Ligninolytic enzyme production by *Polyporaceae* from Lombok, Indonesia

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Polypores were isolated from several forests in Lombok Island, Indonesia and screened for their ability to degrade lignin. Sixty of sixty-five samples isolated were tested using a qualitative plate assay through direct visualization of agar plate decolourisation containing the polymeric dye Poly R-478 (0.02% $^{w}/_{v}$). Fifteen isolates were able to decolourise the dye, indicating a lignin-degrading ability. Spectrophotometric enzyme assays from all selected isolates were carried out to examine the production of ligninolytic enzymes (laccase, lignin peroxidase and manganese peroxidase). Twelve selected isolates produced all three kinds of enzymes tested, but *Hexagonia tenuis* sp. A, *Inonotus patouillardii* and *Stereum* sp. produced only laccase and lignin peroxidase. The importance of this study to support biotechnology in the paper industry is discussed.

Key words: isolation, ligninolytic enzymes, Poly R-478 decolourisation, *Polyporaceae*, screening.

Introduction

Lignin, which is widespread in nature, especially in all-higher plants, is a hydrocarbon aromatic compound. Its complex structure makes lignin very difficult to degrade and therefore it can be persistent in environment. Lignin degradation is important in the pulp and paper industry worldwide, and usually uses chemical substances to breakdown lignins in pulp processing. The process releases hazardous lignin-compound effluents into the environment that are toxic and carcinogenic (Harazono *et al.*, 1996).

One method to overcome the above problem is by using wood-rotting fungi for lignin biodegradation (Bumpus *et al.*, 1985). The fungi, mostly basidiomycetes, are the most efficient lignin degraders in nature (Eriksson *et al.*, 1990) and are also the only organisms able to extensively biodegrade lignin (Kirk and Farrell, 1987). Many studies have indicated that these organisms, especially white rot fungi, may be useful for lignin biodegradation, since their extracellular enzyme production can be important when applied in the pulp and paper industries (Reid and Paice, 1994). Laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) are three types of lignin-degrading enzymes

that have been implicated in the lignin biodegradation (Perez and Jeffries, 1990; Pointing, 1999).

The study of biodegradation of lignin by wood-rotting fungi is limited not only by lack of taxonomy in referring to genetic variety, but also in their potential for industrial use. The studies have mainly focused on *Phanerochaete chrysosporium* (Boominathan and Reddy, 1992). Until now, selectively few species of basidiomycetes have been used in lignin degradation studies, such as *Bjerkandera*, *Pleurotus*, *Polyporus*, *Pycnoporus* and *Trametes* (Addleman and Archibald, 1993; Paice *et al.*, 1993; Eggert *et al.*, 1996). Species of *Polyporaceae* that may have potential capacity for use in the pulp and paper industry can possibly be found in Indonesia, since the country has a great number of fungal species (Anonymous, 1994). Studies are therefore required in order to determine not only variations in fungal species and lignin degradation capabilities, but also to establish species or strains that may be suitable for biotechnological applications. This paper concerns polyporous fungi from several forests in Lombok, West Nusa Tenggara (western part of Lesser Sunda Islands), Indonesia, not only to assess their potential as lignin degraders, but also to acquire some promising isolates that can be applied in pulp and paper industry.

Methods and materials

Fungi

We explored several forests (Senaru, Torean, and Mt. Pusuk Forest in Sembalun) in Lombok, Indonesia to collect *Polyporaceae* and 65 fruit bodies were studied. All specimens were isolated by using a method of Hunter-Cevera and Belt (1992) and maintained on Corn Meal Agar at 27 C.

Screening for ligninolytic enzyme production

Before further studies were performed, the ability of isolates to decolourise a polyaromatic dye, Poly R-478, were tested by a qualitative plate test method through direct visualization of agar plate medium decolourisation. The screening medium contained the following per litre of distilled H_2O: 0.60 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.4 g K_2HPO_4, 0.22 g $(NH_4)_2C_4H_4O_6$, 20 g sorbose, 15 g No.1 technical agar, 10 ml mineral solution and 0.02% $^{w}/_{v}$ Poly R-478; the pH was set to 4.5. Mineral solution (per litre of distilled H_2O) consisted of 7.4 g $CaCl.2H_2O$, 1.2 g $FeC_6H_5O_7$, 0.7 g $ZnSO_4.7H_2O$, 0.5 g $MnSO_4.4H_20$, 0.1 g $CoCl_2.6H_2O$ and 10 g Thiamine-HCl (Glenn and Gold, 1983; Field *et al.*, 1992; Paterson and Bridge, 1994). A 1 cm^2 mycelium plug were transferred to screening medium plates for 21 days incubation at 27 C. Diameters of clearing zone formation around the mycelial plugs were

measured for 21 days. Isolates, which formed clearing zones, were selected for enzyme assays.

