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Biodegradation of Lignin in Oil Palm Fronds by White Rot Fungi

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ABSTRACT

Oil palm fronds are a by-product from the harvest of the fresh fruit from oil palms. Nowadays, oil palm fronds are used as a source of roughage for ruminants. However, the effective use of the fronds is limited by poor nutrient composition, mainly due to their high lignin and low protein levels. White rot fungi, which degrade cellulose, hemicellulose and lignin, are widely used to increase the digestibility of agro-residues. In this research, 63 fungal isolates of white rot fungi in Thailand were sampled and screened for their production of ligninolytic enzymes by the agar plate screening method. The results showed that 48 isolates could produce laccase, manganese peroxidase and/or lignin peroxidase. A total of 27 isolates that exhibited high ligninolytic enzyme activity were selected to study the lignin degradation in oil palm fronds by solid-state fermentation. It was found that only 7 isolates: OP04, OP06, OP13, OP16, OP47, OP53 and OP61 could reduce lignin in oil palm fronds from 30% down to 15˜18% within 30 d at 30 °C and OP06 showed the highest laccase and manganese peroxidase activities (mean ± SD) of 0.896 ± 0.02 and 0.799 ± 0.07 U/g of dry weight, respectively, where one unit (U) of enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of substrate per minute.

Keywords: lignin, oil palm fronds, white rot fungi, ligninolytic enzymes, laccase

INTRODUCTION

Oil palm is one of the most important economic crops in Thailand, involving many industries and especially, the palm oil processing industry. Currently, oil palm plantations have an area of 579,644 ha (OAE, 2008). Oil palm fronds are the most abundant by-product from oil palm plantations; they are available daily throughout the year when the palms are pruned during the harvesting of bunches of fresh fruit for the production of oil. Oil palm fronds have great potential for use as a feed for ruminants (Hassan *et al*., 1994). However, poor nutrient composition due to high amounts of lignin (15˜56% w/w) and low protein content (2˜6% w/w) limits their effective use (Hassan *et al*., 1994; Liang, 2006; Wanrosli *et al*., 2007). The white rot fungi, mostly basidiomycetes, are the most efficient degraders of lignin in nature (Smith and Thurnston, 1997). Elisashvili *et al*. (2008) reported that these fungi secrete one or more extracellular enzymes that are essential for lignin degradation, such as laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP). Furthermore, white rot fungi have the ability to bio-transform fibrous agro-residues into value-added products through their extracellular enzyme activities (Wood and Fermor,

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1982). The purposes of the present study were to evaluate the growth of white rot fungi on oil palm fronds and their potential in lignin degradation for enhancing the value of lignocellulosic biomass.

MATERIALS AND METHODS

Isolation and cultivation of white rot fungi

Various kinds of mushroom in the white rot fungal group were collected from different sites in Thailand. A small portion of each white rot sample was transferred onto PDA plates added with chloramphenicol (0.05%) and incubated at 30 °C for 7 d. After 7 d, fungal cultures were transferred on the same medium until pure colonies were obtained. The culture was maintained on PDA agar slants and stored at 4 °C. The culture was subcultured once every month.

An agar plug (7 mm diameter) from each fungal colony grown on a PDA plate for 5 d was transferred to a new PDA plate and incubated at 30 ± 2 °C and 37 ± 2 °C. The fungal colony diameter was measured every day until the colony was distributed across the agar plate.

Screening for ligninolytic enzyme production

The agar plate screening for ligninolytic enzyme production was performed using ABTS (2,2 -azinobis(3-ethyl-benzthiazoline-6-sulphonic acid) agar medium, manganese-agar medium (Steffen *et al.*, 2000) and Sabouraud agar added with 3,3 -dimethoxybenzidine. Each plate was inoculated with one agar plug (7 mm diameter) from a fungal colony grown on a PDA plate. The cultures were incubated at 30 ± 2 °C for 5 d. The formation of a dark-green ring around the fungal colony on the ABTS-plates indicated the presence of peroxidases and/or lacasses. Mn-plates were evaluated for the formation of black and dark-brown flecks of manganese oxide (MnO_2) caused by the action of Mn-peroxidase. The formation of a brown color around the colony indicated a positive result for lignin peroxidase caused by 3,3 -dimethoxybenzidine that was converted from its reduced form (colorless) into its oxidized form (brown color). The zone of the enzyme (cm) divided by the colony diameter (cm) was measured to evaluate the enzyme activity of each fungal isolate.

