and Dobson 1997; Dittmer et al. 1997**)**. The presence of copper did not affect fungal growth since the biomass dry weights at different times were the same in the presence and in the absence of copper (Palmieri et al. 2000).

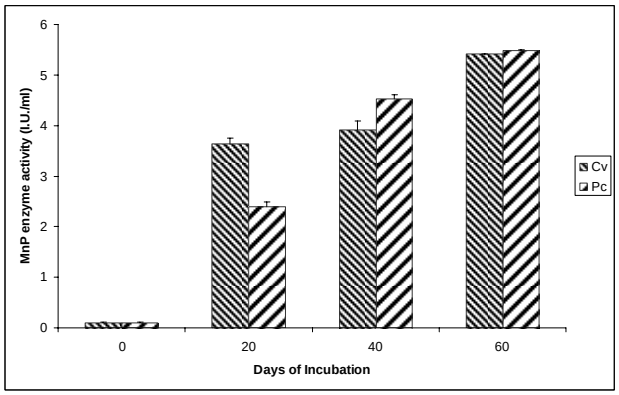

Fig. 2. Manganese peroxidase activities over days of incubation with *C. versicolor* (Cv) and *P. coccineus* (Pc). Results were means of six independent samples with three enzymes assays per sample and standard deviation. The MnP activity for *C. versicolor* at 0, 20, 40 and 60 days of incubation was determined to be 0.096±0.012, 3.641±0.114, 3.908±0.187 and 5.410±0.008. Meanwhile for *P. coccineus* was 0.089±0.022, 2.400±0.097, 4.523±0.082 and 5.487±0.004.

Manganese peroxidase activities

The peroxidase activities of the extract from both fungi increased gradually over days of treatment (Fig. 2). $MnSO_4$ was added to the reaction medium, which confirmed the presence of Mn-dependent peroxidases (MnP). A basal oxidation of $MnSO_4$ indicated that some Mn-dependent peroxidases could be present in the wood extracts or that they contain enough Mn^{2+} to initiate MnP catalyzed reactions (de Souza-Cruz et al. 2003). Results observed from the graph (Fig. 2) showed that manganese peroxidase activities in wood chips biotreated with *P. coccineus* and *C. versicolor* are comparable at Day 60. However manganese peroxidase activities in enzyme extract from *C. versicolor* predominated over its laccase activities (Fig. 1). This indicated that lower copper content presence in enzyme extract from *C. versicolor* than in *P. coccineus*.

Previous studies had shown that the production of manganese peroxidase by the white rot fungus *Lentinus edodes* was suppressed by a high nitrogen concentration in the medium, whereas under these conditions laccase production reached its maximum level (Buswell et al. 1995). However, from this study *C. versicolor* and *P. coccineus* produced both the highest level of laccase and peroxidase-manganese peroxidase activities in the cultured *Acacia mangium* wood chips. Similar results were obtained by Youn et al (1995) during cultivation of *Pleurotus ostreatus* in a protein-rich medium and by Heinzkill et al (1998) during cultivation of *Panaeolus sphinctrinus*, *Panaeolus papilionaceus*, and *Coprinus friesii* in soybean medium containing surplus nitrogen. The different effects of nitrogen on enzyme production can be explained by the natural substrates of the coprophilic and saprophytic fungi used. Wood, which contains little nitrogen, was the substrate when the ligninolytic enzymes of most white rot fungi are produced (Heinzkill et al. 1998).

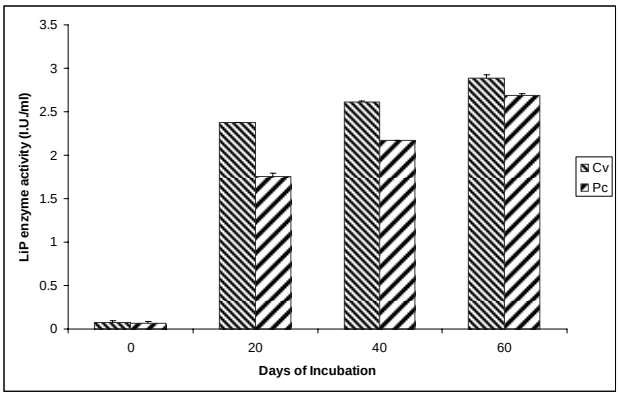

Fig. 3. Lignin peroxidase activities over days of incubation with *C. versicolor* (Cv) and *P. coccineus* (Pc). Results were means of six independent samples with three enzymes assays per sample and standard deviation. The LiP activity for *C. versicolor* at 0, 20, 40 and 60 days of incubation was determined to be 0.076±0.017, 2.373±0.006, 2.617±0.003 and 2.885±0.037. Meanwhile for *P. coccineus* was 0.065±0.018, 1.756±0.035, 2.169±0.004 and 2.688±0.022.

