Nutritive composition of oil palm empty fruit bunch fibers treated with mycelia culture of Lingzhi (*Ganoderma lucidum*) as a potential ruminant feedstuff

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Abstract

Efforts were made to reduce the wide gap between requirement and availability of local feed by having a better utilization strategy of unconventional feed resources. Malaysia is one of the world's largest palm oil producers. The process of extraction of palm oil produces empty fruit bunch (EFB), which is considered as waste product. Biological delignification using white rot fungi (WRF) was reported to enhance the feeding values of agricultural by-products used in ruminant rations by fungal degradation of lignin. Steam sterilization before fungi inoculation is known to affect substrate composition. Thus, this study was conducted to evaluate the effect of steam sterilization of oil palm EFB fibres on the nutritive composition of oil palm EFB fibres treated with mycelia culture of *Ganoderma lucidum* at different incubation periods as a potential ruminant feedstuff. Shredded oil palm EFB fibres were prepared as sterilized substrate in polypropylene bags and inoculated with mycelia culture of *G. lucidum*. The uninoculated sterilized substrate was compared with unsterilized substrate. All inoculated substrates were incubated starting from week 2, 4, 6, 8, 10 and 12 with 4 replications for each period. All samples were analysed for their nutritive compositions. Result showed that only crude protein and cellulose content were significantly changed after steam sterilization process where crude protein was reduced from 6.07% to 1.97% and cellulose increased from 22.35% to 27.52%. All nutritive composition analysed (dry matter, organic matter, crude protein, ash, cellulose, hemicellulose and lignin) for oil palm EFB fibres treated with *G. lucidum* showed significant changes across incubation period. Its lignocellulose components such as lignin, hemicellulose and cellulose were significantly reduced by 21%, 20% and 35%, respectively after 12 weeks of incubation. These results suggest that oil palm EFB fibres treated with mycelial culture of *G. lucidum* have potential to be utilized as ruminant feedstuff. *Ganoderma lucidum* was proven to have the ability in delignification of lignin in oil palm EFB fibres. Exploitation of this biological treatment could possibly maximize the use of locally available agro-industrial by-products to increase the production of local cost-effective feed for ruminants.

Keywords: Ruminant feed, feed composition, oil palm by-product, empty fruit bunch, *Ganoderma lucidum*

Introduction

Livestock industry is one of Malaysia's important industries as it contributes to agricultural development. It supplies meats, which are the most important sources of animal protein in the diets of the Malaysian population (Kaur, 2010). The ruminant sub-sector showed progress in recent years. However, its self-sufficiency level (SSL) which refers to the ability of the local production to meet the local demand is still less than 30%. This indicates that ruminant products from local sources are still inadequate to meet the local demand following the concomitant increase in population and consumption. Low supply by local farmers also resulted in high dependency on beef and mutton imports from other countries (DVS, 2014).

One of the major challenges, which prevented Malaysia's livestock production from growing, is the high cost of imported animal feeds. The cost is approximately USD 607 million (RM 2.5 billion) per year. High dependency on these imported feeds causes price fluctuations and high cost of animal production. Various strategies have been introduced to reduce the feed cost as it accounts for about 70% of production cost (Wan Zahari *et al*., 2009).

At present, Malaysia is one of world's largest palm oil producers and exporters where Malaysia produced 18.86 million metric tonnes of palm oil, which contributed to 30% of the global palm oil supply (USDA, 2018). In the process of extracting palm oil, an oil palm mill produces a number of by-products and wastes such as empty fruit bunches (EFB), palm oil mill effluent (POME), palm fibre and palm kernel shell (Yusoff, 2006). On average, for every tonne of fresh fruit bunch (FFB) processed, about 230-250 kg (23-25%) are contributed by empty fruit bunches (EFB) (Madaki, 2015). It was reported that about 19 million tonnes of EFB are produced annually and they increase with the increasing palm oil production (Goh *et al.*, 2010). This large quantity of EFB cannot be used effectively and are discharged from the mill after removal of the fruit. Previously, the EFB are either incinerated or applied to the field as a way of disposal. However, these practices have created environmental pollution. New strategy needs to be looked into to better utilize EFB to minimize pollution and contribute to more sustainable wastes management in agriculture (Igwe and Onyegbado, 2007).