Oil palm frond fermentation

Solid-state fermentation (SSF) was carried out in 250-mL flasks containing 20 g of oil palm fronds (sized approximately 2 mm) moistened with distilled water to adjust the moisture content to about 70%, followed by autoclaving at 121 °C for 15 min. One plate of fungal mycelium grown on PDA agar was blended with 10 mL of 0.85% NaCl solution in a Waring blender for 15 s and this suspension was used as the inoculum for solid-state fermentation. The sterilized substrate was inoculated with 20% (v/w) of fungal mycelium suspension and was incubated at 30 ± 2 °C for 30 d. Control flasks were incubated without fungal samples under the same conditions. Samples were harvested at 5 d intervals over 30 d. Six grams of each sample was analyzed for pH, moisture content (AOAC, 1990), glucosamine (for fungal growth determination according to the method of Van de Loo, 1976) and residual lignin (Van Soest *et al.*, 1991). The remaining contents of each flask were suspended in 50 mL sodium acetate buffer (50 mM; pH 5.0) and incubated on a rotary shaker (150 rpm) at 4 ± 2 °C for 1 h. Solids were separated by filtering through a cheesecloth and then, the filtrate was centrifuged (4 °C; 7,000 rpm; 15 min). The supernatant was used for determination of enzymatic activity. Each experimental treatment was repeated in triplicate.

Enzyme assay

Laccase (EC 1.10.3.2) activity was determined colorimetrically by modification of Rodriguez *et al.* (1999). The increase in the absorbance of 436 nm caused by the oxidation of

ABTS at 30 °C was measured. The reaction mixture contained 0.2 mM ABTS, 20 mM sodium acetate (pH 5). Manganese peroxidase (EC 1.11.1.13; MnP) activity was determined colorimetrically by the method of Heinfling *et al*. (1998). The increase in the absorbance of 469 nm caused by the oxidation of 2,6-dimethoxyphenol (2,6-DMP) at 30 °C was measured. The reaction mixture contained 1.0 mM 2,6-DMP, 0.1 M sodium acetate (pH 4.5), 0.1 mM H_2O_2 and 1.0 mM $MnSO_4$. Lignin peroxidase (EC 1.11.1.14; LiP) activity was determined using veratryl alcohol (3,5-dimethoxybenzyl; VA), as described by Tien and Kirk (1988). The increase in the absorbance of 310 nm caused by the oxidation of VA at 30 °C was measured. The reaction mixture contained 10 mM VA, 50 mM d-tartaric acid buffer (pH 3.0) and 0.5 mM H_2O_2. One unit (U) of enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 µmol of substrate per minute.

RESULTS AND DISCUSSION

Isolation of white rot fungi and fungal growth

In total, 63 isolates of white rot fungi were identified and were designated as OP01 to OP63. All fungal isolates could grow on PDA agar both at 30 °C and 37 °C. However, most of the isolates that grew at 37 °C had growth rates of less than 0.2 cm/day (data not shown), indicating that temperature had an effect on fungal growth. Tripathi and Yadav (1992) reported that most white rot fungi are mesophiles and could grow in the temperature range 15˘35 °C.

Screening for ligninolytic enzyme production

Extracellular ligninolytic enzyme activity was found in 48 of the 54 white rot fungi isolates and the other 6 isolates did not show any ligninolytic enzyme activity. There were 9 of the 63 isolates that could not grow on the screening medium. Laccase activity was found in 44 isolates, while manganese peroxidase (MnP) activity and/or lignin peroxidase (LiP) activity was observed in some isolates (Figure 1). It should be noted that only 7 isolates of white rot fungi showed positive results for MnP activity, despite the fact that MnP is the most common lignin-modifying peroxidase produced by almost all white rot fungi (Hofrichter, 2002). This may have been due to the short incubation period (5 d) of cultures, since the other fungal isolates exhibited MnP activity after 7 d of incubation. The 27 isolates that exhibited high growth and produced high enzyme activity of one or more ligninolytic enzymes (evaluated based on the zone of enzyme per colony diameter) were selected for further cultivation on oil palm fronds. The selected isolates were OP03, OP04, OP06, OP08, OP10, OP12, OP13, OP15, OP16, OP18, OP21, OP29, OP34, OP35, OP36, OP37, OP39, OP43, OP44, OP46, OP47, OP50, OP52, OP53, OP57, OP59 and OP61.

Degradation of lignin by selected white rot fungi

The lignin content in the oil palm fronds was monitored during 30 d of solid-state fermentation. In addition, the growth and ligninolytic enzyme activity (laccase, MnP, LiP) of the 27 selected fungal isolates were determined to verify the relationship between lignin degradation and the production of ligninolytic enzymes. The lignin degradation in oil palm fronds caused by each selected white rot fungi is shown in Figure 2. The results indicated that OP04, OP06, OP13, OP16, OP47, OP53 and OP61 were the isolates that had higher ability in lignin reduction than the other isolates, as these 7 isolates were able to decrease the lignin content in the oil palm fronds from 30% down to 15˘18% within 30 d. Even so, there was no significant *($P > 0.05$)* difference in lignin degradation between the 7 isolates (Table 1). Although the lowest lignin content was obtained on day 30 of the fermentation, the highest activity levels of laccase, MnP, and LiP with most fungal isolates were observed on day 25.