Lignin peroxidase activities

Peroxidase activity is detected through the use of oxidation of veratryl alcohol in the extract in the presence of H_2O_2. The addition of hydrogen peroxide (500μM) in the reaction mixture stimulated the conversion but higher concentration of supplemented hydrogen peroxide inhibited the conversion (Ohkuma et al. 2001). As shown in Fig. 3 below, lignin peroxidase activities increased as days of treatment with *C. versicolor* and *P. coccineus* became longer. The highest value of lignin peroxidase activities for *C. versicolor* was 2.885 U/mL and 2.688 U/mL for *P. coccineus* had been obtained from the extract biotreated for 60 days.

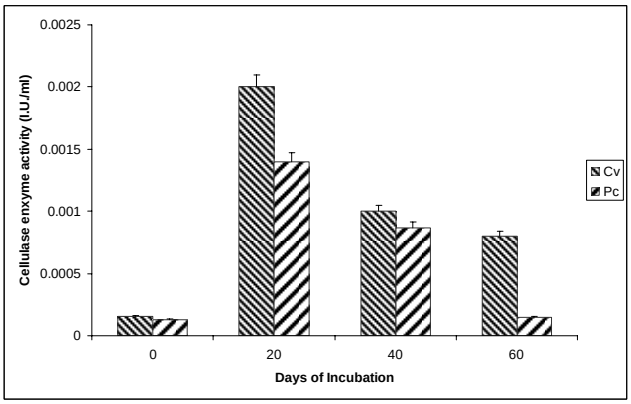

Fig. 4. Cellulase activities over days of incubation with *C. versicolor* (Cv) and *P. coccineus* (Pc). Results were means of six independent samples with three enzymes assays per sample and standard deviation. The cellulase activity for *C. versicolor* at 0, 20, 40 and 60 days of incubation was determined to be 0.000153±0.014, 0.002±0.021, 0.001±0.011 and 0.0008±0.010. Meanwhile for *P. coccineus* was 0.000127±0.008, 0.00140±0.008, 0.00087±0.004 and 0.00015±0.007.

conditions in *Rigidoporus lignosus* (Galliano et al. 1991), *Ceriporiopsis subvermispora* (Lobos et al. 1994), *Lentinula edodes* (Buswell et al. 1995), and *Agaricus bisporus* (Wood 1980; Perry et al. 1993).

Both fungi showed similar activities pattern of lignin peroxidase and manganese peroxidase but exhibited higher level of activity in manganese peroxidase. This is because greater concentration of more than 0.1mM $MnSO_4$ was found to severely inhibit the oxidation of veratryl alcohol (Mater and Field 1998). In this study, the concentration of $MnSO_4$ was more than 0.1mM. In manganese peroxidase reaction, this acted as substrate, therefore this was the reason why manganese peroxidase activities in both fungi extract gave higher activities compared to lignin peroxidase activities. A low rate-limiting (noncompetitive) concentration (0.033mM) of Mn(II) stimulates veratryl alcohol oxidation indicates that Mn(II) helped to protect the enzyme from H_2O_2 inactivation by completing the catalytic cycle (Mater and Field 1998). It was also observed that the veratryl alcohol (0.1mM) incubated with the enzyme without Mn(II) could delay the H_2O_2 inactivation of the enzyme as measured by Mn(II)-oxidizing ability (Mater and Field 1998).

The present study showed *C. versicolor* lignin peroxidase activities gave higher value than *P. coccineus* lignin peroxidase activities. This observation was expected since both the substrates would have to compete for the same oxidized heme group. Besides that high phenolic compound from *P. coccineus* enzyme extract inactivates lignin peroxidase activity since enzyme extract from *P. coccineus* was darker in color than the other fungus. In addition the lower lignin peroxidase activities in both fungi in comparison with laccase activities might be due to inhibition of veratryl alcohol oxidation by aromatic fungal metabolites or lignin-derived products.