The widespread use of EFB as livestock feed is still constrained by its low digestibility as it is a lignocellulosic material which comprises of major components such as cellulose, hemicellulose and lignin (Lasure and Zhang, 2004). That is why an alternative approach has been proposed to improve degradability of EFB fibres or any possible agricultural by-products for ruminant feeding which is by using lignocellulolytic fungi.

Researchers have reported that treatment of biological delignification using white rot fungi (WRF) through fungal degradation of lignin enhanced feeding values of many locally available agricultural by-products for ruminant rations (Tabi *et al.*, 2008; Eriksson *et al.*, 1990). White rot fungi, a wood-decaying basidiomycete, are believed to be the most effective lignin-degrading microbes in nature, as they secrete lignin-modifying enzymes (LME), namely lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac). These WRF can be exploited to improve the nutritional value of fodder for ruminant nutrition (Srivastava *et al*., 2011). However, in this study, before the fungi were inoculated to EFB substrate, steam sterilization or sanitation was done to kill the microbes present. According to Ramos (2003), steam power has the potential to degrade (thereby pretreat) the complex structure of lignocellulosic material.

Thus, the objectives of this study were (i) to evaluate the effect of steam sterilization of oil palm empty fruit bunches (EFB) fibres on the nutritive composition and (ii) to evaluate the nutritive composition of oil palm EFB fibres treated with mycelial culture of *Ganoderma lucidum* at different incubation periods as a potential ruminant feedstuff.

Materials and Methods

Isolation of Ganoderma lucidum

Mushroom (*Ganoderma lucidum*) sample was obtained from a local mushroom farm located in Penampang, Sabah, Malaysia. Pure culture of the fungus was isolated using potato dextrose agar (PDA) by standard tissue culture technique from the basidiocarp (Scrase, 1995). Stock culture (slant) was then prepared in PDA, and stored at 4°C for further use.

Preparation of oil palm EFB fibres, inoculation and incubation

Shredded oil palm empty fruit brunches (EFB) fibres with an average length of 5 cm were obtained from a local palm oil refinery mill located in Lahad Datu, Sabah, Malaysia. The freshly obtained fibres were sun-dried (to less than 10 wt. % moisture content) until further use to reduce its moisture content and prevent the growth of moulds. The dried EFB fibre samples (100 g each) were soaked in distilled water overnight to allow them to absorb water, toasted, and inserted into polypropylene bags. Four bags of unsterilized EFB were separated while the other bags were then sterilized using autoclave machine (Tomy, SX-700) at 121°C with 15 p.s.i. for 15 min. The autoclaved EFB were left to cool to room temperature (25-28°C) before inoculated with *G. lucidum*.

The sterilized oil palm EFB fibres were inoculated with 16 plugs (5 mm) of 7-day old *G. lucidum* mycelia. Four bags of uninoculated EFB fibres were served as control, and the inoculated fibres were incubated at 2, 4, 6, 8, 10 and 12 weeks (Table 1). The experiment was conducted in four replications, which made a total of 24 experimental units excluding the control. The experimental units were arranged in completely randomized design (CRD) in room conditions at 25-28°C with 80-85% relative humidity stored in the dark.

After each incubation period, 24 bags of the incubated or treated EFB were harvested for sampling. All four replicates from each treatment were pooled and homogenized. The whole samples from respective treatments were then oven-dried at 70°C until constant weight. The dried samples were cut into smaller sections (1-5 mm) and kept in sealed bags for further analysis. The same procedure was performed to the unsterilized and sterilized EFB fibres samples (control).

Table 1. Experimental treatments

Sample	Incubation period (week)
Oil palm EFB[1] fibres only (Unsterilized) - control	0
Oil palm EFB fibres only (Sterilized) - control	0
Oil palm EFB fibres (Sterilized) + mycelial culture	2
Oil palm EFB fibres (Sterilized) + mycelial culture	4
Oil palm EFB fibres (Sterilized) + mycelial culture	6
Oil palm EFB fibres (Sterilized) + mycelial culture	8
Oil palm EFB fibres (Sterilized) + mycelial culture	10
Oil palm EFB fibres (Sterilized) + mycelial culture	12

[1]EFB: empty fruit bunches.

Nutritive composition analysis

All replicate samples as shown in Table 1 were analysed in triplicate for dry matter (DM), ash, organic matter (OM) and crude protein (CP) following the method described by Association of Analytical Communities (AOAC, 1995). Fibre composition, namely, neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) content were analysed according to methods described by Van Soest *et al.* (1991). The hemicellulose content was calculated as the difference between NDF and ADF. Cellulose content was calculated as the difference between ADF and ADL.

