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INVESTIGATION OF OIL PALM EMPTY FRUIT BUNCHES IN BIOSODA PULPING BY TROPICAL WHITE-ROT FUNGI, *Ganoderma australe* (FR.) PAT.

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ABSTRACT

Ganoderma australe, a white-rot fungus, is well-known as a decomposer of logs and stumps in the Malaysian forest. We investigated *G. australe* (KUM60848) strain for production of ligninolytic enzymes i.e. lignin peroxidase (LiP) and laccase, and hydrolytic enzymes, i.e. cellulase and xylanase, using oil palm empty fruit bunches (EFB) in solid-substrate fermentation (SSF). EFB was shown to support good mycelial growth for *G. australe* during 28 days of solid-substrate fermentation. All tested enzyme activities demonstrated highest activities of LiP (0.18±0.02 U/ml), laccase (1.92±0.03 U/ml), cellulase (0.72±0.11 U/ml) and xylanase (0.42±0.01 U/ml) throughout the 28 days of incubation. Compared to an untreated control, the pre-treated EFB yield increased to a maximum of 18% during biopulping. Fourteen days of SSF had the highest degree of material dissolved, as shown by pulp yields and the optimum values (29.8 Nm/g tensile index and 2.73 Kpa m^2/g burst index) acceptable to obtain paper sheets.

Key words: white-rot fungi, *lignocellulolytic* enzymes, biopulping, oil palm wastes

INTRODUCTION

Fungi are the main organisms responsible for wood biodegradation. The white-rot fungi are able to degrade lignin and carbohydrates efficiently. In Peninsular Malaysia, one of common white-rot species encountered is *Ganoderma australe* (Mohamad-Hasnul *et al.,* 2012). To degrade lignin, the white-rot fungi produce extracellular oxidative enzymes such as laccase, lignin peroxidase (LiP), manganese peroxidase (MnP) and low molecular mass compounds that mediate the action of these enzymes (Kirk and Cullen, 1998). This non-specific oxidative system makes white-rot fungi useful for biotechnological applications, for instance, in the pulp and paper industry for pitch control, biopulping and biobleaching (Akhtar *et al.*, 1998). In recent years, incubation of wood chips with white-rot basidiomycetous fungi has been studied as a pre-treatment step in mechanical or chemical pulping.

Biopulping is a process of treating wood chips with lignin-degrading fungi prior to pulping. Pre-treatment of wood with lignin-degrading fungi, especially white-rot fungi, enables modification of the wood and lignin, with the aim of easing fibre separation and lignin removal in the mechanical or chemical pulping process, respectively. Various species of white-rot fungi have been used in biopulping: *Ceriporiopsis subvermispora* has proven to be very competitive both on softwoods and hardwoods (Ferraz *et al.*, 2007). White-rot fungi and their extracellular enzymes (especially ligninases and xylanases) are used for the treatment of wood chips prior to pulping. While ligninases attack the lignin content of wood, xylanases degrade hemicellulose and make the pulp more permeable for the removal of residual lignin (Singh *et al.*, 2010). The list of benefits reported for using this bio-treatment includes energy saving during defibration, an increase in the rate of delignification, a decrease in alkali consumption and improvement of pulp strength properties (Akhtar *et al.*, 1998; Mendoça *et al.*, 2002).

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Oil palm (*Elaeis guineensis*) is the most important species in the *Elaeis* genus belonging to the family Arecaceae. One hectare of oil palm plantation can produce about 50–70 tonnes of biomass residues (Malaysian Palm Oil Board, 2006). Oil palm biomass is classified as lignocellulosic residue that typically contains 50% cellulose, 25% hemicellulose and 25% lignin in its cell wall (Gu *et al.*, 2000). This biomass is suitable as a raw material for the production of pulp and paper, composites, carbon products and for chemical extraction. There is increasing interest in using oil palm EFB to produce pulp and paper in Malaysia (Gurmit *et al.*, 1999; Rushdan, 2002; Kamarudin *et al.*, 2008; Rosnah *et al.*, 2010). With its large cultivation of oil palm (4.85 million hectares), empty fruit bunches are available in sufficient quantity (an estimated five million tons a year, dry weight) (Kamarudin *et al.*, 2008). The EFB are composed of 45–50% cellulose and about equal amounts (25–35%) of hemicellulose and lignin (Deraman, 1993), a potential raw materials for pulp and paper.

