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The Effect of Physical and Biological Pre-treatments of Oil Palm Fronds on *in vitro* Ruminal Degradability

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ABSTRACT

Physical pre-treatment of the oil palm frond (OPF) is known to loosen the lignocellulose while the biological pre-treatment is capable in degrading the lignin, making the substrates more accessible for rumen microbes. This study aimed at assessing the efficacy of physical, biological and combination of both pre-treatments of OPF on the *in vitro* ruminal degradability. Five different samples of OPF pre-treatments were used in this study; OPF was subjected to the physical pre-treatment (POPF), OPF to the biological pre-treatments using an enzyme extract of each *Ganoderma lucidum* (BGL) and *Lentinula edodes* (BLE), respectively. Another two samples were subjected to a combination of physical and biological pre-treatments of *G. lucidum* (CGL) and *L. edodes* (CLE) respectively. The control was non-treated OPF. Two fistulated Katjang goats consuming 440 g/kg OPF and 897 g/kg commercial pellet daily on dry matter basis were used as rumen fluid donors. *In vitro* incubation was carried out at 39°C for 24 hours. Proportions of volatile fatty acid were measured at the end of incubation by gas chromatography. Results showed that concentrations of lignin following all pre-treatment methods were significantly

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lower ($p<0.05$) at 150(POPF), 90(BGL), 119(BLE), 100(CGL) and 120(CLE) g/kg DM as compared to the FOPF (190 g/kg). After 24 hours of incubation, the cumulative gas of all treatment groups differed significantly from FOPF. Both BGL and CGL showed significantly higher propionate and butyrate concentrations as well as apparent rumen degradable carbohydrate with 6.57 mg and 6.54 mg, respectively as compared to the FOPF. It appeared that BGL and CGL resulted in higher lignin degradation that increased the *in vitro* rumen degradability. In conclusion, biological pre-treatment with enzyme extract of WRF, either alone or in combination are promising to improve the quality of OPF.

Keywords: Biological pre-treatment, *in vitro* ruminal degradability, lignin, oil palm frond, physical pre-treatment, white rot fungi

INTRODUCTION

Oil palm industry generates abundant amounts of by-products including oil palm fronds (OPF). The industry produces approximately 83 million tons of OPF annually. Oil palm frond has been widely utilized as ruminant feed as an alternative to grasses or roughages when feed is in short supply. Whole OPF consists of petiole and leaflets, which are fed to ruminants following chopping, and usually in combination with other feedstuff as total mixed rations. Indeed, adding the OPF in the diet of beef and dairy cattle could support live weight gain between 0.6 and 0.8 kg per day and milk yield of about 22 litre per day (Wan Zahari et al., 2003). Despite the previous reports of OPF as a promising source of roughage for ruminants, the use of OPF for ruminant feeding is still limited. In addition, OPF cannot be fed solely as animal feed due to its poor nutritive values with low metabolisable energy of 4.9 to 6.5 MJ/kg dry matter (DM) (Dahlan, 2000). The lignin content of OPF is also high at an average of 205 g/kg DM (Abdul Khalil et al., 2006) and can impair OPF intake and digestibility. These problems have encouraged lots of studies on investigating different technologies to improve the feeding value of OPF.

To date, some physical pre-treatments have been developed to upgrade the OPF, which include pressing using conventional sugarcane machine (Zahari et al., 2012), pelleting, grinding, chopping and steaming. Wan Zahari et al. (2003) reported that pelleting, grinding and steaming processes increased the intake and digestibility of OPF hence, improved the feed intake and growth performance of cattle. Furthermore, physical pre-treatment on OPF is considered practical, cost effective, easily operated, apart from low risk of health and safe.

The OPF could also be improved by chemical reactions using alkaline solutions such as sodium hydroxide (NaOH), ammonia (NH_3) and urea. This chemical pre-treatment requires maintenance of low temperature and pressure as compared to other pre-treatment strategies (Mosier et al., 2005). In addition, treatment with NaOH is also detrimental to the palatability of OPF in cattle, thus, urea pre-treated rice and wheat straws are considered safer, more palatable and cost-effective than NaOH

pre-treatment (Walli, 2010). However, there are some factors that should be considered for successful urea pre-treatment which are urea level, moisture content and duration of pre-treatment.

