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Biodegrading Ability and Enzymatic Activities of Some White Rot Fungi on Kenaf (*Hibiscus cannabinus*)

(Kebolehan Pereputan Biologi dan Aktiviti Enzim Beberapa Kulat Reput Putih ke Atas Kenaf (*Hibiscus cannabinus*))

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ABSTRACT

Lignocellulosic materials consist of lignin walls and cellulose fibrils that are bounded into lignin matrix preventing enzymatic activities to occur efficiently. Natural microorganisms such as fungi have the ability to break down this matrix and make the lignocellulosic components more accessible to enzymes. We report on the ability of four white rot fungi: Oxyporus latemarginatus, Rigidoporus vinctus, Phanerochaete chrysosporium *and* Coriolus versicolor*, to degrade kenaf biomass. Fungi were inoculated separately onto kenaf medium and weight loss was determined after four weeks of incubation period. We observed* O. latemarginatus *as the fastest-growing fungus when compared with the rest and thus recorded the highest in biomass weight loss (3-fold higher). Filtrates from the fermentation were assayed for ligninase activity. All species produced high levels of lignin peroxidase (LiP), about the same amount of laccase except for* P. chrysosporium *and very low levels of manganase peroxidase (MnP). When analyzing for cellulase activities, all four species produced similar amounts of endoglucanase, exoglucanase and β-glucosidase. Because of its consistently fast growth and high enzymatic activities,* O. latemarginatus *stands as a superior candidate in biological pretreatment of lignocellulosic biomass.*

Keywords: Basidiomycetes; biological pretreatment; cellulose; enzyme

ABSTRAK

Bahan-bahan lignoselulosa terdiri daripada dinding lignin dan gentian halus selulosa yang disempadani lignin matriks menghalang aktiviti enzim berlaku dengan cekap. Mikroorganisma semula jadi seperti kulat mempunyai keupayaan untuk memecahkan matriks ini dan menjadikan komponen lignoselulosa lebih mudah diakses oleh enzim. Kami melaporkan keupayaan empat kulat reput putih: Oxyporus latemarginatus, Rigidoporus vinctus, Phanerochaete chrysosporium *dan* Coriolus versicolor *untuk merendahkan biojisim kenaf. Kulat telah disuntik secara berasingan ke dalam medium kenaf dan kehilangan berat telah ditentukan selepas empat minggu tempoh pengeraman. Kami mengamati* O. latemarginatus *sebagai kulat yang berkembang pesat berbanding dengan kulat lain dan sekaligus mencatat penurunan berat biojisim tertinggi (3 kali ganda lebih tinggi). Hasil tapisan daripada penapaian telah dicerakinkan untuk kegiatan ligninase. Semua spesies menghasilkan peroksidase lignin (LiP) pada tahap tinggi, lakase pada jumlah yang hampir sama kecuali* P. chrysosporium *dan peroksidase manganase (MnP) pada tahap sangat rendah. Apabila menganalisis aktiviti selulase, kesemua empat spesies menghasilkan eksoglukanase, endoglukanase dan β-glukosidase pada jumlah yang sama. Oleh sebab pertumbuhannya yang cepat dan tekal dan aktiviti enzimnya yang tinggi,* O. latemarginatus *berpeluang menjadi calon terbaik dalam prarawatan biologi biojisim lignoselulosa.*

Kata kunci: Basidiomycetes; enzim; prarawatan biologi; selulosa

INTRODUCTION

Alternative fuels such as fuels from highly abundant plant biomass are needed to reduce current human dependency on petroleum. One promising biofuel is ethanol, mainly manufactured from lignocelluloses and especially for use as fuel for automobiles (Holmgren & Sellstedt 2008; Ozturk 2010; Ozturk et al. 2006; Shi et al. 2009). There are several steps in the conversion of lignocellulosics to ethanol. The pretreatment step is for the purpose of breaking down the lignin and to release the crystalline structure of cellulose. The hydrolysis step uses a combination of enzymes to transform cellulose into glucose and the fermentation step uses microbes to ferment glucose into ethanol (Sun & Cheng 2002). Of the three steps, pretreatment continues to be the main recalcitrant factor to achieve an efficient and commercially viable process in treating raw biomass. Besides, pretreatment is often singled out as the culprit in the high cost of ethanol production, costing almost one third of the total production cost (NREL 2000). Therefore, it is important to find new improved ways for the pretreatment step.