Our study evaluated the extracellular enzyme production of *G. australe* growth as a result of solid substrate fermentation of EFB, which could be useful in biopulping. To achieve this goal, the *G. australe* strain was evaluated for the extracellular lignocellulolytic enzyme activity during solid-substrate fermentation, and for the effect of biopulping of EFB on paper properties.

MATERIALS AND METHODS

Sample collection

Shredded oil palm empty fruit bunch were obtained from a local palm oil processing company, Seri Ulu Langat Palm Oil Mill, Dengkil, Selangor, Malaysia. The sample was collected in autoclaved plastic bags and stored at 4–8ºC. The EFB was thoroughly washed to ensure it was dust free, and then dried in an oven at 60ºC until a constant weight is obtained.

Ganoderma australe culture

Mycelia culture of the studied *G. australe* (KUM60848) was obtained and maintained in the Mushroom Research Centre, University of Malaya. This strain was used based on its ability to produce lignocellulolytic enzymes (Mohamad-Hasnul *et al.*, 2007).

Solid-substrate fermentation to profile extracellular enzymes

Fresh cultures of *G. australe* were maintained on malt extract agar (MEA) at 25 ± 2ºC in dark conditions. In this study, we used solid substrate fermentation of dry oil palm EFB. The contents were autoclaved (15 min, 121ºC, 15 psi). After cooling, the contents were inoculated into 10% (3.0 ± 0.5 g) of colonized wheat grain and then incubated in the dark at 25 ± 2ºC for 28 days. The crude extract was prepared according to Avneesh *et al.* (2003). The solid-substrate cultures were sampled at seven days intervals, starting from day zero until day 28. The mixture was then homogenized at a speed of 11000 rpm for 5 min. Filtered solution was then centrifuged at 9000 rpm for 24 min at 4ºC. Finally, the designated supernatant was used for the enzyme assay.

Enzymatic activity assay

All enzyme assays were spectrophotometrically determined and performed in triplicate at room temperature. The assays contained 0.2 ml supernatant in a final volume of 3 ml. Lignin peroxidase (LiP) activity was determined according to Tien and Kirk (1984). The assay solution contained 3mM veratryl alcohol (0.2mM final concentration), 100mM sodium tartrate buffer at pH 3.0 and 7.5mM H_2O_2 (0.5mM final concentration). LiP activity was measured by oxidation of veratryl alcohol to veratryldehyde in the presence of H_2O_2 at pH3.0. The reaction was initiated using 0.2ml of 7.5mM H_2O_2. The enzyme activity was measured at λ=310nm after 5 min. One unit of enzyme activity was expressed as 1µmol veratraldehyde (VAD) released per minute.

Laccase activity was assayed according to Leonowicz and Grzywnowicz (1981). The assay solution contained 0.1mM syringaldazine (4-Hydroxy-3,5-dimethoxybenzaldehydeazine) in 50% ethanol. The substrate was dissolved in 50% ethanol and 50mM sodium citrate buffer (pH 4.8). The enzyme solution was mixed with buffer at room temperature. 0.1mM syringaldazine (substrate) was then added and mixed with a vortex mixer to start the reaction. The initial rate of colour change was measured on a spectrophotometer at a wavelength of λ=525nm. One unit was defined as the enzyme producing one unit of absorbance change/min.

Carboxymethycellulase activity was assayed according to Miller (1959). The assay solution contained a 1% (w/v) solution of sodium carboxymethyl cellulose (medium viscosity) in 50 mM sodium citrate buffer (pH4.8), 3.0 ml of Dinitrosalicylic (DNS) acid reagent, 1.0 ml of Rocchele salt (40%) and 50 mM sodium citrate buffer (pH4.8). The activities were expressed as 1 µmol glucose released/min/g substrate, defined as the change in absorbance at 575 nm.

Xylanase activity measurement was based on the sugar reaction in presence of dinitrosalicylic (DNS) reagent (Miller, 1959). Pure xylose was used as the standard. About 1.8 ml substrate solution was

mixed with 0.2 ml of enzyme solution. The mixture was mixed and incubated for 1h at 40ºC in a water bath with moderate shaking. The reducing sugar released in the reaction was determined using DNS method. The change in absorbance was monitored at 575 nm. One unit of enzyme activity was expressed as 1µmole of xylose released per minute.