Cellulase activities

Cellulase enzyme is responsible in releasing cellobiose from long cello-oligomers and cellulose, to be further converted to glucose by the action of beta-glucosidases (Kirk and Cullen 1998). From the hydrolytic enzymes produced in *C. versicolor* and *P. coccineus* cultures, cellulases were initially assayed using DNS assay method described by Bernfeld (1955) with some modifications. This study showed that cellulase activities declined as days of treatment with *C. versicolor* and *P. coccineus* increased (Fig. 4). The lowest activities of both *C. versicolor* (0.00080 U/mL) and *P. coccineus* (0.00015 U/mL) were noticed at 60 days enzyme extract. The results showed that as the treatment period was increased, the amount of cellulase was reduced in the cultures.

As for white-rot fungi, cellulase synthesis is induced by cellulose and repressed by glucose (Eriksson and Hamp 1978). Since in the presence of glucose, laccase production seemed to be induced (Eggert et al. 1995) and in turn cellulase production was repressed, the present study demonstrated parallel relationship between laccase and cellulase activities. It is also found that cellulase activities (Fig. 4) produced from *P. coccineus* cultures showed lower activities rate than *C. versicolor* cultures. This indicates that *P. coccineus* had the capacity to degrade lignin preferentially with limited attack on cellulose. This preferential degradation is useful in an environmentally friendly biotechnological delignification process in paper pulp manufacturing (Camarero et al. 1998).

Cellulose degradation by fungi is generally a result of the induction of a family of cellulolytic enzymes which could be classified into three major classes: endoglucanases, exoglucanases (cellobiohydrolases) and glucosidases (Knowles et al. 1987). It was well established that the degradation of cellulose by white rot fungi, similar to that of other fungal cellulases, was carried out by a multicomponent enzyme complex in which the individual components interacted synergistically to degrade cellulose to glucose. The precise mechanism/s of cellulase induction is not known. The most accepted view of the induction process was that the organisms produce a basic level or a constitutive amount of cellulase that subsequently produce soluble hydrolysis products of cellulose that function as inducers. Cellobiose, a product of cellulase

action, both induced and inhibited cellulase of *P. chrysosporium* (Eriksson and Hamp 1978).

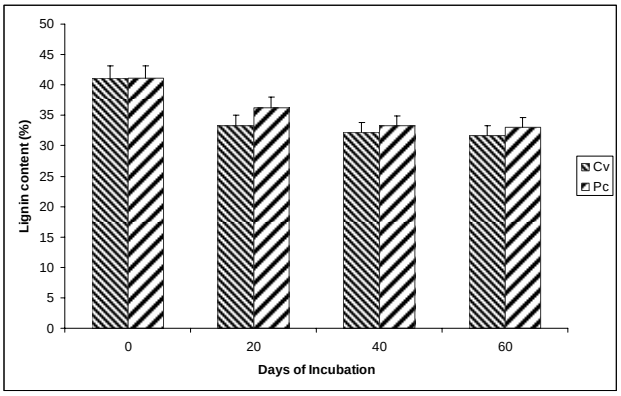

Fig. 5. Percentage of lignin content over days of biopulping using *C. versicolor* (Cv) and *P. coccineus* (Pc). Results are means of two independent samples with two enzymes assays per sample.

Lignin determination

In the present study, the starting lignin content in *A. mangium* wood chips was determined at 41% (w/w). The lignin was reduced by fungal treatments to values of 32% (w/w) after biotreated with *C. versicolor* and 33% (w/w) with *P. coccineus*. Wood chips biotreated with *C. versicolor* showed slightly higher percentage in lignin loss of 9% (w/w) than wood chips biotreated with *P. coccineus* of 8% (w/w) after the 60 days biopulping treatment period (Fig. 5). The low selectivity for lignin degradation of *P. coccineus* and *C. versicolor* at prolonged fermentation stages showed that both species were selective lignin degraders which are suitable for biopulping process.

Conclusion

The present study was successfully done to extract and characterize the ligninolytic enzymes such as lignin peroxidase, manganese peroxidase, laccase and cellulase from the white rot fungus *P. coccineus* and *C. versicolor* during biopulping of *A. mangium* wood chips. The results from this study had showed the suitability of both fungi in the pretreatment of biopulping process with increase activities of laccase, manganese peroxidase and lignin peroxidase over the cultivation period. The study revealed that laccase production from *P. coccineus* was the highest compared to other ligninolytic enzymes produced indicating it was induced in a nitrogen rich condition and high copper content which is a well-known inducer for laccase.