Statistical analysis

All data obtained were analysed by using one-way analysis of variance (ANOVA). Their means were then compared by using the Duncan's multiple range test (DMRT) at significance level of 5% ($p<0.05$). Regression analysis (linear model) was performed to analyse the trends of changes of nutritive composition across incubation period (week). All statistical analyses were performed using the Statistical Analysis System (SAS) 9.4 (2007).

Results and Discussion

Effect of steam sterilization on nutritive compositions of empty fruit bunch fibres

The nutritive composition between the two controls unsterilized and steam sterilized (uninoculated) oil palm EFB fibres were shown in Table 2. As for unsterilized EFB, its crude protein was in contrast to that reported by Mahlia *et al*. (2000). The lignin was similar with Ariffin *et al.* (2008) and Baharuddin *et al.* (2011) while cellulose and hemicellulose content measured were varied. Cellulose content was similar as recorded by Singh *et al*. (2008). Since EFB fibres are readymade within any mill operation, their open structures with high moisture (60-65%) and residual oil contents are easily contaminated by fungal growth found to have detrimental effects such as deterioration in its properties (Simarani *et al*., 2009). Therefore, different sources of EFB fibres from different mills might have different chemical composition depending on their handling.

It was observed that the crude protein content had significant change after the steam sterilization where it was significantly reduced from 6.07% to 1.97%. The crude protein reduction was from the results of damages of individual amino acids, which causes reactions between amino acids, and between amino acids and carbohydrates that reduce the availability and biological quality of the protein (Ritskes-Hoitinga and Hedrich, 2004).

Table 2. Nutritive composition of oil palm empty fruit bunch (EFB) fibres for unsterilized, steam sterilized (uninoculated) and steam sterilized inoculated with mycelial culture of *Ganoderma lucidum* at different incubation period

Com-position[1] (%)	Incubation period (week) Mean ± Standard error								p-value
	0[2]	0[3]	2	4	6	8	10	12	
DM	26.23^{c} ± 1.37	28.25^{c} ± 0.7	27.15^{c} ± 1.0	29.1^{c} ± 0.84	27.34^{c} ± 0.63	33.57^{b} ± 1.08	40.96^{a} ± 1.0	39.57^{a} ± 0.92	<.0001
OM	97.18^{a} ± 0.19	96.93^{a} ± 0.51	91.12^{c} ± 0.86	92.28^{c} ± 0.05	92.56^{c} ± 0.81	94.75^{b} ± 0.48	96.92^{a} ± 0.38	96.12^{ab} ± 0.57	<.0001
CP	6.07^{a} ± 0.36	1.97^{bc} ± 0.15	1.37^{d} ± 0.14	1.42^{cd} ± 0.17	2.19^{b} ± 0.09	1.97^{bc} ± 0.09	1.59^{cd} ± 0.14	1.91^{bcd} ± 0.10	0.001
Ash	2.83^{c} ± 0.19	3.07^{c} ± 0.50	8.88^{a} ± 0.87	7.73^{a} ± 0.05	7.44^{a} ± 0.81	5.24^{b} ± 0.48	3.08^{c} ± 0.38	3.88^{bc} ± 0.57	<.0001
Cell.	22.35^{e} ± 1.0	27.52^{ab} ± 0.6	28.98^{a} ± 0.34	28.77^{a} ± 0.96	26.93^{ab} ± 0.37	26.17^{bc} ± 0.67	24.73c^{d} ± 0.75	22.91^{de} ± 0.53	<.0001
Hem.	18.19^{a} ± 0.57	16.58^{ab} ± 0.34	16.51^{ab} ± 0.95	16.02^{abc} ± 1.04	15.63^{bcd} ± 0.63	14.46^{bcd} ± 0.28	14.09^{cd} ± 0.75	13.30^{d} ± 0.95	0.0023
Lignin	12.46^{a} ± 0.5	11.26^{ab} ± 0.83	10.36^{b} ± 0.53	9.85^{bc} ± 0.38	8.55^{cd} ± 0.09	8.26^{d} ± 0.51	7.90^{d} ± 0.30	7.29^{d} ± 0.24	<.0001

[1]DM: Dry matter, OM: Organic matter, CP: Crude protein, Cell.: Cellulose, Hem.: Hemicellulose
[2]Unsterilized, [3]Uninoculated,
^{a,b,c,d,e} Means with different superscript within row differ significantly ($p<0.05$) .