Biopulping of oil palm EFB after solid substrate fermentation by *G. australe*

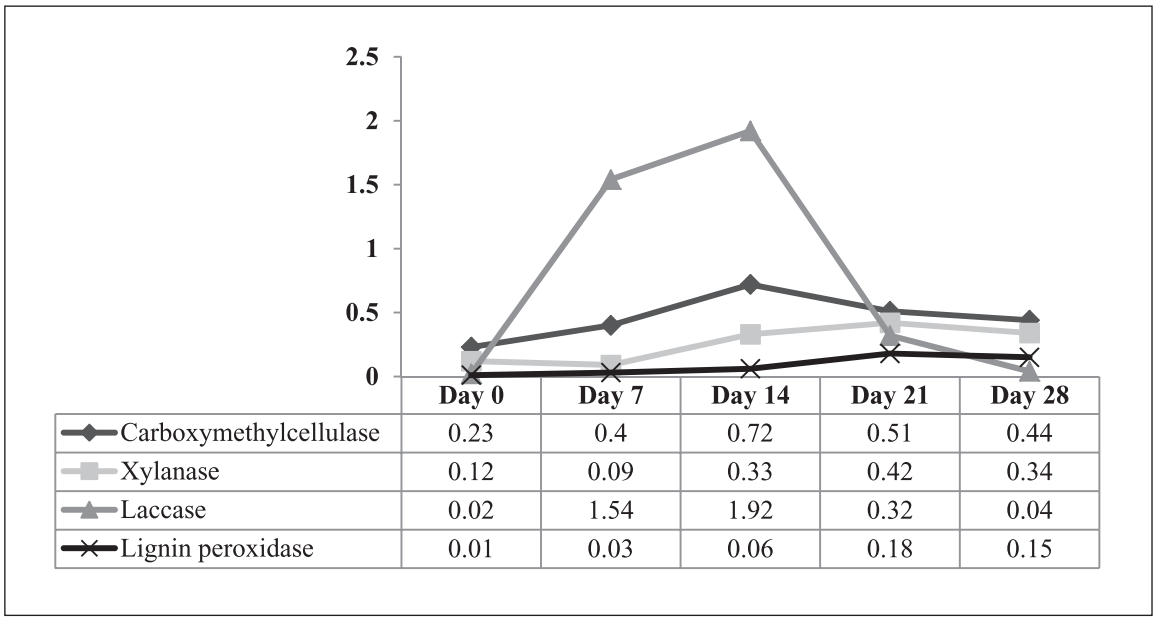
After 14 and 21 days of fermentation by *G. australe*, the EFB was pulped by soda pulping process. A control pulping was also performed on EFB without any pre-treatment. Pulping trials were carried out in a Weverk Rotating digester. The pulping conditions employed were: Maximum cooking temperature: 170ºC; EFB to liquor ratio: 1:7 (w/v); Amount of NaOH: 25% of EFB dry weight (Wanrosli *et al.,* 2004). The total pulp yield was calculated as the sum of the screened pulp yields and the sieves. The physical strength of the pulp hand sheets was determined according to the TAPPI Test Method (T 220 sp-96 and T 441 om-89) and Malaysian Standard (MS ISO 1924-2:1999, MS ISO 2758:2002 and MS ISO 5626:1999).

RESULTS

The enzyme activity of *G. australe* was evaluated during solid-substrate fermentation of EFB. For the periods varying between seven and 28 days, EFB supported mycelial growth of *G. australe* well. During the treatment periods, the fungus rapidly colonized EFB, with formation of an abundant mycelia mass after the first week of incubation. In this study, pre-treated EFB samples were extracted with extraction buffer solution to recover the extracellular enzymes produced during the fungal growth. Results indicated that *G. australe* produced LiP, laccases, cellulases and xylanases during the treatment period. Extracellular enzymes produced during this experiment were extracted and quantified.

LiP activity was observed over the 28 days of pre-treatment (Fig. 1). The maximum LiP activity was observed on day 21, with 0.18±0.02 U/ml. The amount of laccase activity recovered was higher than LiP throughout the treatment period, with a maximum 1.92±0.03 U/ml on day 14. With increasing incubation period, the amount of laccase produced by *G. australe* decreased to 0.04±0.02 U/ml (after 28 days of treatment).

The cellulase enzyme was also assayed apart from ligninolytic enzymes in order to assess the applicability of the fungi test strain for biopulping purposes. The results showed that the maximum cellulase activity was 0.72±0.11 U/ml on day 14 (Fig. 1). This showed that the *G. australe* strain produced low cellulase activity throughout the incubation period. Even though the cellulase activity was slightly higher compared to LiP activity, its production was significantly lower compared to laccase on days 7 and 21. As for xylanase, the maximum activity of 0.42±0.01 U/ml was observed on day 21of EFB pre-treatment.