In the hydrolysis step, enzymes such as cellulases are often introduced into the pretreated biomass. However, lignin and hemicelluloses presence made it difficult for

the enzymes to reach cellulose, resulting in a less effective hydrolysis. Thus, preremoval of lignin and hemicelluloses in the pretreatment processes is highly desirable and can significantly increase hydrolysis efficiency (Hakalaa et al. 2005). Existing pretreatment methods include steam explosion and the use of dilute acid, alkali and oxidant (Shi et al. 2009). However, these are chemicals both environmentally not friendly and costly. A more desirable option to chemicals is a pretreatment method that uses microorganisms such as fungi, which possess certain enzymes that can naturally breakdown lignin found in lignocellulosic biomass. Because microbes are readily available and can multiply on its own in simple processes using fundamental equipment, they provide an attractive alternative over the much sought physicochemical methods, which are more costly (Shi et al. 2009).

Microorganisms such as the brown, white and soft-rot fungi have been used to breakdown lignin and hemicellulose in waste materials such as agricultural residues and even for cleaning of chemically uploaded textile waters (Schurz 1978; Seker et al. 2006). Brown rots mainly attack cellulose, while white and soft rots attack both cellulose and lignin (Fan et al. 1987). These basidiomycetes fungi have developed specialization in consuming organic materials. White rot fungi are known to degrade lignin to a great extent and at a fast pace when compared to any other group of organisms (de Koker et al. 2000). Lignin degradation by white rot fungi is thought to involve in delignifying enzymes including lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Eriksson et al. 1990). Previous work has indicated that using white rot fungi as a delignification agent in pretreating plant materials could reduce lignocellulose defiance to enzymatic hydrolysis (Zhang et al. 2007). Several white rot fungi have been investigated on different lignocellulosic biomass to evaluate their delignification efficiencies (Shi et al. 2009). This could improve present technology in energy production from plant-based materials.

Biological pretreatment using natural organisms has been gaining attention, reason being more emphasis has been placed on natural properties and environmentally friendly methods. There are many species and types of organisms and their capabilities are yet to be discovered. Consequently, more studies are awaited to be done on various species for understanding their degrading ability. In this paper, growth and degrading ability of four different fungi species on kenaf biomass has been compared.

Materials and Methods

Fungi Cultivation

The white rot fungi, *Oxyporus latemarginatus*, *Rigidoporus vinctus*, *Phanerochaete chrysosporium* and *Coriolus versicolor* used in this study were obtained from the culture center of the Forest Research Institute Malaysia (FRIM). The asexual stage was kept in Potato Dextrose Agar (PDA) slants and sub-cultured every 2 months. For inoculation, they were grown in PDA slants at 28°C for 7 days, after which the asexual spores are normally produced. Fungal spores at the concentration of 10^7spores/mL were prepared in sterilized distilled water for use in pretreatment.

Biomass Pretreatment

Kenaf (*Hibiscus cannabinus* L.) cultivar V36 was obtained from the Malaysian Agricultural Research and Development Institute (MARDI), Malaysia. Whole stalks of kenaf were chipped into small particles using a chipper mill, dried to 5% moisture content and screened to even size. The chips were powdered using a pulverizer and powder passed through a 0.85 mm screen. A total of 5 g of the powdered kenaf was then transferred into a conical flask. Ten milliliters of synthetic medium with composition per liter of 0.01 g $MgCl_2$, 0.002 g KH_2PO and 0.0005 g of $CaCl_2$ were taken using pipet and spread evenly on the powder surface. Conical flasks were closed with aluminum foil to avoid moisture loss and contamination and then autoclaved at 121°C for 20 min. A total of 10 mL of inocula was added into the kenaf medium and left at room temperature for 4 weeks. Sixteen conical flasks were prepared; each species had four replicates. Treatment was repeated thrice at different times.

