EFFECTS OF FEEDING PRE-TREATED OIL PALM FROND WITH FILAMENTOUS FUNGI ON GROWTH PERFORMANCE, DIGESTIBILITY, AND MEAT QUALITY OF GOAT

By

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Oil palm frond (OPF) poor nutrient composition limits the effective use of OPF for ruminant feeding due to the high amount of lignin (15-56% w/w). However, OPF has great potential as the source of dietary energy for ruminants, as it contains about 80% of the structural carbohydrates on a dry matter basis. Lignin is chemically challenging to be degraded due to a complex three-dimensional network formed by various ether and carbon-carbon linkages among the lignin subunits and structural carbohydrates. The most effective and actively used biological pre-treatment method for fibrous by-products conversion by lignin degradation is fungal pre-treatment. The present study aims to improve the nutritional value of OPF to be the ruminant feed.

The enzyme activity of the filamentous fungi was determined. The synergistic effect of the fungi was also assessed to enhance the ligninolytic

enzyme

activity of the fungi. The *in vitro* and *in vivo* ruminal degradability of OPF pre-treated with enzyme extract was also assessed. The OPF pre-treatment was done by comparing between the silage pre-treatment with overnight pre-treatment by four different enzyme extract. In this study, eight filamentous fungi were identified which are F49 (*Marasmius palmivorus* MN871732), F02 (*Mucor fusiformis* KY560315), F77 (*Penicillium citrinum* MK312421), F32 (*Trichoderma asperellum* MK928414), F14 (*Schizophyllum commune* MN856258), F39 (*Phanerina mellea* or Ceriporia mellea MK432982), F03 (*Aspergillus neoellipticus* MG596660) and F65 (*Syncephalastrum racemosum* MH857909).

This study found that the enzyme activity production of the desired enzyme was best shown by *Phanerina mellea*. The enzyme extract pre-treatment by *Phanerina mellea* (STB) showed highest *in vitro* gas production after 24 h incubation with the value of 17.92 mL/250 mg DM. In animal trials, the pre-treatment of OPF with enzyme extract from *Phanerina mellea* and *Schizophyllum commune* improved the body condition score (BCS) from 0 - 25% to 75% - 100%. The serum biochemical profiles were similar between each diet which is in the standard value of healthy goats. The evaluation of meat and carcass in this study showed that the inclusion of the pre-treated ensiled OPF potentially improved the carcass performance of the goats and keep the good meat quality.

Keywords: Enzyme pre-treatment, Goat, Oil palm frond, Silage, White rot fungi.

SDG: GOAL 02: Zero Hunger, GOAL 09: Industry, Innovation and Infrastructure, GOAL 12: Responsible Consumption and Production.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KESAN MEMBERI MAKAN PRA-RAWATAN PELEPAH KELAPA SAWIT DENGAN MENGGUNAKAN KULAT BERFILAMEN TERHADAP PRESTASI TUMBESARAN, PENCERNAAN, DAN KUALITI DAGING KAMBING

Oleh

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Komposisi nutrien Pelepah kelapa sawit (OPF) yang lemah mengehadkan penggunaan OPF yang berkesan untuk pemakanan ruminan disebabkan oleh jumlah lignin yang tinggi (15-56% b/b). Walau bagaimanapun, OPF mempunyai potensi besar sebagai sumber makanan bertenaga untuk ruminan, kerana ia mengandungi kira-kira 80% struktur karbohidrat berdasarkan bahan kering. Lignin secara kimia sukar untuk dicerna disebabkan oleh rangkaian tiga dimensi kompleks yang dibentuk oleh pelbagai hubungan eter dan karbon-karbon antara subunit lignin dan struktur karbohidrat. Kaedah pra-rawatan biologi yang paling berkesan dan aktif digunakan untuk penukaran produk simpanan berserat oleh degradasi lignin ialah pra-rawatan kulat. Kajian ini bertujuan untuk menambah baik nilai pemakanan OPF untuk dijadikan makanan ruminan.

Aktiviti enzim kulat berfilamen ditentukan. Kesan sinergistik kulat juga dinilai untuk meningkatkan aktiviti enzim ligninolitik kulat. Kajian ini juga menilai kebolehdegradasian ruminal *in vitro* dan *in vivo* OPF pra-rawatan dengan ekstrak enzim. Pra-rawatan OPF dilakukan dengan membandingkan antara pra-rawatan silaj dengan pra-rawatan semalaman oleh empat ekstrak enzim yang berbeza. Dalam kajian ini, lapan kulat berfilamen telah dikenalpasti iaitu F49 (*Marasmius palmivorus* MN871732), F02 (*Mucor fusiformis* KY560315), F77 (*Penicillium citrinum* MK312421), F32 (*Trichoderma asperellum* MK928414), F14 (*Schizophyllum commune* S928414), F39 (*Phanerina mellea* or *Ceriporia mellea* MK432982), F03 (*Aspergillus neoellipticus* MG596660) dan F65 (*Syncephalastrum racemosum* MH857909).