	Day 0	Day 7	Day 14	Day 21	Day 28
Carboxymethylcellulase	0.23	0.4	0.72	0.51	0.44
Xylanase	0.12	0.09	0.33	0.42	0.34
Laccase	0.02	1.54	1.92	0.32	0.04
Lignin peroxidase	0.01	0.03	0.06	0.18	0.15

Fig. 1. *Ganoderma australe* KUM60848 enzyme activity (U/ml).

Untreated control and pre-treated EFB were submitted to pulping for 14 and 21 days under the conditions previously described. A fourteen day period of bio-treatment was chosen based on the highest enzyme activities of laccase produced by *G. australe*. The 21 day period was chosen based on the highest activity of LiP and xylanase, whereby the laccase activity was detected at low levels. The selections were made on both selected days to evaluate the paper properties produced at different pre-treatment periods.

The yields of pulp from untreated and pre-treated EFB (14 and 21 days) after cooking process were 30.3% and 35.7% (14 days); 28.5% (21 days) (w/w) of the original EFB added, respectively (Table 1). SSF at 14 days showed the highest degree of material dissolved, as indicated by yields. The pre-treated EFB has increased the yield to a maximum of 18% (at 14 days) and decreased to 6% (at 21 days) compared to untreated EFB.

The paper properties are presented in Table 2. The pulping process influenced the paper properties i.e. grammage, thickness, tensile index, burst index and the number of folds. The effect of a 14 and 21 day period of fungal pre-treatment with EFB on fibre strength in paper was measured as tensile and burst index. At 14 days of pre-treatment, the tensile index was reduced by 11%, whereas the burst index was higher by 14% when compared to untreated EFB. On the other hand, after 21 days of pre-treatment, the tensile index was reduced by 6% and the burst index by 13%. The decrease in paper strength caused by *G. australe* was at its lowest on day 14. Furthermore, the thickness and number of folds were higher compared to untreated EFB.

Table 3 shows the optimal results obtained in this work, as well as those of other authors, for EFB pulp, wheat straw and olive wood. As observed, the values contributed by Jiménez *et al.* (2009) and Wanrosli *et al.* (2004) for tensile index and burst index are quite inferior to those obtained in this work; in the work of Jiménez *et al.* (2009) the values are 170ºC, 70 min and 15% soda; in the work of Wanrosli *et al.* (2004) to the values are 160–162ºC, 63–64 min and 21.7–23.4% of soda, versus 170ºC, 120 min and 25% soda used in this work.

DISCUSSION

White-rot fungi produce various enzymes involved in lignin degradation, but also produce cellulases, xylanases and other hemicellulases (Hatakka, 1994, 2001). According to Hatakka (2001) and Wong (2009), the enzymes involved in lignin degradation are lignin peroxidase, laccase, manganese peroxidase, versatile peroxidase and H_2O_2-forming enzymes such as glyoxal oxidase and aryl alcohol oxidase.

Table 1. The effect of biopulping by *G. australe* KUM60848 on pulp yield and alpha cellulose content of EFB, at 14 and 21 days

Days of treatment	Yield (%)	Alpha cellulose (%)
14	35.7	79.1
21	28.5	77.4
Untreated EFB	30.3	72.0

Table 2. The effect of biopulping of EFB using *G. australe* KUM60848 on paper properties, at 14 and 21 days

Days of SSF fermentation	Grammage (g/m^2)	Thickness (µm)	Tensile index (Nm^2/g)	Burst index (kPa m^2/g)	No of folds
14	61.9	125±2.21	27.0±2.81	2.53±0.197	12±3.92
21	60.1	108±2.20	28.5±0.99	1.87±0.086	2±0.91
Untreated EFB	60.8	119±2.22	30.2±1.32	2.22±0.095	5±1.46

Table 3. Comparison of various pulps beaten from non-wood

Type of pulp	Yield (%)	Tensile index (Nm/g)	Burst index (kPa m^2/g)
Pre-treated EFB beaten soda pulp	35.70	29.81	2.73
EFB soda-anthraquinone pulp beaten (Jiménez *et al.*, 2009)	39.0	59.63	4.17
EFB soda pulp (Wanrosli *et al.*, 2004)	41.0	35.75	
Wheat straw beaten soda pul (Jiménez *et al.*, 2002)	53.15	45.70	1.81
Olive wood kraft beaten pulp (Díaz *et al.*, 2005)	25.56	39.0	1.95

The low levels of LiP activity detected in the EFB showed that LiP might be of minor significance in the delignification of EFB. However, the low detectable level might also be caused by interference that could possibly have occurred upon assaying LiP in extracts recovered from cultures grown on lignocellulosic materials (Liew *et al.,* 2011). Moreover, a typical assay based on oxidation of veratryl alcohol suffers from the interference of dissolved aromatic compounds present in wood extracts (Archibald, 1992).