Studies have shown that biological pre-treatment with white rot fungi (WRF) can effectively degrade lignin and enhance digestibility of various lignocellulosic biomass (Hassim et al., 2012; Metri et al., 2018). In addition, the biological pre-treatment improves nutrient digestibility of poor quality roughages including wheat straw (Tuyen et al., 2012), rice straw (Sharma & Arora, 2010), sugarcane bagasse (Tuyen et al., 2013) and OPF (Rahman et al., 2011). In fact, *G. lucidum* and *L. edodes* could lessen and degrade approximately 40% of lignin content in the OPF (Rahman et al., 2011). However, the consequence of biological pre-treatment using WRF is time consuming which leads to dry matter losses (Rahman et al., 2011) due to the fungal metabolism. Nevertheless, study has shown that this limitation can be overcome if the WRF pre-treatment is done by using enzyme extract of that WRF as compared to the WRF mass. Indeed, the dry matter loss in wheat straw has been reported to reduce after using the enzyme extracts isolated from *Trametes versicolor*, *Bjerkandera adusta* and *Fomes fomentarius* (Rodrigues et al., 2008). This finding indicated that pre-treatment of wheat straw with enzyme extract isolated from WRF degraded the lignocellulose without the unnecessary polysaccharide consumption.

Although a number of studies has been conducted on physical and biological pre-treatments of OPF, combination of physical and biological pre-treatments using an enzyme extract of each *G. lucidum* and *L. edodes* have not yet been applied to OPF. Therefore, this study was carried out to assess the efficacy of physical, biological and the combination of both pre-treatments of OPF on *in vitro* ruminal degradability. The physical pre-treatment was done by pressing the OPF using conventional sugarcane machine, whereas the biological pre-treatment was done by pre-treating OPF with enzyme extracts from two types of WRF (*G. lucidum* and *L. edodes*).

MATERIALS AND METHODS

Oil Palm Fronds

Fresh OPF from 7-year old palm trees were used which were obtained from a palm plantation located in Felda Kemahang, Kelantan, Malaysia. The petiole of the OPF with leaflet was chopped off at approximately 2 metres length, which was 1/3 of the whole OPF length. The petioles were stored under shade at ambient temperature of 28−30^{o}C and relative humidity of 75–95% prior to pre-treatment. Sample weight and moisture content were recorded every 24 hours in a storage condition.

Physical Pre-treatment of OPF

Fresh whole OPF were taken with leaflets and chopped between 50 and 60 cm length. Then, they were pressed using a conventional sugarcane pressing machine

with three heavy duty steel rollers with specification of power 1500 watt and speed at 1400 RPM (Prasad et al., 2012; Zahari et al., 2012). The chopped OPF were inserted into the chamber and the spinning rollers pressed the petioles and leaflets to obtain the sample. The sample of whole OPF pressed fibre was dried at 60°C overnight prior to the chemical and *in vitro* analyses.

Biological Pre-treatment of OPF with Enzyme Extracts from WRF

Fungal Strains. Two white rot fungi, *G. lucidum* strain ATCC 64251 and *L. edodes* strain ATCC 52998 were cultivated in potato dextrose agar (PDA) plate at 30°C for 7 days. The fungi were then transferred into fresh PDA plate and incubated for another 7 days at 30°C. Seven-mm-diameter plugs were cut from each of fungal colony grown on purified culture, placed into a new PDA agar plate and cultured for 5 days to observe mycelia growth.

Enzyme Extraction

The enzyme extraction was performed in quadruplicate, as described by a published study (Dias et al., 2010). Enzymatic extracts were obtained from solid culture media containing 15 g of ground OPF with 22.5 mg of glucose in 250 mL Erlenmeyer flasks (Dinis et al., 2009). After that, 45 ml of deionised water was also added into the flask.

All flasks were autoclaved at 121°C for 20 min prior to treating OPF with WRF. After cooling, three plugs (10 mm in diameter) were taken from the isolated fungus and added to the sterile flask containing the ground OPF. The flask was then incubated at room temperature (37°C) for 45 days (Azmi et al., 2016). The content of culture flask was suspended in 150 ml of deionised water and the flask was placed on a rotary shaker (100 rpm) for 3 hours. Enzyme extracts were filtered (Whatman GF/A) and 0.06 g polyvinyl polypyrrolidone (PVPP) was added before centrifugation at 12000 × *g* for 10 minutes. The aliquots were used for determination of enzyme activity according to Azmi et al. (2016).