However, manganese peroxidase activities of *C. versicolor* predominated over its laccase activities. These results lead to the assumption that copper content in *C. versicolor* was lower than the other fungi. In this study, manganese peroxidase activity from both fungi presents higher level of activities than its lignin peroxidase activities. Nevertheless, cellulase activities from both fungi demonstrated the contrary pattern. Apart from that, it was observed that cellulase productions are repressed by presence of glucose. Finally, lignin content in wood chips biotreated with both fungi shown reduction after the pretreatment with the respective fungi indicating the usefulness of the pretreatment step in biopulping process.

Acknowledgment

This research project was funded by the UNIMAS Fundamental Research Grant: 1(36)/316/2002(53).

References

Akhtar M, Kirk TK, Blanchette RA (1996) Biopulping: An overview of consortia research. In: Biotechnology in the pulp and paper industry. Facultas-Universitätsverlag, Berggasse 5, A- 1090 Wien, Austria

Akhtar M, Blanchette RA, Kirk T K (1997) Microbial delignification and biomechanical pulping. Adv Biochem Eng Biotechnol Springer-Verlag Berlin, Heidelberg 57:159-195

Akhtar M, Blanchette RA, Myers GC, Kirk TK (1998) An overview of biochemical pulping research. In: Young R, Akhtar M (eds) Environmentally friendly technologies for the pulp and paper industry, Wiley, New York

Akhtar M, Scott GM, Swaney RE, Shipley DF (2000) Biomechanical pulping a mill-scale evaluation. Resour Conserv Recycl 28:241–252

Ali M, Sreekrishnan TR (2001) Aquatic toxicity from pulp and paper mill effluents: a review. Adv Environ Res 5(2):175–196

Bernfeld P, (1955) Amylases, α and β. Meth Enzymol 1:149-158

Buswell JA, Cai Y, Chang ST (1995) Effect of nutrient and manganese on manganese peroxidase and laccase production by *Lentinula* (*Lentinus*) *edodes*. FEMS Microbiol Lett 128**:**81-88

Camarero S, Sarkar S, Ruiz-Dueñas FJ, Martinez MJ, Martinez AT (1998) Description of a versatile peroxidase involved in the natural degradation of lignin that has both manganese peroxidase and lignin peroxidase substrate interaction sites. J Biol Chem 274:10324-10330

Collins PJ, Dobson ADW (1997) Regulation of laccase gene transcription in *Trametes versicolor*. Appl Environ Microbiol 63**:**3444–3450

Cullen D (1996) Recent advances on the molecular genetics of ligninolytic fungi. J Biotechnol 53:273-289

De Souza-Cruz PB, Freer J, Siika-Aho M, Ferraz A (2003) Extraction and determination of enzymes produced by *Ceriporiopsis subvermispora* during biopulping of *Pinus taeda* wood chips. Enzyme Microb Tech 34:228-234

Dittmer JK, Patel NJ, Dhawale SW, Dhawale SS (1997) Production of multiple laccase isoforms by *Phanerochaete chrysosporium* grown under nutrient sufficiency. FEMS Microbiol Lett 149:65-70

Eggert C, Temp U, Eriksson K (1995) The ligninolytic system of the white rot fungus *Pycnoporous cinnabarinus*: Purification and characterization of the laccase. Appl Environ Microbiol 62:1151-1158

Eriksson KE, Hamp SG (1978) Regulation of endo -1, 4-β-glucanase production in *Sporotrichum pulverulentum*. Eur J Biochem 90:183-190

Ferraz A, Guerra A, Mendonca R, Masarin F, Vicentim MP, Aguiar A, Pavan PC (2008) Technological advances and mechanistic basis for fungal biopulping. Enzyme Microbial Technol 43:178–185

Galliano H, Gas G, Seris JL, Boudet AM (1991) Lignin degradation by *Rigidoporus lignosus* involves synergistic action of two oxidizing enzymes: Mn peroxidase and laccase enzyme. Microbial Technol 13:478-482

Gold MH, Alic M (1993) Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Microbiol Rev 57**:**605-622

Hatakka A (1994) Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. FEMS Microbiol Rev 13:125-135