Enzyme preparation

Two plugs of inocula (1 × 1 cm^2 each), taken from 7 day-old cultures of selected isolate, were transferred to a 100-ml flask containing 15 ml of liquid basal medium as described previously (Paterson and Bridge, 1994) with no addition of agar. After incubation at room temperature and 140 rpm (until the cultures reached their optimal growth rate), the culture was centrifuged (10000 g, 4 C, 10 min), and the supernatants were recovered for ligninolytic enzyme assays.

Enzymatic activity assay

All enzyme assays were determined spectrophotometrically by using a Perkin Elmer Lambda 3B UV/VIS and performed in triplicate at room temperature. The assays contained 0.25-1 ml supernatant (depending on the maximum activity obtained) in a final volume of 5 ml and were performed in 1 cm cuvettes.

Laccase (Lac) activity was assayed according to a modification method of Arora and Sandhu (1985) and Kirk and Kelman (1965). The assay solution contained 1.76 mM catechol as substrate in 10 mM citrate phosphate buffer (pH 5.5). The activities are expressed as ΔOD/min/ml, defined as the change in absorbance at 395 nm due to catechol oxidation.

Lignin-peroxidase (LiP) activity measurement was based on the oxidation of veratryl alcohol (VA) to veratraldehyde in the presence of H_2O_2 (Bonnen *et al.*, 1994). The assay solution contained 2 mM VA in 25 mM tartrate buffer, pH 2.5 and was initiated by the addition of 0.4 mM H_2O_2. The change in absorbance was monitored at 310 nm. One unit of enzyme oxidizes 1 $\mu mol.min^{-1}$ VA to veratraldehyde (ε_{310} = 9,300 $M^{-1}cm^{-1}$).

Mangaan-peroxidase (MnP) activity was determined according to Bonnen *et al.* (1994). The assay solution contained 1 mM guaiacol, and 1 mM $MnSO_4$ in 10 mM citrate phosphate buffer (pH 5.5). The reaction was initiated by the addition of 50 μM H_2O_2. After incubation at room temperature for 10 minutes, the change in absorbance due to the oxidation of guaiacol was monitored at 465 nm (ε_{465} = 12,100 $M^{-1}cm^{-1}$).

Results and discussion

Isolation

This study involved 65 fungal specimen belong to five families of *Polyporaceae* s.l., which are *Corticiaceae*, *Ganodermataceae*,

Table 1. *Polyporaceae* used in this study, growth rates and clearing zone formation.

Code	Taxa	Family	Mycelial growth (mm/day)[a]	Clearing zone formation
346	*Antrodiella* sp. *	*Polyporaceae*	ND	-
359	*Antrodiella* sp.	*Polyporaceae*	0.27	-
387	*Antrodiella* sp.	*Polyporaceae*	0.61	-
392	*Antrodiella liebmannii*	*Polyporaceae*	6	+
355	*Bjerkandera adusta* *	*Polyporaceae*	ND	-
408	*Coriolopsis floccosa*	*Polyporaceae*	0.48	-
348	*Coriolopsis glabrorigens*	*Polyporaceae*	1.69	+
362	*Cyclomyces setiporus*	*Hymenochaetaceae*	0.27	-
413	*Cyclomyces setiporus*	*Hymenochaetaceae*	0.75	-
360	*Flavodon flavus*	*Polyporaceae*	0.79	-
331	*Fomitopsis carneus*	*Polyporaceae*	1.7	-
411	*Ganoderma applanatum*	*Ganodermataceae*	0.52	-
386	*Ganoderma lucidum*	*Ganodermataceae*	1.89	-
374	*Gloeoporus chroceo-*	*Corticiaceae*	0.72	-
393	*Gloeoporus chroceo-*	*Corticiaceae*	0.91	-
372	*Gloeoporus chroceo-*	*Corticiaceae*	3.15	+
329	*Hexagonia tenuis* sp. A	*Polyporaceae*	1.05	+
364	*Hexagonia tenuis* sp. B	*Polyporaceae*	1.68	+
358	*Inonotus patouillardii*	*Hymenochaetaceae*	0.48	+
345	*Lenzites* sp.	*Polyporaceae*	0.87	-
407	*Loweporus inflexibilis*	*Polyporaceae*	P	-
409	*Loweporus roseoalbus*	*Polyporaceae*	2.66	-
349	*Microporellus obovatus*	*Polyporaceae*	0.44	-
382	*Microporus affinis*	*Polyporaceae*	4.59	-
395	*Microporus vernicipes* *	*Polyporaceae*	ND	-
311	*Microporus xanthopus*	*Polyporaceae*	2.75	-
347	*Nigrofomes melanoporus*	*Polyporaceae*	0.05	-
412	*Nigroporus vinosus*	*Polyporaceae*	0.51	-
318	*Oligoporus subcaesius*	*Polyporaceae*	0.36	-
397	*Perenniporia* sp.	*Polyporaceae*	2.00	+
384	*Perenniporia ochroleuca*	*Polyporaceae*	0.58	-
384A	*Perenniporia ochroleuca*	*Polyporaceae*	0.77	-
325	*Phellinus discipes*	*Hymenochaetaceae*	0.29	-
330	*Phellinus lamaensis*	*Hymenochaetaceae*	0.42	+
341	*Polyporus udus*	*Polyporaceae*	1.92	+
312	*Poria* sp.	*Polyporaceae*	0.22	-
316	*Poria* sp. *	*Polyporaceae*	ND	-
320	*Poria* sp.	*Polyporaceae*	0.77	-

