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Enhancing the utilization of oil palm fronds as livestock feed using biological pre-treatment method

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Abstract. Despite its advancement in industrial sectors, the economy of South East Asia is still largely dependent on its agricultural sector. The implication of the situation is the large amount of agricultural by-product produced. The agricultural by-products saw limited utilization and are usually disposed via several methods. Indeed, the limited utilization especially as animal feed is due to the presence of lignin which limits its effectiveness. Several methods have been explored to manipulate the lignin content of the by-products. The most cost-effective way of manipulating the lignin content is via biological pretreatment using white rot fungi. In this experiment, one of the abundant agricultural by-products in Malaysia, oil palm fronds (OPF), was used to be pretreated with fungi to enhance its nutritional value and assess its potential as animal feed. Firstly, several fungi were isolated from rotten OPF and were identified. The identified fungi are *Trichoderma harzianum*, *Fusarium solani*, *Trichoderma asperellum*, and *Trichoderma koningiopsis*. Their enzymes activity were tested and pitted against conventional biological pretreatment agent, the white rot fungi *Lentinula edodes* and *Ganoderma lucidum*. The results showed the isolated *Trichoderma harzianum* and *Fusarium solani* exhibited optimal enzyme activity profile as pretreatment agent compared to the white rot fungi in term of enhancing the utilization of the agricultural by-product as livestock feed. Indeed, effective utilization of agricultural by-product as livestock feed will reduce the feeding cost of livestock industry as it constitute a major portion of the production cost.

1. Introduction

The countries in the South East Asia region possess a large disparity in terms of development and endowment. In recent times, the region undergone rapid growth in term of development resulting its economy to flourish significantly. The growth of manufacturing sector, which earned the region its nickname 'Factory Asia' have successfully highlight the region as a mix of manufacturing global value chains (GVCs). This growth have resulted in strong increment of growth domestic product



(GDP) in which most country managed to average close to 5% per year over the 2000 to 2016 period. Apart from that, the economic growth also drives the population growth at 1.3% per year over the same period.

Alongside the growth in industrial sector of the region, South East Asia also observed an improvement in food security. The undernourishment rate decline from 31% in early 1990s to less than 10% by 2014-2016. For a region that houses 9% of total world population, South East Asia holds 8% of the world undernourished population. This situation is further reinforced by the different development levels of the countries within the region [1]. The improvement is driven by the development of agricultural and fisheries sectors.

Apart from improving the food security of the region, the agricultural and fisheries sector also contributed in the nations' income. This is evident by the involvement of the nations in the international agro food trade. The point is further strengthen by the data by FAO [1] that shows shift in production share of agricultural product from rice to oil palm which represents a higher value product. Indeed, agricultural still maintains its place as the primary economic source for the South East Asia despite the industrialization of the region.

The increasing production of palm oil in the region means increased in the production of agricultural by-product. Oil palm Fronds, which is also known by its acronym, OPF, is the main by-product of palm oil plantation. It is obtained via the process of felling, pruning and harvesting. Malaysia produces 30 million tonnes of OPF annually from its 45 million hectares plantation area [2].

The OPF's usage is common as animal feed in its various forms such as freshly chopped, silage, pellets and cubes [2]. Apart from that, OPF also saw its usage as source of roughage for ruminants. However, the effective use of OPF is hindered by its high lignin content and low protein content [3]. The lignin is the main component of lignocellulosic biomass. It acts as structural support and functions to impart strength to plant tissues. The lignocellulosic component also protects the cellulose from enzymatic degradation. This is problematic as it prevents effective degradability of cellulose and hemicellulose OPF by the rumen microbes [4].

Fungi provided a suitable means of pre-treatment method in reducing the lignin content of the OPF. The other methods such as alkali pre-treatment and steam treatment are considered as least technological mature and expensive process [5]. Furthermore, the addition of chemicals such as strong alkali may inhibit subsequent chemical processes [6]. Bacterial pre-treatment is omitted due to its slower reaction time compared to fungi [7]. Previous study showed 12% increase in ruminal degradability of OPF pre-treated with white rot fungi.

In this study, fungi from rotten OPF will be isolated and identified. Next, their enzymes activities will be determined and compared with conventional lignolytic white rot fungi to identify the alternative to the white rot fungi.

2. Materials and Methods

2.1. Isolation and cultivation of fungi

The fungi were collected from rotten OPF in oil palm plantation, Taman Pertanian Universiti, Universiti Putra Malaysia. The Fungi were then cultured on potato dextrose agar (PDA) repeatedly until pure cultures were obtained.