Laccase was detected as the main oxidative enzyme in the extracts. In co-operation with other ligninolytic fungal enzymes such as LiP, manganese peroxidase and versatile peroxidase, laccase oxidizes lignin and, together with the polysaccharides cellulose and hemicellulose, comprises the principal components of wood (Sjöström, 1993). According to Regis *et al.* (2008), *G. australe* also produced laccase during biodegradation of *Eucalyptus globolus* with values reaching 60 IU kg^{-1} of wood (after 45–60 days of incubation).

In paper manufacturing, fungal treatment has been suggested because it can reduce the energy cost of refining pulp. Although fungal cell walls may be beneficial to the paper product, the use of xylanases may reduce the treatment time of pulps (Ken *et al.,* 1988). Xylanases may also yield a range of desirable pulp characteristics by selectively hydrolyzing certain xylan components. Total removal of xylan from fibre is not necessarily desirable, because xylan contributes significantly to fibre strength, and thus to paper quality (Clark, 1978).

The application of white-rot fungi to EFB is an attractive method, which increases the pulp yield and improves the properties of hand sheets produced. Values of yield and burst index for the beaten pulp of olive wood were lower than those values obtained for EFB beaten pulp, independently of the beating grade which is different from one pulp to another. However, the value of the tensile index is higher for wheat straw soda beaten pulp in comparison with the EFB beaten pulp, except for the higher value given by Wanrosli *et al.* (2004). Finally, it is possible to conclude that the EFB pretreated with white-rot fungi produces beaten pulps the burst properties of which can compete with and exceed pulps of two singular raw materials i.e. wheat straw, widely known and used globally, and olive wood, a single raw material with a ligneous structure such as hardwood and softwood.

Based on these results, we found that the *G. australe* evaluated in this work showed good potential for the production of extracellular lignocellulolytic enzymes. In the experiment concerning biopulping of EFB, we showed that the properties of paper were influenced by the pre-treatment of *G. australe*. The values contributed by Jiménez *et al.* (2009), and Wanrosli *et al.* (2004), for tensile index and burst index are quite inferior to those obtained in this work; the work by Jiménez *et al.* (2009) operated at 170ºC, 70 min and 15% soda; the work by Wanrosli *et al.* (2004) operated at 160-162ºC, 63-64 min and 21.7-23.4% of soda, versus 170ºC, 120 min and 25% soda used in this study.

Values of yield and burst index for the pulp beaten of olive wood was lower than those values obtained for EFB pulp beaten, independent of the beating grade which differ from one pulp to another. However the value of tensile index is higher for wheat straw soda pulp beaten, versus the EFB pulp beaten except for the higher value given by Wanrosli *et al.* (2004). A comparison of Burst Index along with the other agricultural residues considered (Jiménez and López, 1993; Jiménez *et al.,* 1993; Díaz *et al.,* 2005), reveals the following: The Burst Index for EFB (14 days incubation); 2.53 kPa.m2/g was higher than those for vine shoots and olive trimming residues, which revealed a maximum value of 0.99 kPa.m2/g and 0.58 kPa.m2/g respectively. The 14 days incubation was similar to those for sunflower stalks (1.62-3.22 kPa.m2/g) and cotton plants stalk (2.09-4.15 kPa.m2/g), but lower than those for sorghum stalks (4.2-5.3 kPa.m2/g). It can be observed that the only parameter where pre-treatment of EFB with *G. australe* showed a clearly better performance was the burst index during 14 days of incubation. Burst Index and Resistance to Folding (No. of Folds) were higher for 14 days compared to 21 days incubation, thus indicating that duration of incubation of 14 days with *G. australe* showed better properties for paper production from EFB. Therefore, it is possible to conclude that the pretreated EFB with *G. australe* give pulp beaten whose burst properties can compete and overcome the pulps of two raw materials i.e. wheat straw and olive wood.

On the other hand, it was also noticeable also that the strain culture conditions applied may not favour the production of ligninolytic enzymes. Nevertheless, the limited detectable amount of LiP should not be neglected, because it is equally important in causing significant delignification. In conclusion, we found that the *G. australe* strain investigated in this work has potential as a lignocellulolytic organism; some further process optimization is needed to use this fungus as an alternative in biotechnological applications, such as biopulping.

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