Oil Palm Frond Pre-treatment with Enzyme Extracts from WRF

Pre-treatments of OPF with each of the enzyme extracts were performed in triplicate. About 8 ml of the culture media of enzyme extracts were collected into 250 ml Erlenmeyer flask containing 70 ml citrate buffer (50 mM; per liter of distilled water: 10.5 g $C_6H_8O_7.H_2O$ and 14.7 g $C_6H_5Na_3O_7.2H_2O$; pH5.0) and 11 g of OPF. The flasks were autoclaved at 121°C for 20 minutes. After cooling, 20 ml enzymatic extract, 6.7 ml MnSO4 and 1 ml H_2O_2 were added into the flasks. The flasks were then put in a forced air oven at 40°C for 5 days (Hassim, 2012). Oil palm frond residues were obtained after filtration through paper filter. Samples were immediately dried in a forced air oven at 60°C, ground to pass a 1 mm screen (Retsch, Cutting mill, model SM1, Haan, Germany) and stored in airtight flasks at room temperature for later chemical analysis and *in vitro* incubation with rumen fluid.

Experimental Design

The experimental design was completely randomized with three replicates per treatment. The non-treated OPF (FOPF) was considered as control group in this study. The treatments included physical pre-treatment of OPF (POPF), biological pre-treatment of OPF with enzyme extracts from two WRF which were *G. lucidum* (BGL) and *L. edodes* (BLE) and combination of physical and biological pre-treatments of *G. lucidum* (CGL) and *L. edodes* (CLE) respectively.

Chemical Analyses

Proximate analysis. All samples used in the current study namely the control sample (FOPF), POPF, BGL, BLE, CGL, CLE and goat concentrates were analysed for chemical compositions (g/100g DM). Dry matter content of samples was determined by oven-drying at 105°C for 24 hours based on standard analytical method (Association of Analytical Chemistry [AOAC], 1990). Ash content was determined by combustion at 550°C for 4 hours in a muffle furnace. Determination of crude protein (CP) involved three different stages. First stage was the digestion process of the sample with concentrated sulfuric acid (H_2SO_4), followed by distillation process with sodium hydroxide (NaOH) by Kjeldahl system and finally titration against acid. The amount of nitrogen (N) found were converted to crude protein (CP= N*6.25. Crude fibre (CF) was determined by washing and boiling the sample in H_2SO_4 and NaOH using fibre bag technique. Ether extract was determined by extraction with petroleum ether. All experiments were done in triplicate.

Lignocellulose Content. The samples of FOPF, POPF, BGL, BLE, CGL and CLE were weighed 1 g per fibre bag. Insoluble fibres in the samples included cellulose, hemicellulose and lignin (Van Soest, 1963) were measured as neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) based on the Gerhardt application fibrebag-system protocol. All experiments were done in triplicate.

Neutral Detergent Fibre Analysis. Fibre bags were dried for 1 hour at 105°C and were allowed to cool in desiccator for 30 min. Approximately 1 g of sample was weighed in fibre bags. Neutral detergent fibre solution was prepared using EDTA ehtylenediamine tetra acetic acid-disodium salt and disodium tetra borate-decahydrate, dodecylsulphate-sodium salt, 2-ethoxyethanol, sodium dihydrogenphosphate and heat-stable α-amylase. The NDF (%) were determined as follow using the blank value as shown in equation below:

$$\text{NDF (\%)} = \frac{(m_3 - m_1) - (m_4 - m_5)}{m_2} \times 100$$

where m_1 – weight of fibre bag (g), m_2 – initial sample weight (g), m_3 – weight of crucible with dried fibre bag and sample residue after digestion, m_4- weight of crucible with ash (g), m_5 – blank value of empty fibre bag (g)

Acid Detergent Fibre (ADF) Analysis. Acid detergent fibre solution was prepared by diluting N-cetyl-N, N, N-trimethyl-ammoniumbromide in sulphuric acid. The protocol of ADF analysis was similar to the NDF analysis.

Acid Detergent Lignin (ADL) Analysis. For the ADL procedure, the ADF procedure was used as a preparatory step. However, the components of cellulose and lignin were not eluted from the feed by the acid detergent solution. The cellulose was therefore dissolved with 72% sulphuric acid in order to receive the crude lignin (ADL). Hemicellulose was calculated as NDF – ADF and cellulose as ADF – ADL (Van Soest, 1963).