In addition, cellulose content also significantly changed after the steam sterilization where it increased from 22.35% to 27.52%. According to Ramos (2003), steam sterilization was reported to be efficient in partially hydrolysing hemicellulose, modifying the lignin, increasing access to surface area, decreasing the crystallinity of cellulose and its degree of polymerization. This hemicellulose removal part is due to the organic acid generated by the hemicelluloses-acetyl groups cleavage. The evolved acid could have hydrolysed the hemicellulose and altered the lignin structure. The removal of hemicellulose from the microfibril was believed to expose the cellulose surface (Shamsudin *et al*., 2012). However, in the present study, only the cellulose was significantly changed, but not hemicellulose and lignin content. This was similar to Shamsudin *et al*. (2012), where there was only least effect on the chemical compositional changes after steam sterilization at low temperature (121ºC) and in contrast, at high temperature (140-260ºC) where 18% increase in cellulose, 16% reduction in hemicellulose and 10% reduction in lignin. Ariffin *et al*. (2008) reported 7% increase in cellulose, 9% reduction in hemicellulose and 14% reduction in lignin after steam sterilization at high temperature (140-240ºC). Their study also showed that the differences of the cellulose, hemicellulose and lignin content in steam-sterilised EFB (low and high temperatures) were 3.2, 1.3 and 1%, respectively.

Therefore, the steam sterilization method was effective in the partial removal of hemicellulose and degradation or

modification of lignin thereby increased in cellulose only with high temperature (140-260^{o}C) steam sterilization with high pressure for several minutes. When high pressure steam was used for sterilization, the heat would cause the moisture in the EFB to expand then hydrolyse part of the EFB component (Ramos, 2003). Besides that, the moisture introduced by the steam acted chemically to break down gums and resins into soluble and insoluble oils which loosened the fibrous EFB structures for ease of attack by enzymes later (Shamsudin *et al*., 2012).

It can be observed from the Table 2, where other nutritive components of sterilized EFB fibres such as dry matter, organic matter and ash did not change significantly after the steam sterilization. That was due to the recalcitrant lignin component of the EFB which did not degrade after the sterilization, as it acts like a glue by filling the spaces between and around cellulose and hemicellulose (Tina 1998).

Nutritive composition of empty fruit bunch fibres treated with mycelia culture of Ganoderma lucidum

It was observed that the mycelia fully colonized the bags on week 2 and the mycelia density increased until the last incubation period. Mushroom strains have short cultivation period because they have high levels of enzyme activity (Gao *et al.,* 1993). Good colonization of mycelia means effective fungal treatment (Kenealy and Dietrich, 2004).

The nutritive composition of oil palm EFB fibres treated with mycelial culture of *G. lucidum* at different incubation periods is presented in Table 2. Based on the results obtained, dry matter content only showed changes starting from week 8 (33.57%) where it significantly increased, reaching 39.57% at week 12 of incubation. In contrary, a study conducted by Eriksson *et al.* (1990) on the enzymatic degradation of wood found that along with the production of lignocellulolytic enzymes, substrate was rapidly degraded, thus reducing its dry matter content. However, according to Sun *et al*. (2011), as incubation period increased, the dry matter content of treated substrate also increase as a result from the dry weight of mycelia hyphae. These matured mycelia also have their dry matter which contributes to the high or increasing dry matter content of the treated EFB fibres. Therefore, dry matter content of treated EFB fibres increased as incubation period increased.

The organic matter content of uninoculated EFB (96.93%) was higher compared to inoculated EFB (91.12%) at week 2. However, the organic matter of inoculated EFB increased significantly at week 8 (94.75%) and week 10 (96.92%) then remained unchanged until 12 weeks (96.12%) of incubation period. The organic matter content was related to the nitrogen and carbon content of the treated EFB fibres. All of the elements were essential for the *G. lucidum* mycelial growth and substrate lignin delignification which explained the decrease in organic matter content of treated EFB in week 2 (Eriksson *et al.*, 1990). While the significant increase in organic matter content as the mycelia have organic compounds and carbohydrates as their major components (Bonnen *et al*., 1994).