+: presence of clearing zone; -: absence of clearing zone;
ND = none detected; P = poor growth;
a = mean values from 5 replicates; * specimen that could not be isolated;
Poria spp. have not determined yet.

Table 1. (continued).

Code	Taxa	Family	Mycelial growth (mm/day)[a]	Clearing zone formation
323	*Poria* sp.	*Polyporaceae*	13	-
328	*Poria* sp.	*Polyporaceae*	0.98	-
335	*Poria* sp.	*Polyporaceae*	0.78	-
339	*Poria* sp.	*Polyporaceae*	8.67	-
342	*Poria* sp.	*Polyporaceae*	P	-
371	*Poria* sp.	*Polyporaceae*	P	-
373	*Poria* sp.	*Polyporaceae*	2.67	-
388	*Poria* sp.*	*Polyporaceae*	ND	-
315	*Poria* sp. A	*Polyporaceae*	1.06	+
326	*Poria* sp. B	*Polyporaceae*	9.75	+
381	*Poria* sp. C	*Polyporaceae*	4.88	+
394	*Rigidoporus dextrinoideus*	*Polyporaceae*	0.74	-
406	*Rigidoporus vinctus*	*Polyporaceae*	0.26	-
338	*Schizopora flavipora*	*Polyporaceae*	0.99	-
356	*Skeletocutis nivea*	*Polyporaceae*	0.51	-
369	*Skeletocutis nivea*	*Polyporaceae*	0.27	-
319	*Stereum* sp.	*Stereaceae*	0.85	-
405	*Stereum* sp.	*Stereaceae*	7.8	+
383	*Stereum ostrea*	*Stereaceae*	0.16	-
410	*Tinctoporellus epimiltinus*	*Polyporaceae*	0.72	-
365	*Trametes* sp.	*Polyporaceae*	P	-
361	*Trametes* sp. A	*Polyporaceae*	2.05	+
379	*Trametes* sp. B	*Polyporaceae*	4.59	+
351	*Trametes versicolor*	*Polyporaceae*	2.54	-
390	*Trichaptum* sp.	*Polyporaceae*	0.24	-
332	*Trichaptum durum*	*Polyporaceae*	1.78	-
385	*Trichaptum durum*	*Polyporaceae*	8.67	-

Hymenochaetaceae, *Polyporaceae* and *Stereaceae* (Table 1). It was not possible to determine all of genera studied, such as *Antrodiella*, *Lenzites*, *Poria*, *Stereum* and *Trametes*, to species level.

Antrodiella sp. (346), *Bjerkandera adusta* (355), *Microporus vernicipes* (395) and two *Poria* spp. (code number 316 and 388) could not be isolated. This may have been due to unsuitable culture conditions or poor viability of the fungi (Moore-Landecker, 1990). However, the isolation method used in this study, was successfully used to isolate 92.3% of all samples collected from several forests.