kajian ini mendapati bahawa penghasilan aktiviti enzim yang dikehendaki adalah terbaik ditunjukkan oleh *Phanerina mellea*. Pra-rawatan ekstrak enzim oleh *Phanerina mellea* (STB) menunjukkan pengeluaran gas *in vitro* yang lebih tinggi selepas pengeraman 24 jam dengan nilai 17.92 mL/ 250 mg DM. Dalam percubaan pada haiwan, pra-rawatan OPF dengan ekstrak enzim daripada *Phanerina mellea* dan *Schizophyllum commune* meningkatkan skor keadaan badan (BCS) daripada 0 - 25% kepada 75 - 100%. Profile biokimia serum adalah serupa antara setiap diet yang mana berada dalam nilai standard kambing yang sihat. Penilaian daging dan karkas dalam kajian ini menunjukkan bahawa campuran OPF disilaj pra-rawatan berpotensi meningkatkan prestasi karkas kambing dan mengekalkan kualiti daging yang baik.

Kata Kunci: Kulat reput putih, Kambing, Pra-rawatan enzim, Pelepah kelapa sawit, Silaj.

SDG: MATLAMAT 02: Sifar Kelaparan, MATLAMAT 09: Industri, Inovasi dan Infrastruktur, MATLAMAT 12: Tanggungjawab Penggunaan dan pengeluar

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LIST OF ABBREVIATIONS

%	Percent
%/kg	Percent per kilogram
µl	microliter
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
ADF	Acid detergent fiber
ADG	Average daily gain
ADG	Average daily gain
ADL	Acid detergent lignin
AE	*Aspergillus ellipticus*
aNDF	Adequate neutral detergent fiber
ANOVA	Analysis of variance
BCS	Body condition score
BF	Biceps femoris
BLAST	Basic Local Alignment Search Tool
bp	basepair
BSF	Black soldier fly
BW	Body weight
$C_4H_6O_6$	Acid tartrate
$C_6H_8O_7$	Citric acid
CAS-9	CRISPR-associated protein-9 nuclease system
CF	Crude fiber
CH_4	Methane gas
CMCase	carboxylmethylcellylase

CON	Control group
CP	Crude protein
CRISPR	Clustered regularly interspaced short palindromic repeats
Cu^{2+}	copper
CWH	Chinese wildrye hay
DM	Dry matter
DNA	Deoxyribonucleic acid
DNS	dinitrosalicylic
EE	Ether extract
EFB	Empty fruit bunch
FOPF	Fermented oil palm frond
FVWs	Fruit vegetable wastes
g	Gram
g/d	Gram per day
g/kg	Gram/kilogram
g/kg DM	Gram per kilogram dry matter
h	Hour
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HCL	Hydrochloric acid
HPO_3	Metaphosphoric acid
l/d	Gram per liter
L/kg	Liter per kilogram
LAB	Lactic acid bacteria
LD	Longissimus dorsi

LiP	Lignin peroxidase
LMEs	lignin modifying enzymes
M	Molar
MF	*Mucor fusiformis*
mg	miligram
mgDM	Milligram dry matter
mL	mililitter
mL/kg	Milliliter per kilogram
mm	Millimeter
mm	milimeter
Mn^{2+}	manganese
MnP	Manganese peroxidase
mol/L	Moles per liter
MP	*Marasmius palmivorus*
N	Nitrogen
Na_2HPO_4	Sodium phosphate dibasic
NaOH	Sodium hydroxide
ND	Not detected
NDF	Neutral detergent fiber
nm	nanometer
nM	nanomolar
°C	Degrees celcius
OM	Organic matter
OPF	Oil palm frond

OPT	Oil palm trunk
PC	*Penicillium citrinum*
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PKC	Palm kernel cake
PKS	Palm kernel shell
PM	*Phanerina mellea*
POME	Palm oil mill effluent
PPF	Palm press fiber
ppm	Parts per million
PUFA	Polyunsaturated fatty acid
PVPP	Polyvinylpolypyramidone
RBBR	Remazol brilliant blue R
RS	Rice straw
SC	*Schizophyllum commune*
SD	Standard deviation
SEM	Standard error of mean
SEM	Scanning electron microscope
SFA	Saturated fatty acid
SLH	Sugarcane leaf hay
SR	*Syncephalastrum racemosum*
SSF	Solid-state fermentation
SSL	self-sufficiency level
TA	*Trichoderma asperellum*

TMR	Total mixed ratio
tons/ha	Metric tons per hectare
TPU	Taman Pertanian Universiti
U/mL	Unit per milliliter
UPM	Universiti Putra Malaysia
UV	Ultraviolet
VFA	Volatile fatty acid
VP	Versatile peroxidase
WRF	White rot fungi