***In vitro* Incubation with Rumen Fluid and Analysis.** The *in vitro* incubation with ruminal fluid was performed in syringes (100 ml volumes) according to the published method (Rahman et al., 2011). Rumen fluid was collected from two fistulated male Katjang goats before morning feeding at 0800h. Both goats were fed with OPF and a commercial pellet. The goats were cared for in accordance to the animal ethics guidelines of the Universiti Putra Malaysia (UPM/IACUC/AUP-R039/2016). The rumen fluid was mixed in a mixture (Waring Products Division, New Hartford, USA) for 30 seconds and filtered through four layers of cheesecloths. The rumen fluid (5 mL) was mixed with 20 ml of bicarbonate and phosphate buffer in 100 mL sterile gas-tight syringes containing 0.25 g of each sample (FOPF, POPF, BGL, BLE, CGL and CLE) following the modified method by Menke (1988). Air was removed from the syringes before the tip was closed. All treatments were done in triplicate and incubation was done at 39°C for 24 h in the oven. Syringes were shaken carefully to ensure complete mixing of the incubated contents. Gas production was measured and recorded by reading the scale on the syringe at 0, 2, 4, 6, 8, 10, 12 and 24 h of incubation. Following 24 h incubation, the samples were acidified with 25% metaphosphoric acid in water, centrifuged (10 min, 4°C at 15,000 × *g*) and filtered before the filtrate was used to determine the VFA.

Determination of Volatile Fatty Acid and Apparent Rumen Degradable Carbohydrate

At 0 and 24 h of incubation, 10 ml of rumen fluid was collected for VFA analysis and acidified with 0.2 ml phosphoric/formic acid (10/1, v/v). Samples were centrifuged for 10 min at 15,000 x *g* and the supernatant was recovered for VFA analysis by gas chromatography (Shimadzu GC-14A, Shimadzu Corporation, Hertogenbosch, The Netherland). Apparent rumen degradable carbohydrate (ARDC) was calculated based on the equation below (Rahman et al., 2011):

$$\text{ARDC (mg)} = \frac{\text{Ac}}{2} + \frac{\text{Pr}}{2} + \text{But} * \frac{162}{1000}$$

with 162 the assumed molecular weight of 1 mol fermented carbohydrates (Demeyer, 1991) and Ac, Pr and But expressed as net micro-molar production.

Statistical Analysis

Statistical analyses were performed using Statistical Package for Social Science 20.0 (SPSS software for Windows, release 20.0 SPSS, Inc., Chicago, IL, USA). All parameters were statistically evaluated separately using a one-way analysis of variance (ANOVA) at a significance level of 5% between the controls, physical and biological pre-treatments of OPF. Thus, the null hypothesis was rejected when $P<0.05$.

RESULTS AND DISCUSSION

Chemical Composition of Pre-treated OPF

Chemical compositions of FOPF, POPF, BGL, BLE, CGL and CLE are shown in Figure 1. The content of CF showed significant ($p<0.05$) difference in all pre-treated OPF as compared with FOPF (control), except following physical pre-treatment. However, the physical, biological and combined pre-treatments did not change the cellulose content of the OPF ($p>0.05$). The hemicellulose contents of all pre-treatments decreased significantly ($p<0.05$) compare to control, but no significant ($p<0.05$) differences were observed among pre-treatment. Similarly, the lignin contents of all pre-treated OPFs showed significant ($p<0.05$) decrease as compared with the non-treated OPF ($p<0.05$) and treatments with BGL and CGL showed significant ($p<0.05$) decrease as compared to other pre-treatments. The content of CP was highest in both BGL and CGL (80 g/kg DM), followed by CLE (78.2 g/kg DM), BLE (75 g/kg DM), FOPF (50 g/kg DM) and POPF (47.07 g/kg DM).

In the current study, fresh OPF consisted of 21% hemicellulose, 49% cellulose and 19% of lignin (Figure 1), nearly similar value with the 19.2% hemicellulose (Hong et al., 2012) and 20% lignin reported earlier (Dahlan, 2000), except for the cellulose (31.5%). Previous study also reported higher content of hemicellulose at 30.34% (Hermiati et al., 2013) in OPF obtained from Banten, Indonesia. This may be due to factors such as geographic location, age and climate. In the current study, fresh OPF from 7-year old palm trees were used. Furthermore, handling and different portion of OPF used may also contribute to the difference of chemical composition. In the current study, 1/3 of the whole OPF length was used.