Screening for lignin degradation ability

The screening procedure showed 15 isolates formed clearing zones in agar plates containing Poly R-478 (Fig. 1) with the color of the zones ranging

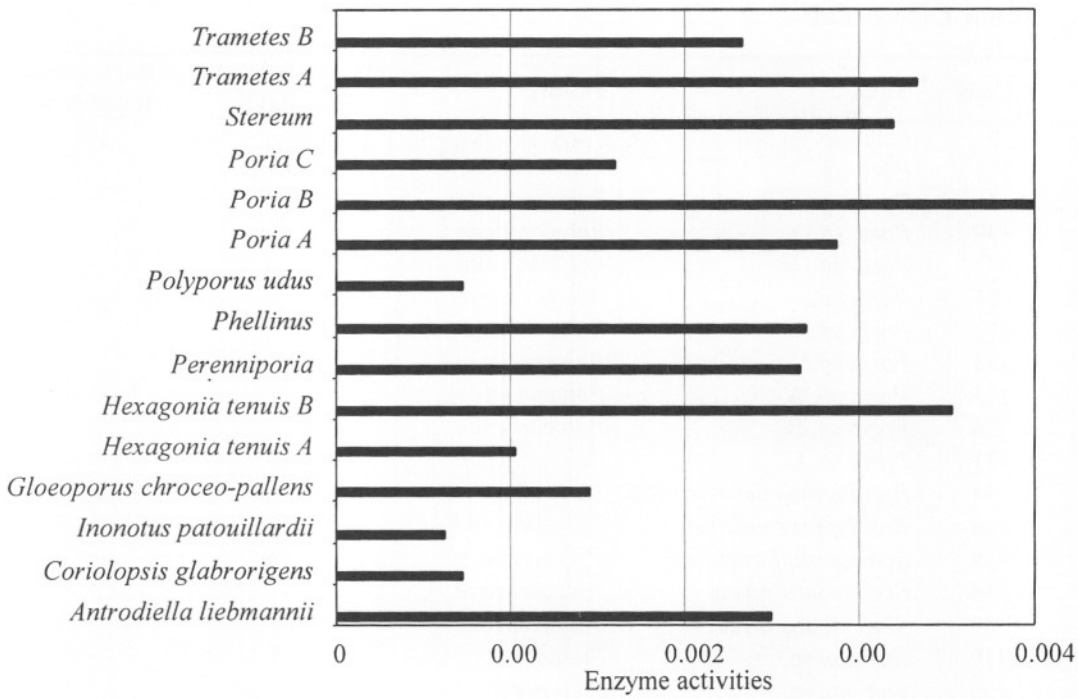

Fig. 1. Laccase Activities (ΔOD/ml).