Naked Eye Observation

Fungal growth was observed using naked eye. Mycelium coverage on the medium surface was observed and the growth performance was scored qualitatively from 0 to 6: 0- hypha not visible; 1- very thin patches of hypha; 2- visible thin mycelium; 3- half of medium surface covered by mycelium; 4- uneven mycelium on surface and spreading to bottom of the flask; 5- full mycelium coverage and spreading to bottom of the flask, 6- thick, full and even mycelium on whole medium surface including the bottom of the flask. Samples were observed once a week for 4 weeks.

Filtrate Preparation

After 4 weeks, the mycelium covering the samples was first washed away by adding 30 mL of sodium acetate buffer, pH 5.0. Conical flasks were shaken gently for 20 min at room temperature. The sample was filtered using a filter paper (Whatman, No.1). Kenaf biomass was oven-dried at 90°C for 3 days and weighed to determine the weight loss. The filtrate was poured into a centrifuge tube and centrifuged at 8500 rpm, 4°C, for 20 min. The supernatants were stored at 4°C for further analysis.

Enzyme Analysis

Lignin Peroxidase (LiP) LiP was determined in reaction mixture containing 0.1 M sodium tartrate (pH3.0), 0.16 mM azure B and 0.5 mL culture supernatant. The reaction was initiated by adding 2 mM H_2O_2. Absorbance was read after 1 min at 651 nm. One unit of enzyme activity was

expressed as Optical Density (O.D) decrease of 0.1 units per min per mL (Arora & Gill 2001).

Laccase Laccase activity was determined in reaction mixture consisting of 1 mM sodium acetate buffer (pH5.0), 0.5 mM 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and 1.5 mL of culture supernatant. The absorbance was read at 420 nm and enzyme activity was defined as amount of enzyme catalyzing production of 1 µmol of colored product per min per mL (Li et al. 2008).

Manganese Peroxidase (MnP) MnP was determined in reaction mixture containing 100 mM sodium tartrate buffer (pH4.5), 0.4 mL $MnSO_4$, 1 mM 2, 6-dimethoxyphenol (2.6-DMP) and 0.1 mL culture supernatant. The reaction was initiated with 0.1 mM H_2O_2. Absorbance was read after 3 min at 469 nm. One unit of enzyme activity was expressed as O.D decrease of 0.1 units per min per mL.

Endoglucanase Endoglucanase activity was assayed using 50 mM KOH-KH_2PO_4 (pH6.2), 2% carboxymethyl cellulose (CMC) and 0.5 mL culture supernatant (Buswell et al. 1996). The mixture was maintained at 50°C for 30 min and reaction was terminated by adding 3 mL of dinitrosalicyclic acid (DNS) reagent and boiled at 100°C for 15 min. The absorbance was recorded at 565 nm. One unit of endoglucanase was expressed by 1 µmol of glucose liberated per mL enzyme per min.

Exoglucanase Determination of exoglucanase was done by measuring the amount of reducing sugar released in reaction mixtures which consisted of 50 mM KOH-KH_2PO_4 (pH6.2), 1% Avicel and 0.5 mL culture supernatant. Mixture was maintained at 50°C for 30 min (Buswell et al. 1996). Three milliliters of DNS reagent were added and mixtures were boiled at 100°C for 15 min. Absorbance was read at 565 nm and 1 unit of exoglucanase was expressed by 1 µmol of glucose liberated per mL enzyme per min.

B-Glucosidase The enzyme was assayed using 50 mM KOH-KH_2PO_4 (pH6.2), 40 mM p-nitrophenol-β-D-glucopyranoside incubated at 40°C for 30 min and terminated by adding 3 mL of 1 M Na_2CO_3 (Cai et al. 1994). The absorbance was recorded at 400 nm. One unit of β-glucosidase activity was determined as 1 µmol of p-nitrophenol liberated per mL enzyme per min.

Data Analysis

Weight loss and enzyme activities were compared among the four fungal species using one-way Analysis of Variance (ANOVA). Further analysis on the differences of enzyme activity between fungi was carried out using Least Significant Difference (LSD) method at 95% confident level.