In this study, the lignocellulose content decreased following physical and biological pre-treatments with *G. lucidum* and *L. edodes*. However, only lignin and hemicellulose contents showed significant decrease following physical pre-treatment, which were consistent with the results of a previous study that demonstrated the 11% decrease of lignin and 13% of hemicellulose following physical pre-treatment with hot compressed water at 200°C (Goh et al., 2012). The report also showed that the cellulose content remained similar as the current study.

Biological pre-treatments either with enzyme extracts from *G. lucidum* and *L. edodes* or in combination with physical pre-treatment did not change the cellulose content but reduced the hemicellulose and lignin contents. By partially removing

the lignin, the accessibility of enzymes to cellulose was markedly improved as lignin acts as physical barrier (Pan et al., 2005). It is expected that the reduction of hemicellulose as well as lignin gives a positive effect on OPF digestibility. *Ganoderma lucidum* and *L. edodes* have been shown to produce ligninolytic enzymes, including the

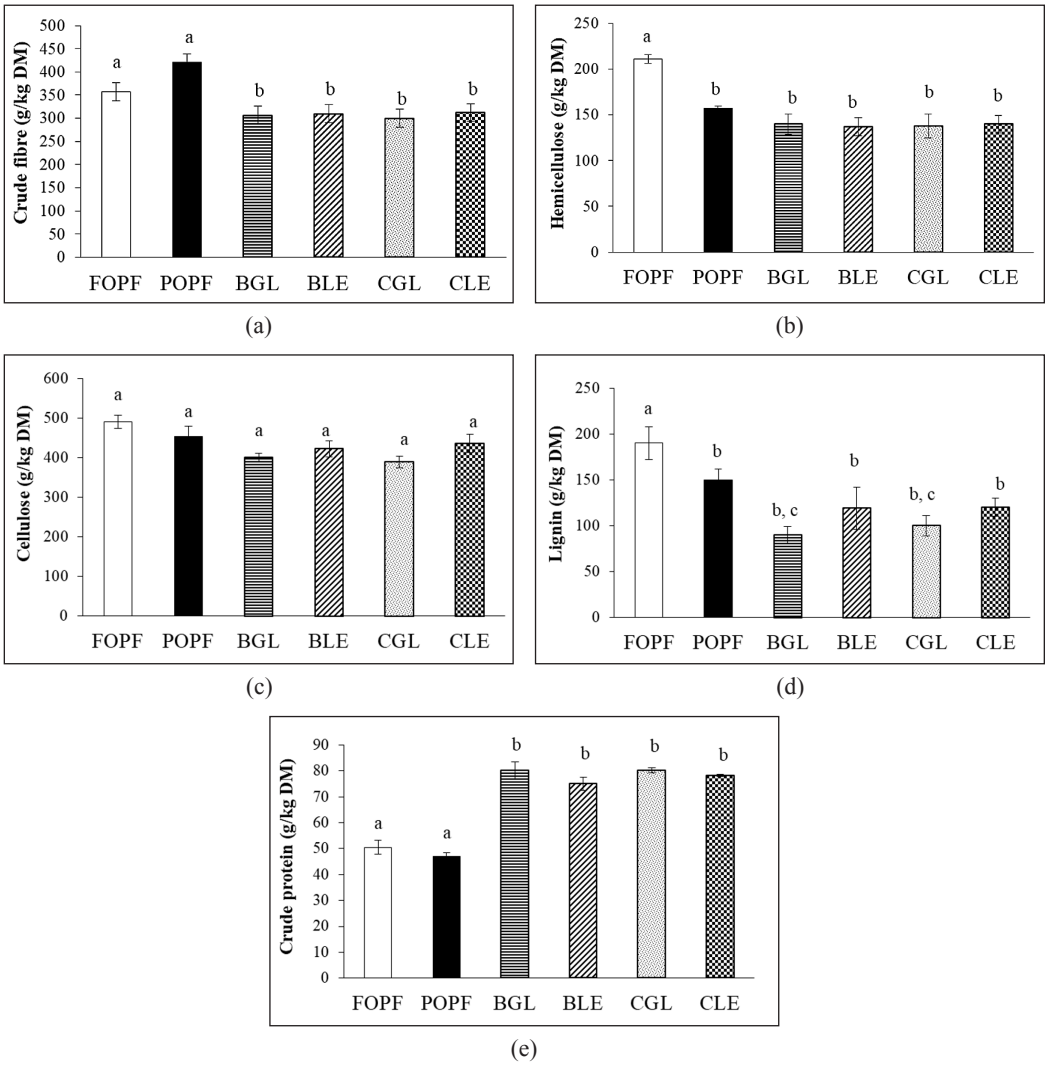

Figure 1. Comparison of chemical compositions of (a) crude fibre, (b) hemicellulose, (c) cellulose (d) lignin and (e) crude protein in non-treated OPF as control, physical pre-treated OPF, biological pre-treated OPF with enzyme extract of *G. lucidum* and *L. edodes* as well as combination of pre-treated OPF with enzyme extract of *G. lucidum* and *L. edodes*

Notes: Bars indicate standard error of mean (n=4). [abc] means in the same row with different superscript are significantly different ($p<0.05$). FOPF: non-treated OPF; POPF: physical pre-treated OPF; BGL: biological pre-treated OPF with enzyme extract of *G. lucidum;* BLE: biological pre-treated OPF with enzyme extract of *L. edodes*; CGL: combination of pre-treated OPF with enzyme extract of *G. lucidum*; CLE: combination of pre-treated OPF with enzyme extract of *L. edodes*

laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) that decompose hemicellulose, cellulose and lignin of OPF by oxidising the phenolic and non-phenolic lignin polymer and mineralise the insoluble lignin (Datta et al., 2017).

Total Gas Production Following 24 hours *in vitro* Incubation

There was a steady increase in the volume of gas produced by all substrates with time of incubation (Figure 2). Total gas productions for all pre-treatment groups (POPF, BGL, BLE, CGL and CLE) were significantly ($p<0.05$) higher than the FOPF. The highest cumulative gas production was in BGL (48 ml) whereas FOPF was the lowest (27.5 ml) following 24 hours incubation.