Isolate OP06 exhibited the highest laccase and MnP activity (based on the triplicate measurements and shown as mean ± SD) of 0.896 ± 0.02 and 0.799 ± 0.07 U/g dry weight, respectively, whereas the highest LiP of 0.353 ± 0.05 U/g dry weight was produced by isolate OP04 (Table 1). Levonen-Munoz *et al.* (1983) stated that the efficiency in lignin removal depends upon the fungal species and varies from one substrate to another. The efficiency in lignin degradation of the selected fungal isolates in this study also seemed to be greater for the fungal species that could produce more than one ligninolytic enzyme. It can be seen from the results in Table 1 that all 7 fungal isolates had high activity of laccase, MnP, and LiP except for isolates OP47 and OP61 in which LiP activity could not be detected. Thus, the culture conditions for fermentation of oil palm fronds by the selected white rot fungi should be investigated further.

CONCLUSION

Using the agar plate screening method, 48 from 54 isolates of white rot fungi were shown to exhibit ligninolytic enzyme production and 9 of the 63 isolates could not grow on the screening medium. The 27 isolates that showed high activity of laccase, MnP or LiP were selected to culture on oil palm fronds. Only 7 isolates˘namely OP04, OP06, OP13, OP16, OP47, OP53 and OP61 could degrade more than 50% of the lignin content in oil palm fronds within 30 d of cultivation. The efficiency in lignin degradation of selected fungal isolates seemed to be related to the ability of the fungi to produce more than one ligninolytic enzyme.

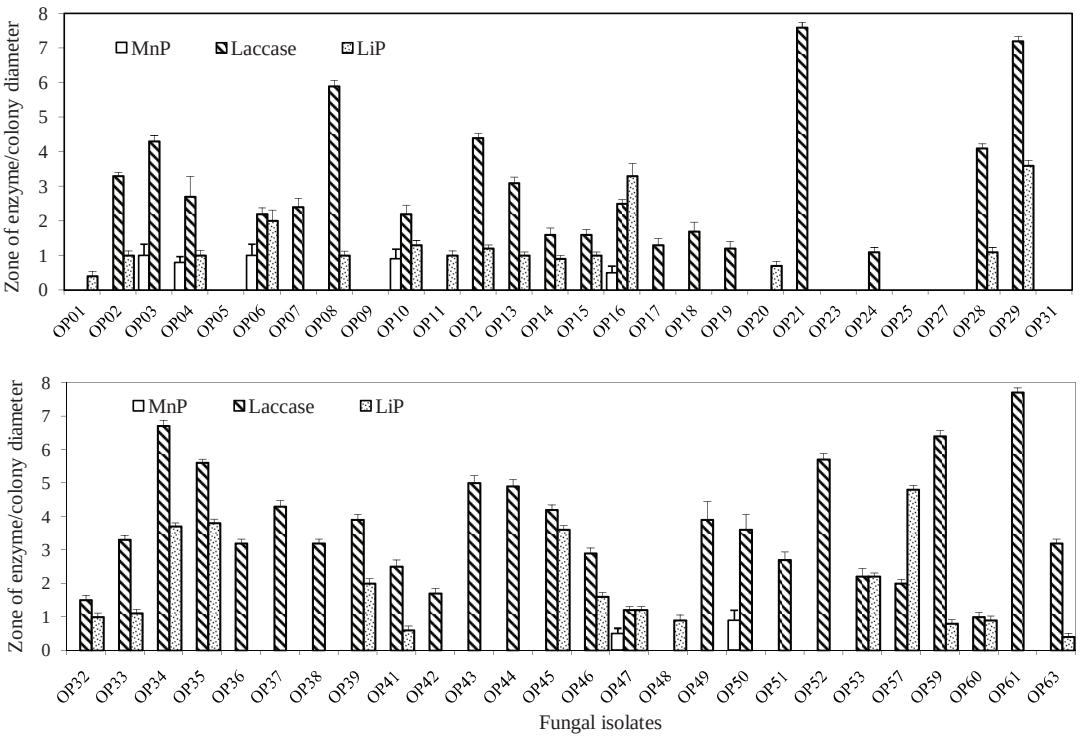

Figure 1 Zone of enzyme/colony diameter of 54 white rot fungi isolates on ABTS, Mn and Sabouraud added to 3,3 -dimethoxybenzidine medium incubated at 30 °C for 5 d. (Columns show mean (n=3); the SD range is represented by the vertical bars.)