2.2. Identification of fungi isolates

The DNA was extracted from the fungi isolates using QIAGEN DNeasy plant kit according to manufacturer's manual. The ITS region of the DNA extracts were amplified using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') as forward primer and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as reverse primer in polymerase chain reaction (PCR). The PCR products were then sent to a

service provider to be sequenced. The sequences were then assembled and aligned using Biology Workbench 3.2 (<http://workbench.sdsc.edu>). The assembled sequences were then compared with sequence in GenBank using Basic Local Alignment Search Tool (BLAST) (<http://www.ncbi.nlm.gov>). Maximum likelihood phylogenetic tree was constructed using MUSCLE alignment and Gblocks alignment curation [8].

2.3. Oil palm fronds fermentation

Solid state fermentation was carried out in 250 ml Erlenmeyer flasks. Each flask contains 15 g grinded chopped OPF collected from oil palm plantation, Taman Pertanian Universiti, Universiti Putra Malaysia. Deionized water measuring 45 ml was added to each flask. Each flask was also added with 22.5mg of glucose solution. The flasks were then autoclaved at 121 °C for 15 minutes. Three 10 mm diameter plugs from each isolated fungi were added to the sterile flask as inoculum. The flask was then covered with cotton ball and aluminium foil was used to seal the flask. The flask was then incubated at room temperature (37°C). For harvesting the enzyme, 150 ml of deionized water was added to each flask and placed inside rotary shaker for 3 hours. The content was then filtered. The filtrate was then centrifuged at 12,000 g at 4°C and the supernatant was collected. The procedure was repeated for 15, 30 and 45 days fermentation for each isolated fungal species [9]

2.4. Enzyme activity determination

2.4.1. Lignolytic enzymes

Laccase activity was determined according to [17] Oxidation of 2.0 mM 2,2' -azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) with 100 mM citrate phosphate (pH 4.0) as buffer. The formation of ABTS cation radical was measured at 420 nm absorbance.

Manganese Peroxidase was determined by measuring the Mn^{3+} -tartrate formation from 0.10 mM $MnSO_4$ with 100mM tartrate buffer at pH 5.0 and 0.10 mM H_2O_2 at 238 nm absorbance. Lignin Peroxidase was determined by measuring the formation of veratraldehyde from veratryl alcohol, 100mM acid tartrate buffer at pH 3.0 and 0.10 mM H_2O_2 at absorbance of 310 nm.

2.4.2. Cellulolytic enzymes

The activity for carboxymethylcellulase (CMCase) was measured in accordance to IUPAC recommendations. 10g/l of carboxymethylcellulose were placed in 50 mM citrate buffer (pH 4.8) at 50 °C for 30 minutes. The reducing sugar released were determined by using dinitrosalicylic acid (DNS) with glucose as standard. The absorbance was read at 575 nm.

The activity for avicelase was measured in accordance to IUPAC recommendations. 10g/l of avicel (cellulose microcrystalline) were placed in 50 mM citrate buffer (pH 4.8) at 50 °C for 3 hours. The reducing sugar released were determined by using dinitrosalicylic acid (DNS) with glucose as standard. The absorbance was read at 575 nm.

2.4.3. Hemicellulolytic enzyme

For xylanase activity measurement, 10g/l of xylan were placed in 50 mM citrate buffer (pH 4.8) at 50 °C for 30 minutes. The reducing sugar released were determined by using dinitrosalicylic acid (DNS) with xylose as standard. The absorbance was read at 575 nm

The average reading for enzyme activity of 15, 30 and 45 days of fermentation were calculated and compared with the enzyme activity of *Lentinula edodes* and *Ganoderma lucidum*.

3. Results and Discussion

The isolation of fungi from rotten OPF yielded nine fungi isolates. These isolates were labelled S1, S2, S3, S4, S5, S6, S7, S8, and S9. Upon Identification of the fungi species, it is found that those isolates were belong to the species *Trichoderma harzianum*, *Trichoderma harzianum*, *Fusarium solani*, *Fusarium solani*, *Trichoderma asperellum*, *Trichoderma koningiopsis*, *Trichoderma koningiopsis* and *Trichoderma asperellum* respectively. This is exhibited phylogenetic tree in Figure 1 and 2 below.