It can be observed that the crude protein content of treated EFB fibres fluctuated across the incubation period and ranged from 1.37% to 2.19%. The crude protein content measured were in the range as reported by Singh *et al.* (2008) and Yojiro and Ayaaki (1990), who also studied the chemical composition of oil palm EFB fibres. It is known that EFB is a high fibrous material with low protein and energy and considered as feeds for ruminants. The reason of crude protein content fluctuated across the

incubation period was because nitrogen available from the EFB fibres substrate was essential for synthesis of mycelia enzymes, such as those related to substrate degradation and cell growth. Specifically, all of the enzymes can be found in the tips of the hyphae as physical requirements for cell growth such as plastic deformation, incorporation of new cell wall and membrane material (Lew, 2011).

Based on Table 2, the ash content showed that it had significantly increased from 3.07% (uninoculated) to 8.88% at week 2 of incubation. The increase in mineral content on week 2 was probably due to culture media (PDA) which came together with the inoculum. Minerals such as phosphorus, magnesium, sulphur, calcium, iron, potassium, copper, zinc, manganese and cobalt, as well as vitamins, were used in the culture media (Alananbeh *et al*., 2014). Starting from week 8 (5.24%), the ash content showed significant decrease until 3.88% at week 12 of incubation. It has been known that inorganic compound was one of the nutritional sources for the growth of fungi other than nitrogen and carbon compounds (Arora and Sharma, 2009). Those inorganic materials available also influenced the mycelia growth and for the production of lignocelluloytic enzymes. The ash content of uninoculated EFB fibres (control) was the same as reported by Kittikun, *et al*. (2000) and Husin (2002) where the amount was 3.02% and 4.3%, respectively. In addition, according to Singh *et al*. (2008), EFB fibers had high potassium, calcium, phosphorus and magnesium content. Therefore, based on the results of dry matter, organic matter, crude protein and ash, it showed that as a saprophytic basidiomycete, *G. lucidum* consumed nutrients from the substrate through its mycelium, obtaining substances necessary for its development, such as carbon, nitrogen, vitamins and minerals (Michael, 2011; Tabi*et al.,* 2008).

It can be observed from Table 2, that the cellulose content was significantly reduced starting from week 6 (26.93%) until reaching the lowest content 22.91% at week 12 of incubation. Similarly, for hemicellulose and lignin content which significantly reduced and reached the lowest value at week 12 of incubation with 13.30% from 16.58% (control) for hemicellulose, while 11.26% (control) to 7.29% for lignin. The uninoculated EFB (control) lignocellulose material content measured is in agreement with Richana *et al.,* (2015) where their cellulose, hemicellulose and lignin content reported were 25.16%, 12.78% and 10.72% respectively. In contrast Ariffin *et al.* (2008), reported that the cellulose, hemicellulose and lignin of untreated EFB fibres were 51.22%, 28.24% and 15.19%, respectively. The difference between chemical composition of EFB fibres may have resulted from different ways of handling EFB wastes in the mill.

From the regression analysis performed to analyse trends of changes for each nutritive composition across incubation period (week), only lignin, cellulose and hemicellulose content showed significant relationship. The trends of changes across incubation period expressed in relative content are shown in Figures 1-3. Based on Figure 1, it showed that EFB fibres treated with *G. lucidum* reduced for 35% (R^2=0.97, p=0.005) in its lignin content across the 12 weeks of incubation period. Lignin cannot be degraded through anaerobic fermentation in the rumen and was shown to be negatively related to digestibility in ruminants (Lara *et al*., 2003). Lignin is the most recalcitrant to degradation and it is linked to both hemicellulose and cellulose forming a physical seal, an impenetrable barrier which prevents the penetration or release of other components (Howard *et al.,* 2003). After delignification of the lignin in EFB fibres by mycelial culture of G. *lucidum*, cellulose and hemicellulose present would be useful

carbohydrates for ruminants. The hemicellulose content was reduced by 20% (R^2=0.78, p=0.005) after 12 weeks of incubation (Figure 2). Other treated EFB components available also could be exposed or be made available for ruminants after the complex of fibres structure was loosened. Based on Figure 3, the cellulose content was reduced by 21% (R^2 = 0.95, p=0.021). Many reports have shown that the extracellular lignocellulolytic enzymes (LiP, MnP and laccase) play significant roles in the degradation of lignin to the cellulose molecules (Conesa *et al.*, 2002; Lara *et al.*, 2003) and the mycelia can use the cellulose fraction as its carbon source (Lara *et al.*, 2003). Bartnicki-Garcia *et al*. (2000) mentioned that carbohydrates are important for the growth of fungi, because the cell wall synthesis depends on glucose supply.