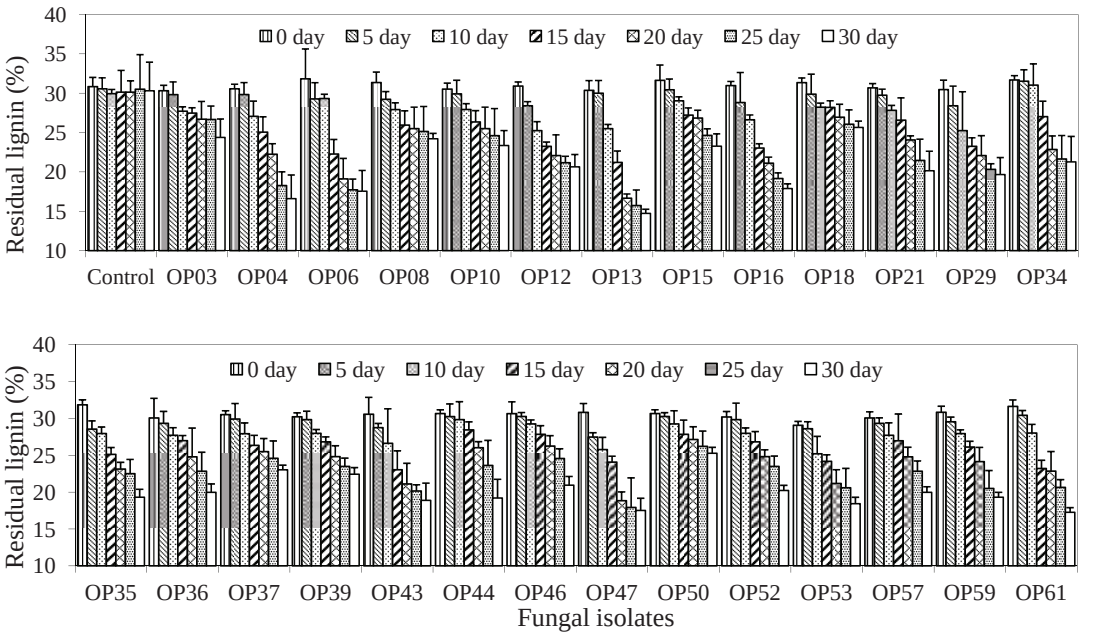

Figure 2 Lignin biodegradation of 27 isolates of white rot fungi resulting from oil palm frond fermentation incubated at 30 °C for 30 d. (Columns show mean (n=3); the SD range is represented by the vertical bars.)

Table 1 Residual lignin and glucosamine (on day 30) and ligninolytic enzyme activity (on day 25) of 7 white rot fungi grown on oil palm fronds and incubated at 30 °C for 30 d (mean ± SD).

Parameter	Fungal isolate						
	OP04	OP06	OP13	OP16	OP47	OP53	OP61
Residual lignin (%)	16.61^{abc}Ø3.00	17.55^{ab}Ø2.64	14.72^{abc}Ø0.54	17.89^{a}Ø0.60	17.51^{ab}Ø1.66	18.43^{a}Ø0.38	17.27^{bc}Ø0.44
Laccase (U/g dry weight)	0.415^{bc}Ø0.04	0.896^{a}Ø0.02	0.298^{c}Ø0.05	0.474^{b}Ø0.08	0.833^{a}Ø0.10	0.348^{c}Ø0.05	0.321^{c}Ø0.05
MnP (U/g dry weight)	0.367^{c}Ø0.04	0.799^{a}Ø0.07	0.315^{c}Ø0.03	0.558^{b}Ø0.05	0.594^{b}Ø0.09	0.699 ^{ab}Ø0.06	0.647^{b}Ø0.16
LiP (U/g dry weight)	0.353^{a}Ø0.05	0.166^{bc}Ø0.04	0.204^{b}Ø0.01	0.212^{b}Ø0.01	n.d.	0.138^{c}Ø0.01	n.d.
Glucosamine (µg/g dry weight)	268.5Ø10.9	181.1Ø13.2	397.4Ø7.5	366.0Ø25.8	282.7Ø11.7	253.3Ø5.8	388.9Ø17.6

U = One unit of enzyme activity, defined as the amount of enzyme catalyzing the oxidation of 1 µmol of substrate per minute.
n.d. = not detected.
^{a,b,c} = mean values in the same row with the same superscript letter are not significantly *(P > 0.05)* different.

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