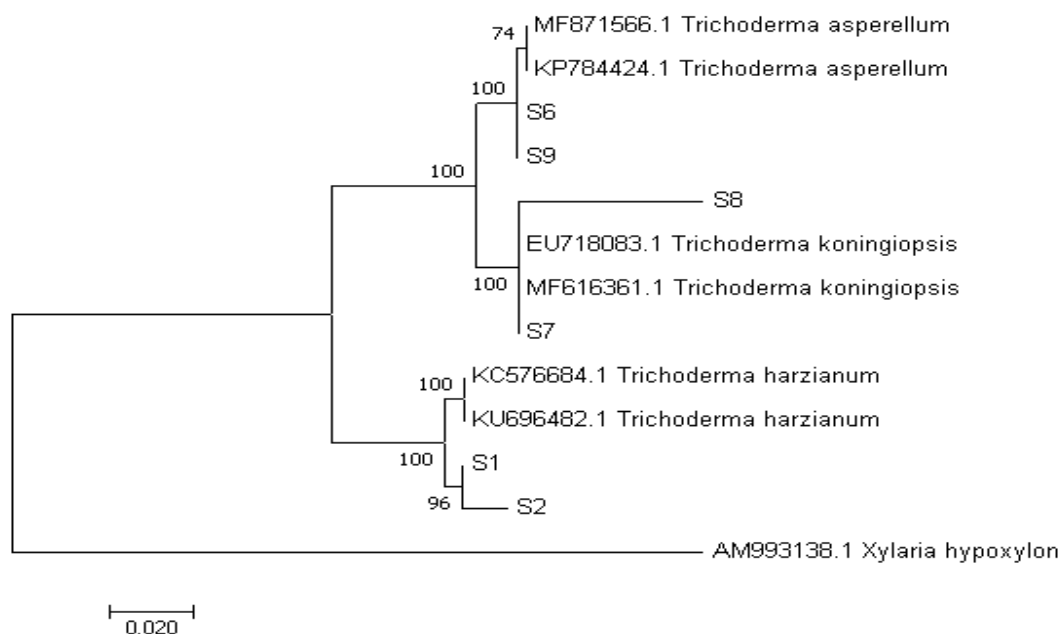


Figure 1. The phylogenetic tree for fungi isolate S1, S2, S6, S7, S8 and S9.

From Figure 1, it is showcase that S6 and S9 occupy the same clade as *Trichoderma asperellum* which identifies them as belonging in the same species. The fungi isolate S7 and S8 occupy the same clade as *Trichoderma koningiopsis* while fungi isolates S1 and S2, belong in the same clade as *Trichoderma harzianum*.

Figure 2 shows that fungi isolates S3, S4, and S5 are all occupying the same clade as the fungi species *Fusarium solani* which effectively identify them as the same species.

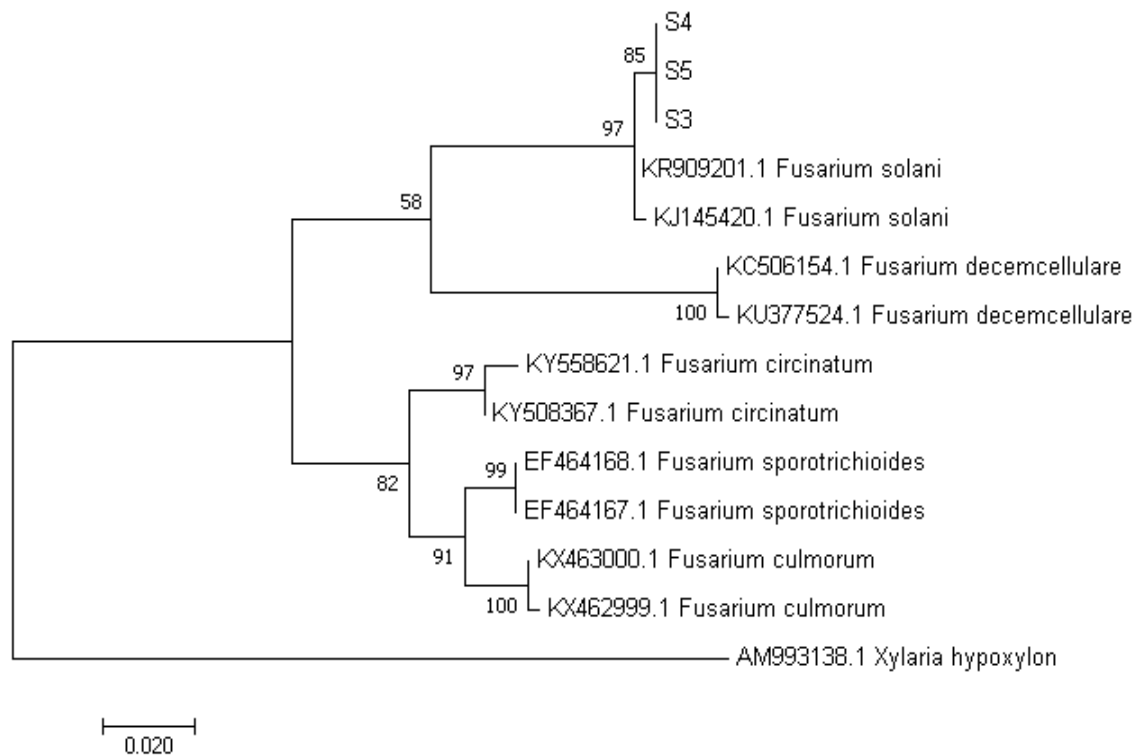


Figure 2. The phylogenetic tree for fungi isolate S3, S4 and S5.