Heinzkill M, Bech L, Halker T, Schneider P, Anke T (1998) Characterization of laccases and peroxidases from wood-rotting fungi (Family *Coprinaceae*). Appl Environ Microbiol 64:1601-1606

Keller FA, Hamilton JE, Nguyen QA (2003) Microbial pretreatment of biomass potential for reducing severity of thermo-chemical biomass pretreatment. Appl Biochem Biotechnol 105:27–41

Kirk TK, Koning JW Jr, Burgess RR, Akhtar M (1993) *Biopulping: A Glimpse of the Future?* Res. Rep. FPL-RP-523. US Department of Agriculture, Forest Service, Forest Products Laboratory, Madison, Wisconsin

Kirk TK, Akhtar M, Blanchette RA (1994) TAPPI PRESS "Biopulping: Seven years of consortia research," TAPPI Biological Sciences Symposium, 57-66, Atlanta, Georgia

Kirk TK, Cullen D (1998) Enzymology and molecular genetics of wood degradation by white-rot fungi. In: Young RA, Akhtar M (eds). Environmentally friendly technologies for the pulp and paper industry. Wiley, New York

Knowles J, Lehtovaara P, Teeri T. (1987) Cellulase families and their genes. Trends Biotechnol 5:255-261

Lobos S, Larrain J, Salas L, Cullen D, Vicuña R (1994) Isozymes of manganese-dependent peroxidase and laccase produced by the lignin-degrading basidiomycetes *Ceriporiosis subvermispora*. Microbiology 140:1691-1698

Messner K, Srebotnik E (1994) Biopulping: an overview of developments in an environmentally safe paper-making technology. FEMS Microbiol Rev 13:351-364

Mater T, Field JA (1998) Characterization of a novel man-ganese peroxidase-lignin peroxidase hybrid isozyme produced by *Bjerkandera* species strain BOS55 in the absence of manganese. J Biol Chem 273:15412-15417

Ohkuma M, Maeda Y, Johjima T, Kudo T (2001) Lignin degradation and roles of white rot fungi: Study on an efficient symbiotic system in fungus-growing termites and its application to bioremediation. RIKEN Rev 42:39-42

Palmieri G, Giardina P, Bianco C, Fontanella B. Sannia G (2000) Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus*. Appl Environ Microbiol 66:920-924

Perry CR, Matcham SE, Wood DA, Thurston CF (1993) The structure and function of laccase protein and its synthesis by the commercial mushroom *Agaricus bisporus.* J Gen Microbiol 139:171-178

Sayadi S, Ellouz R (1995) Roles of lignin peroxidase and manganese peroxidase from *Phanerochaete chrysosporium* in the decolorization of olive mill wastewaters. Appl Environ Microbiol 61:1098-1103

Scott GM, Akhtar M, Lentz MJ, Kirk TK, Swaney R (1998) New technology for papermaking: commercializing biopulping. TAPPI Journal 81:220-225

Scott GM, Akhtar M, Swaney RE, Houtman CJ (2002) Recent development in biopulping technology at Madison, Wisconsin. In: Viikari L, Lantto R (eds) Progress in biotechnology, vol 21. Biotechnology in the pulp and paper industry: eighth ICBPPI meeting. Elsevier, Amsterdam, pp 61–72

Singh D, Chen S (2008) The white-rot fungus *Phanerochaete chrysosporium* conditions for the production of lignin degrading enzymes. Appl Microbiol Biotechnol 81:399–417

Singh P, Sulaiman O, Hashim R, Peng LC (2010) Biopulping of lignocellulosic material using different fungal species: a review. Rev Environ Sci Biotechnol DOI 10.1007/s11157-010-9200-0

Srinivasan C, D'Souza TM, Boominathan K, Reddy CA (1995) Demonstration of laccase in white rot basidiomycetes *Phanerochaete chrysosporium* BKM-F1767. Appl Environ Microbiol 61:4274-4277

Sykes M (1994) Environmental compatibility of effluents of aspen biomechanical pulps. TAPPI Journal 77:160-164

Wood DA (1980) Production, purification and properties of extracellular laccase of *Agaricus bisporus.* J Gen Microbiol 117:327-338

Youn HD, Kim KY, Maeng JS, Han YH, Jeong IB, Jeong G,. Kang SO, Hah YC (1995) Single electron transfer by an extracellular laccase from the white-rot fungus *Pleurotus ostreatus*. Microbiology 141:393-398