The *in vitro* gas production has a good correlation with *in vivo* digestibility of ruminant feed (Bhatta et al., 2007; Liu et al., 2011). Therefore, gas production technique is often used to evaluate the ruminal degradability of feed mixtures. The current study reported an increase in gas production with increasing time of incubation as reported by Rahman et al. (2011). However, the amount of gas production in biological pre-treatment was lower than the previous finding (Rahman et al., 2011), probably due to the use of different fungal strain and different mechanism of action. Indeed, biological pre-treated OPF using enzyme extracts from *G. lucidum* resulted in higher gas production in the current study as compared to the non-treated and other treatments.

The differences between the gas volume in controls and treatment samples may be attributed to the high content of soluble carbohydrate in dietary treatments especially in biological pre-treated OPF using enzyme extracts from *G. lucidum*. To date, there is no report on the response of OPF *in vitro* gas production and rumen fermentation following pressing method using conventional sugarcane machine as well as combined pre-treatment. Physical pre-treatment using sugarcane machine was considered practical, cost effective and easily operated as it didn't require high energy and it was suitable to be operated in small-scale farms. Physical pre-treatment also increased the gas production as compared to the non-treated OPF, indicating increased ferment ability. This was observed in the previous study that other physical pre-treatment, such as steam explosion was proven as a promising biomass pre-treatment which improved the nutritional contents of the fibrous feeds (Goh et al., 2012) and increased the fibrous feed digestion (Viola et al., 2008). Benefits of pre-treatment to ruminants feed include a shift of lignocellulosic biomass to fermentable sugars, allowing the access of cellulose to the microbial enzymes and convert the carbohydrate polymers into fermentable sugars (Saritha et al., 2012). Biological pre-treated OPF using enzyme extracts from *G. lucidum* resulted in higher gas productions as compared to non-treated OPF. The result suggested that WRF used were able to decrease the lignin content in the OPF as well as increase carbohydrate availability for ruminal degradation.