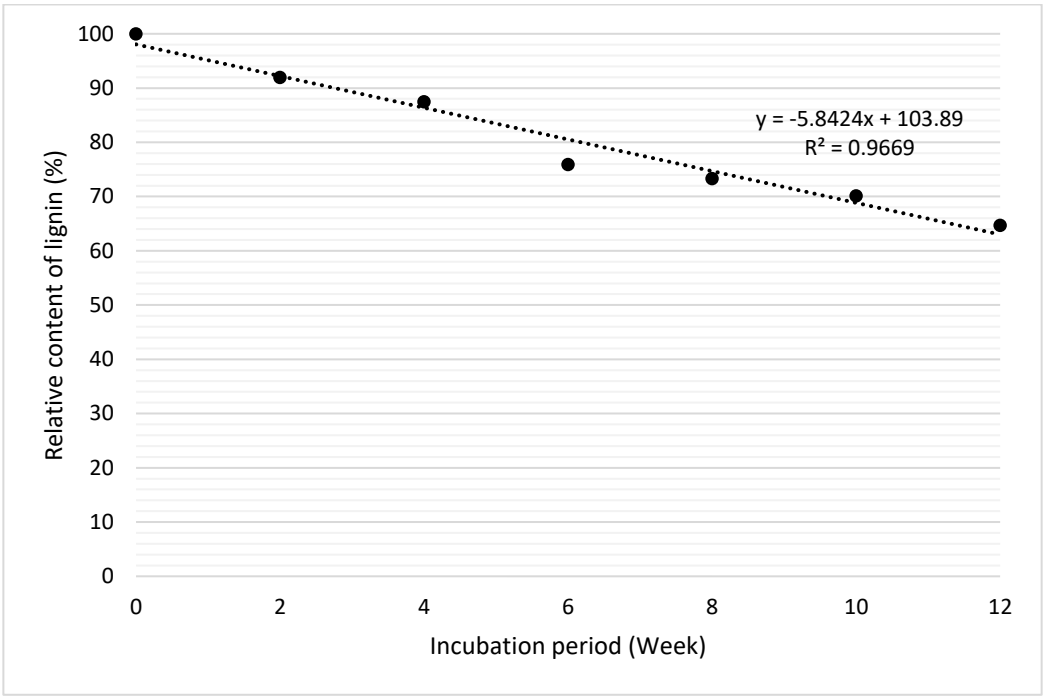

Figure 1. Relative content of lignin across incubation period

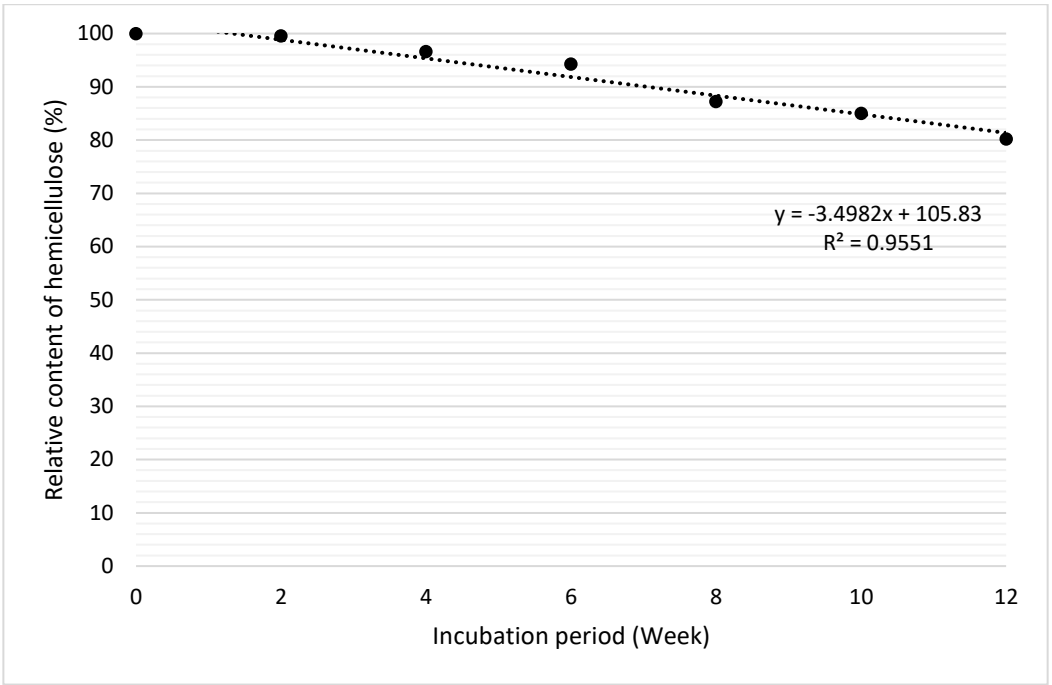

Figure 2. Relative content of hemicellulose across incubation period

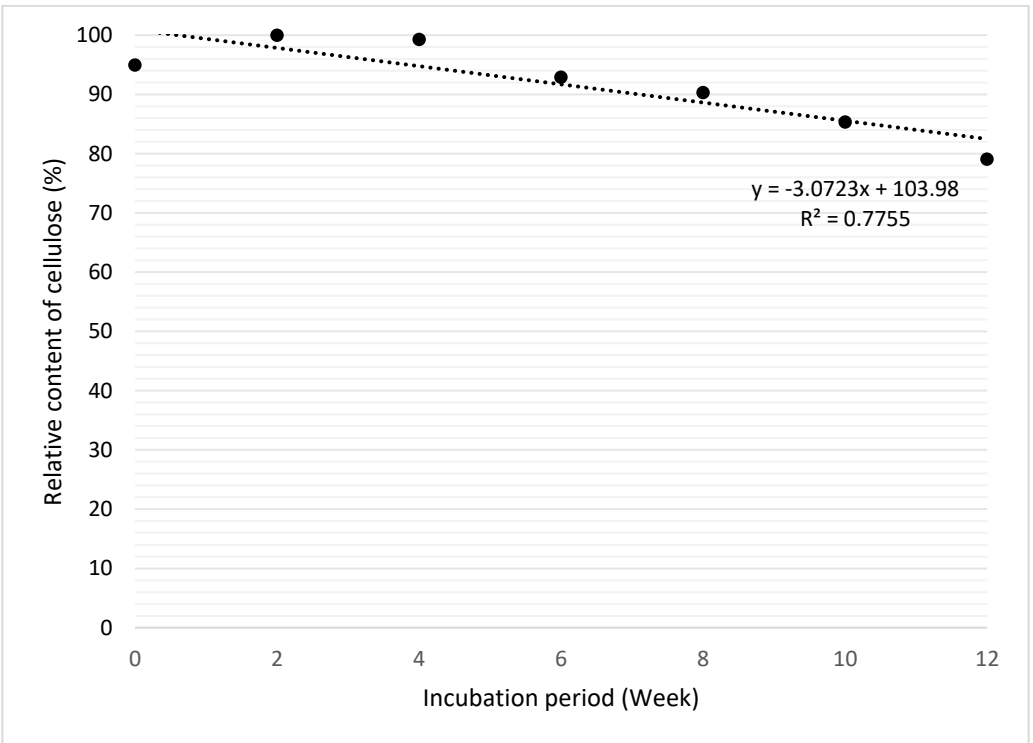

Figure 3. Relative content of cellulose across incubation period

Conclusion

In conclusion, only crude protein and cellulose content of EFB fibres were significantly changed after steam sterilization at 121°C (low temperature). All nutritive components showed significant changes after been treated with *G. lucidum* at different incubation periods. Lignocellulose components of EFB fibres were significantly reduced across the incubation period after treated with mycelia culture of *G. lucidum* where at 12th week, cellulose was reduced for 21%, hemicellulose for 20% while 35% for lignin. *Ganoderma lucidum* was proven to have the ability in delignification of lignin in oil palm EFB fibres. It was found that the lignin content significantly reduced on the week 2 of mycelia culture inoculation which suggests that 2-week incubation is sufficient to reduce the lignin content of EFB. Furthermore, 6 weeks of incubation is recommended for lower lignin with higher crude protein contents. Exploitation of this biological treatment possibly will maximize the use of locally available agro-industrial by-products to increase the production of local cost-effective feed for ruminants. Full utilization of this lignocellulosic material not only will partially solve the ruminant feed problem, but it also can reduce environmental pollution from oil palm mills.

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