Results and Discussion

Observations on Fungal Growth

Powdered kenaf was inoculated with different fungi species in conical flasks and left for four weeks at room temperature. The four fungi species tested were *Phanerochaete chrysosporium* (PC), *Rigidoporus vinctus* (RV), *Oxyporus latemarginatus* (OL) and *Coriolus versicolor* (CV). The progress of fungi were observed and recorded each week using a simple scoring system created from 0 (hypha not visible) to 6 (thick, full and even mycelium on whole medium). Hyphal growth on kenaf medium varied among the species (Figure 1). At week 1, OL showed visible formation of mycelium on the

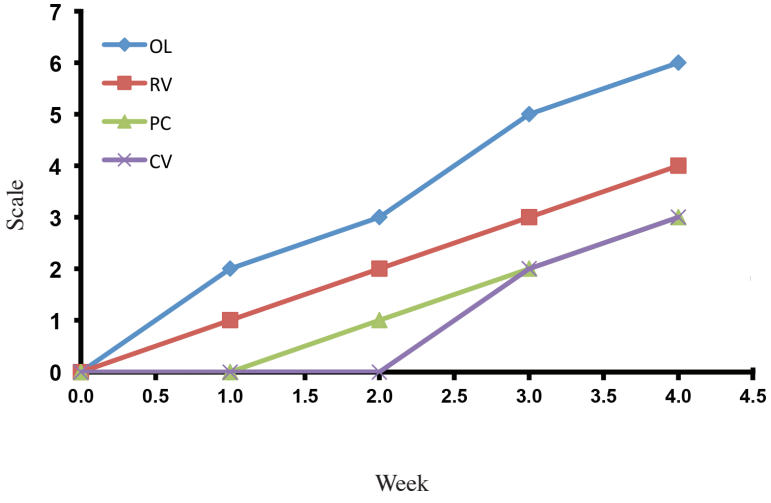

FIGURE 1. Naked eye observation on the progression of white rot fungi mycelia covering kenaf powder in conical flasks over an incubation period of four weeks. Scoring was done on the scale of 0 (no hyphae) to 6 (full mycelium coverage on the medium). ♦ *Oxyporus latemarginatus* (OL), ■ *Rigidoporus vinctus* (RV), ▲ *Phanerochaete chrysosporium* (PC), X *Coriolus versicolor* (CV)

medium; while hypha was hardly seen in the other three species. At week 2, mycelium was readily detectable in OL while in RV and PC, signs of hyphal growth had just started. No hypha were detected in CV during this time. At week 3, all four species showed mycelial development - OL had covered nearly the whole surface and extended to the base of the flask as well, PC and RV showed steady growth as mycelium was seen on the surface; CV however, remained a slow grower. On the final week, OL had covered the whole surface of the kenaf medium with its thick white mycelium. PC managed to cover most or at least half of the surface with its powdery like mycelium. RV also showed improvement where the mycelium was quite thick in some flasks and extended until the bottom of the flask. Although a late starter, CV managed to cover at least half of the medium surface by the fourth week. In summary, CV can be considered a slow grower compared with the other three species. PC grew faster than CV but inconsistently between replicates. OL and RV both had consistent fast growth. OL had the most efficient growth among the four species.

Differences in the color of kenaf powder were also detected. Area that was colonized by white rot fungi turned into light yellow. Original color of the kenaf powder was dark brown. The more mycelia present, the lighter the color of the kenaf powder. Flasks with high mycelium coverage gave a distinct smell indicating an on-going fermentation process.

WEIGHT LOSS

Weight loss indicates degradation of biomass caused by the fungi. When comparing the four fungal species, OL gave the highest loss, which averaged to 27.6%, followed by RV at 13.7% (Figure 2). Although CV was the slowest grower, it recorded a higher weight loss (12.2%) when compared with PC (8.0%). The results for the latter two species were similar to those reported by Halis (2011). OL caused nearly 30% weight loss when inoculated on kenaf biomass making it a desirable wood degrader.