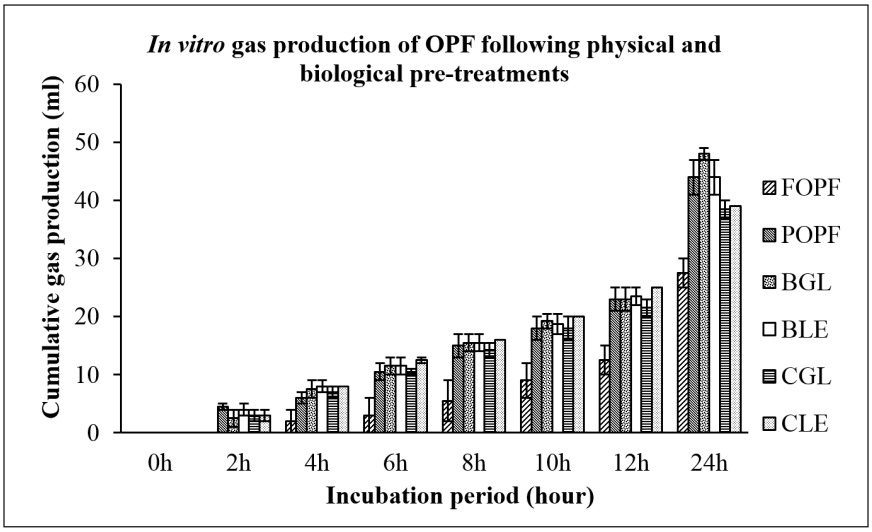

Figure 2. *In vitro* gas measurement (ml) of OPF following physical pre-treatment by pressing using conventional sugarcane machine and biological pre-treatment with enzyme extract of WRF; *G. lucidum* and *L. edodes*

Notes: Data shown are average of triplicates with standard error bars. FOPF: non-treated OPF (control); POPF: physical pre-treated OPF; BGL: biological pre-treated OPF with enzyme extract of *G. lucidum*; BLE: biological pre-treated OPF with enzyme extract of *L. edodes*; CGL: combination of pre-treated OPF with enzyme extract of *G. lucidum*; CLE: combination of pre-treated OPF with enzyme extract of *L. edodes*

Effect of the Pre-treatment on *in vitro* Rumen Fermentation Parameters

The VFA production is shown in Table 1. The levels of acetic, propionic and butyric acids were consistent in all pre-treatment groups and significantly higher ($p<0.05$) than the rumen fluid only (no substrate). In general, the acetic acid showed the highest level followed by the propionic and the butyric acids in all groups (Table 1). Both BGL and CGL were significantly higher ($p<0.05$) in propionic and butyric acids as compared to the FOPF.

There was also an increase in the ARDC of all treatments and the highest ARDC was in BGL with 6.57 mg. The treatments that showed significantly higher ($p<0.05$) ARDC than FOPF were the BGL (6.57$\pm$0.02 mg) and the CGL (6.54$\pm$0.05 mg). Physical, biological and/or combination pre-treatments with enzyme extracts from *L. edodes* failed to change the level of ARDC.

Volatile fatty acids or short chain fatty acids are produced by the anaerobic microbial fermentation in the rumen and supplied an estimated 70-80% of the energy in ruminant (Aluwong et al., 2010). Based on Figure 1, almost 21% of lignin in OPF was lost following pressing method and about 48% of lignin was degraded in OPF pre-treating with enzyme extract of *G. lucidum* and *L. edodes*. The results suggested that the ligninolytic enzymes namely laccase, LiP and MnP decreased the lignin content and in turn improved rumen degradability based on higher VFA production (Table 1). However,

one previous study reported that the lignin loss was not always correlated with VFA production or *in vitro* gas production (Rahman et al., 2011). The same study also demonstrated few WRF including *G. lucidum* that was promising in improving ruminal degradability and the observation agreed with the current study. In the present study, the BGL and CGL produced a significant effect on VFA production and ARDC compared to the FOPF and other pre-treated OPF. The biological pre-treatment of OPF using enzyme extract from *G. lucidum* was observed to be more effective as compared to enzyme extract of *L. edodes* since *G. lucidum* had the optimal enzyme activity for pre-treating the OPF (Azmi et al., 2016). The lignolytic potential needs to be at the highest in order to degrade the lignin content and allow the accessibility of rumen microbes to the cellulose and hemicellulose (Azizi-Shotorkhoft et al., 2016). Meanwhile, it is recognised that low level of cellulose and hemicellulose enzyme activity can maintain the structures of both cellulose and hemicellulose. Hence, this allows the rumen microbes to digest the cellulose and hemicellulose for VFA production.