The next part of the experiment shows the average enzyme activity of the fungi isolates during the 15, 30 and 45 days incubation period. The reading of the enzymes activity were exhibited by the figure below

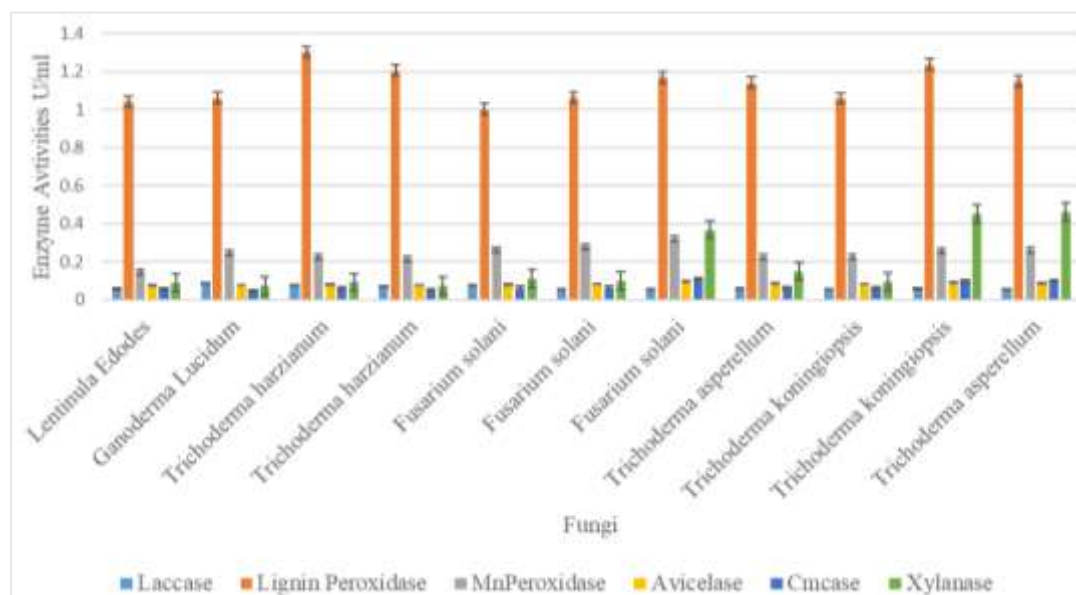


Figure 3. The average enzymes activities of the isolated fungi with *Lentinula edodes* and *Ganoderma lucidum* as controls with error bars representing standard deviation.

The graph shows activities for all the enzymes tested. In general, the fungi exhibited high lignin peroxidase activity. This is due to addition of nitrogen and glucose as energy source, apart from that, the presence of low molecular weight compound [10]. Indeed, the media content which consists of OPF, glucose and ammonium sulphate is conducive for the production of lignolytic enzymes.

From the graph, it is also exhibited that the isolated fungi shows comparable enzyme activity as *Ganoderma lucidum* and *Lentinula edodes* which both considered as white rot fungi belonging to the phylum basidiomycetes. They are known as excellent degrader of fungi [11]. Although the isolated fungi are from the phylum ascomycetes, several member of the phylum also have been recorded to exhibit lignolytic activity especially from the genus *Fusarium* and *Treichoderma* [12].

Based on the enzymes activity, all the isolated fungi shows potential as pretreatment agent to improve the utilization of the agricultural by product as livestock feed. The high lignolytic activity, allows effective degradation of lignin content of the oil palm fronds. The lignin content in OPF limits the physical access of rumen's microbe to the fermentable cellulose and hemicellulose. Furthermore, the low cellulolytic and hemicellulolytic enzymes activity allow the cellulose and hemicellulose to remain intact to be metabolized by the rumen's microbe into fatty acid [13].

Indeed, the utilization of agricultural by product will not only lower the cost of feeding of the livestock in which the cost substitutes a large portion of the production cost of livestock [14]. It also minimizes the competition for plantation area between crops for human use and crops for livestock use.

The delignification potential of the enzyme extract also opens up the wider possibility for the utilisation of the lignocellulosic biomass. One of the other possibilities is the application in enhancing the yield of the second generation bioethanol as the fermentation process can be done more effectively due to removal of the lignin content. Hence, it provides easier access to fermentation substrate [15].

4. Conclusion

The isolated fungi showed enzyme activity profile that is suitable for the purpose as pretreatment agent for the agricultural by product to be used as animal feed. All the fungi isolated produces high lignolytic enzymes and low cellulolytic and hemicellulolytic enzyme. With these enzyme extracts, the lignin content of the agricultural by-product can be reduced with minimal disruption of the cellulose and hemicellulose content.

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