Determination of weight loss gives a general idea on the performance of a fungus. The species with a better performance can be roughly identified from here. Weight loss indicates fungi with good degrading ability, supporting observation via naked eye, which can sometimes be misleading as what observed above between PC and CV. Both visual observation and weight loss determination showed general performance of the fungal species and can be used in selecting potential fungi for further analysis.

LIGNINASE ACTIVITY

Three types of enzyme assays were conducted to detect ligninase activity in the four fungal species. All species had high levels of lignin peroxidase (LiP) activity, with OL having the highest level (Figure 3). RV showed the highest activity for laccase, which was significantly different from OL and PC. CV had lower laccase activity compared with RV and PC showed only traces of it. Manganese peroxidase (MnP) activity detected was quite low in all four fungal species when compared with the other two enzymes. PC showed the highest activity but the difference with the other three species was insignificant.

White rot fungi can be classified into different groups based on their ability and characteristic in expressing enzyme activities. CV is one of the best-known fungus that produces all three lignolytic enzymes. Even though it is well-known that PC is an example of white rot fungus that does not produce laccase, there are evidences that low consistent amount of laccase has been detected in some strains (Srinivasan et al. 1995). Laccase activity was also detected here in OL but in low amounts. MnP activity was the lowest compared with the other enzymes and it stayed consistently low in all the four species tested. This might be due to the reason that in order for lignin to degrade, either LiP or MnP production is needed in association with

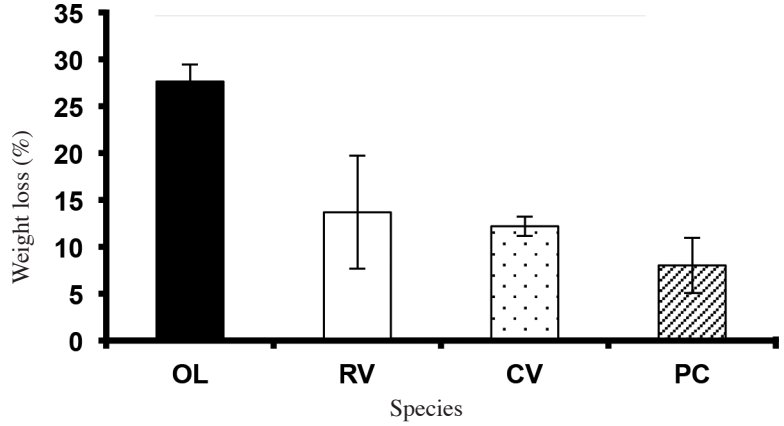

FIGURE 2. Weight loss of kenaf treated with four species of fungi. A total of four flasks were prepared for each species and fungal treatment was repeated three times. Weight loss was averaged among all the replications. OL-*Oxyporus latemarginatus*, RV-*Rigidoporus vinctus*, PC-*Phanerochaete chrysosporium*, CV-*Coriolus versicolor*