A tremendous amount of works has been done on the techniques and pre-treatment strategies in order to improve the value of OPF. The physical pre-treatment has been sometimes abandoned due to its poor quality in increasing the nutritive value of roughage. However, in the present study, the physical treatment applied was practical because it only required conventional sugarcane machine which was economically feasible and environmental-friendly. Using pressing method combined with enzyme extract of WRF may be an alternative way to improve the quality of OPF and increase VFA as well as ARDC. Meanwhile, biological pre-treatment of OPF with enzyme extract of *G. lucidum*, either alone or in combination gives better results in terms of lignocellulosic content, gas production as well as the VFA. Previous studies also reported that *G. lucidum* showed substantial ability to degrade lignin (58%) and hemicellulose (74.8%), but not cellulose in wheat straw (Ćilerdžić et al., 2017). This indicates that *G. lucidum* is the best fungus to be used in pre-treatment in various fibrous by-products which contain high lignin. Some factors which are likely to contribute to the good finding in *G. lucidum* compared to other WRF include the production of a significant amount of ligninolytic enzyme by *G. lucidum*, the enzyme extract from *G. lucidum* matched easily with OPF and enzyme extract from *G. lucium* are not easily denatured to the temperature or any condition during pre-treatment.

Although the combination of pressing method with biological pre-treatment has not been documented yet, different combination strategy for OPF was clearly reported (Metri et al., 2018). It has been shown that combination of few WRF inoculation, namely *Ceriporiopsis subvermispora*, *G. lucidum*, *L. edodes*, *Phlebia brevispora* and *Pleurotus eryngii* for 3 or 9 weeks with enzyme supplementation was not successful in increasing the rate of gas production and

ARDC in OPF except for the high dose of commercial enzyme product, Hemicell to *Pleurotus eryngii* inoculated OPF (Metri et al., 2018).

Table 1
Volatile fatty acid (VFA, mmol/l) production and apparent rumen degradable carbohydrate (ARDC, mg) after 24 hour in vitro ruminal fermentation of pre-treatment and non-treated groups (mean±SE)

Parameters	FOPF	POPF	BGL	BLE	CGL	CLE
Total VFA (mmol/ incubation)	89.03±1.04	92.02±6.64	95.45±0.74	85.17±3.45	95.24±0.40	87.59±1.21
Acetate (mmol)	44.39±0.21^{a}	44.12±2.50^{a}	46.15±0.30^{b}	42.30±0.20^{a}	45.39±0.68^{a}	42.78±1.52^{a}
Propionate (mmol)	13.67±0.17^{a}	14.69±1.18^{a}	15.03±0.08^{b}	13.83±0.16^{a}	15.15±0.08^{b}	13.81±0.29^{a}
Butyrate (mmol)	8.87±0.16^{a}	9.50±0.02^{a}	9.93±0.01^{b}	6.08±2.07^{a}	10.08±0.06^{b}	8.69±0.02^{a}
ARDC (mg)	6.18±0.14^{a}	6.09±0.04^{a}	6.57±0.02^{b}	5.95±0^{a}	6.54±0.05^{b}	5.99±0.15^{a}

Notes: Data shown are average of triplicates. FOPF: non-treated OPF; POPF: physical pre-treated OPF; BGL: biological pre-treated OPF with enzyme extract of *G. lucidum;* BLE: biological pre-treated OPF with enzyme extract of *L. edodes*; CGL: combination of pre-treated OPF with enzyme extract of *G. lucidum*; CLE: combination of pre-treated OPF with enzyme extract of with *L. edodes.* ^{abc} means in the same row with different superscript are significantly different ($p<0.05$)

CONCLUSIONS

In this work, biological pre-treatment with enzyme extract of *G. lucidum* and *L. edodes* showed a similar pattern in degradation of the structural carbohydrates in OPF as well as in increasing the CP content. However, comparing these two WRF, *G. lucidum* seemed more promising for improving the *in vitro* rumen degradability. Biological pre-treatment with enzyme extract of *G. lucidum* combined with physical pre-treatment improved the nutritional values of OPF by decreasing the lignin contents, consequently improving the ruminal digestibility along with high total gas production, high VFA and high ARDC.

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