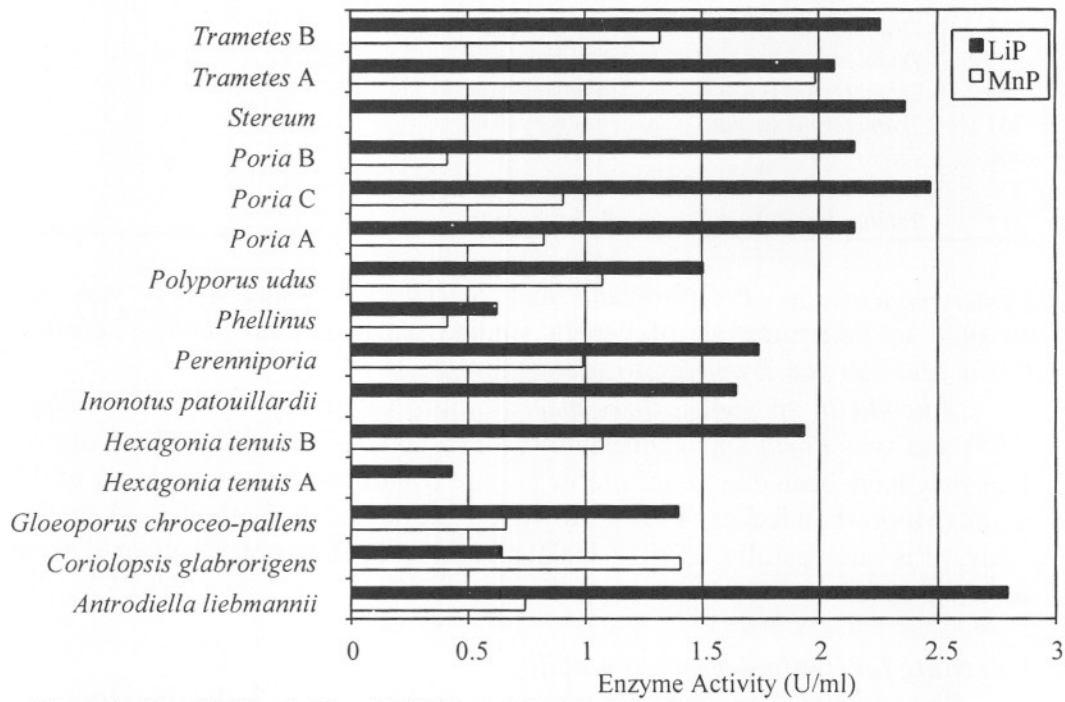

Fig. 2. MnP and LiP activities of 15 Selected Isolates (U/ml).

Table 2. Decolorization percentage of agar medium containing Poly R-478 by 15 selected isolates and their enzymatic test results.

Code	Taxa	Decolorization		Enzyme test		
		50% (day)	100% (day)	Laccase	MnP	LiP
392	*Antrodiella liebmannii*	6*	13	+	+	+
348	*Coriolopsis glabrorigens*	17	a	+	+	+
372	*Gloeoporus chroceo-allens*	11	a	+	+	+
329	*Hexagonia tenuis* A	a*	a	+	ND	+
364	*Hexagonia tenuis* B	14	a	+	+	+
358	*Inonotus patouillardii*	a*	a	+	ND	+
397	*Perenniporia* sp.	14	a	+	+	+
330	*Phellinus* sp.	a*	a	+	+	+
341	*Polyporus udus*	6*	a	+	+	+
315	*Poria* sp. A	a	a	+	+	+
326	*Poria* sp. B	11	12	+	+	+
381	*Poria* sp. C	6	14	+	+	+
405	*Stereum* sp.	10	13	+	ND	+
361	*Trametes* sp. A	10*	a	+	+	+
379	*Trametes* sp. B	7	21	+	+	+

a = not reached the percentage;
* = isolates decolorized the screening medium since the 1st day of incubation.
+ = presence of enzymatic activity; ND = none detected
Percentage of decolorization are given in mean values from 5 replicates.
The percentages are based on diameter of agar plates decolorization occurred during 21-day incubation. Diameters of agar plates are 90 mm.

from reddish-yellow to bright yellow. All of the selected isolates had begun to form clear zones after the 7th day of incubation. Out of these, six isolates (codes: 341,358, 361,392, 329 and 330) had decolorized the medium from the day one (Table 2). This early appearance of clearing zones was uncharacteristic of extensive depolarization, because only *Antrodiella liebmannii* could fully decolorize the screening medium. Furthermore, three isolates (*Hexagonia tenuis* sp. A, *Inonotus pattouillardii* and *Phellinus* sp.) caused less than 50% of depolarisation during the 21-day incubation. The formation of clear zones due to dye depolarization is indicative that test organisms are potential lignin degraders (Crawford, 1981; Field *et al.*, 1992; Paterson and Bridge, 1994. In the present study 15 selected isolates appear to be lignin degraders.

Antrodiella liebmannii, *Poria* sp. B and C, *Stereum* sp. and *Trametes* sp. B) decolorised the agar plates extensively, while in most others decolourisation was relatively inextensive (Table 2). We failed to detect plate decolorisation by *Ganoderma applanatum*, *G. lucidum* and *Trametes versicolor*, although the latter two isolates are well known lignin degraders (Addleman and Archibald,

1993; Pelaez *et al.*, 1995). The isolates showed mycelial growth (Table 1), which means that the isolates can grow in Poly R medium, a synthetic lignin medium. These fungi possibly require different culture conditions for their lignin degradation capabilities. This result also indicates the medium preference of fungi depending on their carbon sources. It has been reported that glucose, which is commonly used in media for fungal growth and ligninolytic studies, can repress laccase activity (Arora and Sandhu, 1985). This may be true for the fungi in this study that failed to decolorize Poly R-478. These fungi utilized sorbose, which is easier to consume, as their carbon source for growth, but do not depolymerize other polymeric compounds. Further studies using different culture conditions are therefore needed.

Enzymatic activity assay

Ligninolytic enzyme activity was detected in all selected isolates, but only 12 isolates demonstrated the simultaneous production of laccase, LiP and MnP (Table 2). This result indicates that such fungi do not require all three enzymes types at one time during the lignin degradation process. As described previously by de Jong *et al.* (1994), most wood-rotting fungi are able to produce three groups of enzymes, but the others produce only one or two groups.