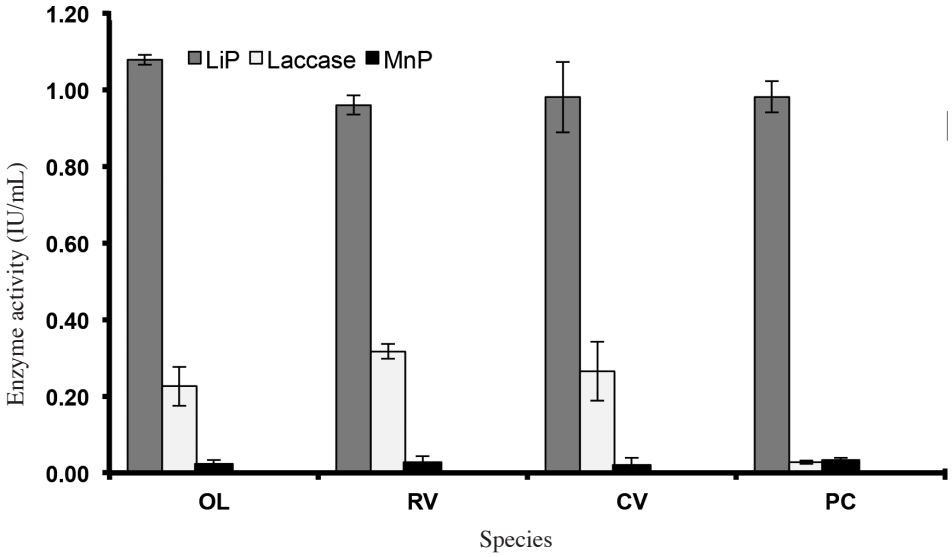

FIGURE 3. Activities of three ligninase enzymes in fungal filtrates after four weeks of incubation in kenaf medium. LiP-lignin peroxidase, MnP-manganese peroxidase. OL-*Oxyporus latemarginatus*, RV-*Rigidoporus vinctus*, PC-*Phanerochaete chrysosporium*, CV-*Coriolus versicolor*

laccase (Arora & Gill 2001). In all the species tested here, LiP production was found at high level indicating that it plays major role in lignin degradation. Besides, there is a particular group of fungi that produces LiP with either one phenoloxidase such as MnP and laccase. More often however, laccase is dominantly produced together with LiP (Tuor et al. 1995). With the exception of PC, we found this is true in other three species studied, whereby their LiP and laccase enzymes were more readily produced. OL and RV have considerably high ligninase activity (Tuor et al. 1995) and are considered as efficient wood degraders (Rosli et al. 2010).

CELLULASE ACTIVITY

Three types of enzyme assays were conducted to determine cellulase activity. The results showed that OL recorded the highest activity for endoglucanase, while PC recorded the lowest (Figure 4). Exoglucanase activity remained high in all four species. There were no significant differences among them according to ANOVA. The reading for β-glucosidase was overall low for all four species, nevertheless, OL showed the highest activity for this enzyme. All white rot basidiomycetes degrade wood polymers cellulose, hemicellulose and lignin at different rates.

CONCLUSION

Enzyme activity and degrading ability of four white rot fungi species was successfully determined. From naked eye observations, *O. latemarginatus* appeared to be the fastest grower on kenaf medium. Kenaf, when treated

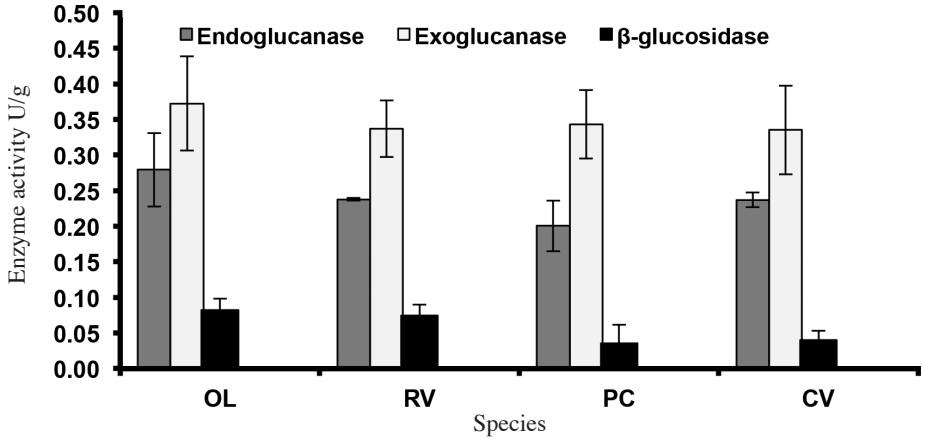

FIGURE 4. Activities of three cellulase enzymes in fungal filtrates after four weeks of incubation in kenaf medium. OL-*Oxyporus latemarginatus*, RV-*Rigidoporus vinctus*, PC-*Phanerochaete chrysosporium*, CV-*Coriolus versicolor*

with *O. latemarginatus* gave the highest weight loss, reaching almost 30%. A comparison with the other three species revealed that the weight loss was about 1/3 of that recorded for *O. latemarginatus*. This species also had very high levels of cellulase and ligninase activities. It can be concluded that among the four species tested, *O. latemarginatus* is the most efficient fungus for degrading kenaf biomass.

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