We detected laccase activity in all of the selected isolates (Table 2 and Fig. 1). This indicates that laccase production is relatively common in the wood-rotting fungi tested. This result is similar to those presented in a review by de Jong *et al.* (1994) who found that laccase is produced by most white-rot fungi, but depending on the culture conditions. The varying laccase activity values of the isolates indicate that the ability to produce laccases vary, and depends on either isolates or strains. These differences between varieties probably result from differences in physiological function (Eggert *et al.*, 1996). The low values for laccase activity reported in this study may have resulted because the fungi were grown at unsuitable conditions, or because of the presence of inhibitors in the growth media (Eggert *et al.*, 1996). It may be necessary that we use an inducer to enhance laccase activities. Several substances can induce laccase production such as Indulin AT, tannic acid (Arora and Sandhu, 1985) and xylidine (Eggert *et al.*, 1996). MnP activity was detected in fewer isolates studied, than laccase and LiP activity. MnP activity appeared frequently among species of *Trametes* and *Poria* and was detected in 80% of the 15 test isolates (Table 2). The highest MnP activity was shown by *Trametes* sp. A (1.984 U/ml). *Inonotus patouillardii*, *Stereum* sp. and *Hexagonia tenuis* A did not show MnP activity. Thus, the three isolates may belong to LiP-Laccase group (white-rot fungi producing LiP and laccase;

Hatakka, 1994), who suggested three main categories of fungi based on enzyme production patterns. The negative MnP result, indicate that these fungi either produce no significant levels of this enzyme or that they require different growth conditions (Paice *et al.*, 1993). The latter is probably true for the *Stereum* strain, which did not show MnP activity in this study, but has previously been shown to be an MnP-producer after 21-day incubation (Pelaez *et al.*, 1995). We also found that *Hexagonia tenuis* B produced MnP, but *Hexagonia tenuis* A did not show MnP activity.

All test fungi produced LiP activity in varying amounts. Apart from *Coriolopsis glabrorigens*, LiP activity was found to be higher than MnP activity (Fig. 3). These results are contradictory to some previous studies that have used the same method of measurement, but failed to detect LiP production in basidiomycetes (Pelaez *et al.*, 1995; Buswell *et al.*, 1996; Schlosser *et al.*, 1997). Although the enzyme activity obtained here were relatively low when compared to other studies (e.g. Perez and Jeffries, 1990; Hamman *et al.*, 1997), the use of veratryl alcohol as a substrate for LiP activity appears to be suitable for the Lombok fungi. It is however, necessary to observe concentrations of the substrate in assay, nutrient limitations, as well as the incubation period that will give optimum values of LiP activity.

Various test fungi produced different combinations of the enzymes, even among the same genera. The different combinations of enzyme production may indicate different abilities of the fungi to degrade media containing lignin, which may be related to strategies for lignin biodegradation by wood-rotting fungi (Reid, 1995).

The use of wood-rotting fungi, especially their enzymes for biopulping, offers many potential advantages. It requires not only relatively low levels of chemicals and low cost and energy demands, but also eliminates the pollution hazards associated with the use of molecular chlorine pulping process (Akhtar *et al.*, 1992; Turner *et al.*, 1992).

Although the regulation and role of the ligninolytic enzymes in lignin degradation is still undetermined, laccase, MnP and LiP are the most common enzymes in biobleaching and biopulping. Hatakka (1994) has suggested that ligninolytic enzymes should be identified from fungi, especially in those species that produce all three important enzymes. One reason is that the practical importance of fungi apparently lacking MnP may be low, although the fungi readily produce LiP. For example, these fungi have not been the best candidates in screening programmes conducted for potential applications.

This study has demonstrated that 12 isolates produced all three enzymes tested (Table 2) and provides twelve isolates that can be investigated for

biopulping applications. However, further research on the culture conditions and enzymatic assay treatments for Lombok Polypores is needed.

The results of this preliminary study provide evidence that many wood-rotting fungal Polypores from Lombok, a small part of Indonesia, have high potential ability in lignin-modifying enzyme production due to their lignin degradation abilities. Many Indonesian wood-rotting fungi remain to be discovered. Hence, it is essential that we conduct similar studies to discover fungal Polypores from all over the country that may give promising results, which are applicable in lignin degradation-dependent industries to support an environmentally friendly paper industry.

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