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background of study

In Malaysia, oil palm plantations use a large part of the land, making oil palm trees the most available plantation. This industry covers 5.6 million hectares (60%) of the total agricultural land which 2.5 million hectares (44.6%) is from Peninsular Malaysia and 3.1 million hectares (55.4%) is from the total plantations in Sabah and Sarawak (MPOB, 2023). This directly contributes in the production of the largest by-product from the agro-industry. The available by-product is abundant and increasingly turned into waste materials that possibly become pollutants. Hence, it is essential to find a solution to turn the by-products into valuable products due to its availability and potential to be a pollutant indirectly will become a value added product that may contribute to the livestock industry. Coincidentally, the livestock industry has a problem in which the cost of feed is the largest share of expenses faced by the farmers (Ghani et al., 2017). Interestingly, the fibrous by-product from agriculture is available and has the potential to be used as animal feed and increase the usage of indigenous feed resources, directly reducing the cost of importing animal feed. The oil palm frond (OPF) is one of the most abundant oil palm plantations fibrous by-products is known to have high natural fiber content (879 g/kg DM) and lignin (200 g/kg DM). OPF is a good candidate to be used as animal feed.

As saprophytic microorganisms, filamentous fungi secrete large quantities of enzymes to be use in decomposition and recycling the complex biopolymers from plant and animal tissues (El-Enshasy, 2007). That roles resulted in increasing interest among researchers to used filamentous fungi in industrial processes. Filamentous fungi are commonly used as cell factory to produce large amount of enzymes for industrial use (Fleißner & Dersch 2010). The application of the enzymes from filamentous fungi includes biomass pre-treatment (Martínez et al., 2018), bioethanol production (Pallín et al., 2024), biogas production (Alexandropoulou et al., 2017), textile industry, dyes decolourization, wastewater treatment, and detoxification (Singh & Singh, 2014). Some members of the filamentous fungi such as white rot fungi (WRF) are excellent degrader of lignin via excretion of extracellular enzymes (Arora et al., 2002). There are previous research involving usage of WRF to pre-treat the agricultural fibrous by-products (e.g. rice straw, wheat straw and sugarcane bagasse) to be used as animal feed (Shrivastava et al., 2011; Eun et al., 2006; Okano et al., 2006). In addition, previous research by Rahman et al. (2011) showed that by elimination of lignin content of OPF increase the ruminal degradability by up to 30%. While research by Hassim et al. (2012) showed increase of 12%, which the difference may be due to different activity of enzyme produced by the WRF used.

This current study aimed to identify the filamentous fungi that have a good combination of high ligninolytic enzyme and low cellulolytic and hemicellulolytic enzyme activity. The increase of the preferred enzyme activity namely laccase, lignin peroxidase (LiP), and manganese peroxidase

(MnP) of filamentous fungi will increase the lignin degradation and the low cellulolytic and hemicellulolytic enzyme activity will reduce the degradation of cellulose and hemicellulose. In the current study, the filamentous fungi were co-cultured to observe the synergistic interaction between fungi. Synergism between two fungal cultures could possibly lead to higher enzyme activity production. However, this effect depends on mode of the interaction between species and the micro environment created to each other. Apart from that, the nutritional condition in the substrate under colonization also plays a role in determining the synergism effect between fungal cultures (Okano et al., 2006).

Removing the lignin content from fibrous by-products has proven to increase the ruminal degradability. However, the usage of single fungal species shows minimal amount in lignin removed. In addition, the lack of knowledge on fungi metabolism results in inconsistent result. Therefore, there is a great interest to have a practical application of enzymatic hydrolysis, in which the enzyme extracted from different fungi were mixed and undergo a test of synergism to further improved their enzymes activity production. Indeed, this could potentially enable more carbohydrate release and allowed rumen microbes to access and fully utilize the carbohydrate polymers (e.g. cellulose and hemicellulose) and improve the ruminal degradability of oil palm fronds.

Other than the enzyme activity assessment, the effectiveness of the pre-treatment was evaluated through *in vitro* ruminal degradability. The *in vitro* gas production, volatile fatty acid (VFA), and the apparent rumen degradable

carbohydrate (ARDC) of the pre-treated OPF were analyzed to see the effect of the pre-treatment. The nutritional composition of the pre-treated OPF was also done to see the nutritional composition of the dry matter (DM), crude fiber (CF), crude protein (CP), ether extract (EE), moisture and Ash. The Van-Soest analysis to determine the cellulose,hemicellulose and lignin composition in pre-treated OPF was also conducted. The effectiveness was also assessed through animal trials in which the goats were fed with a feed formulation that included the OPF pre-treated with enzyme extract from filamentous fungi. The health, feed intake, and body condition performance of the goats were monitored and analyzed. Finally, the goats were slaughtered, and the carcass characteristics and meat quality of the goats were evaluated. The effect of the feeding pre-treated OPF on goats was compared with the goats fed with raw OPF.

1.2 Problem statement

The OPF cannot be used as a sole source of animal feed mainly because of its poor nutritive values and low degradability. Feeding OPF has serious impairment of degradability by rumen microbes due to lignin-(hemi) cellulose bond within OPF. This bond can be broken down by pre-treatment methods such as biological pre-treatment from filamentous fungi. The less effectiveness of lignin degradation by individual enzyme pre-treatment of filamentous fungi triggered researchers to improve the enzyme activity production by fungi co-culture (the interaction between two or more fungi produced synergistic interaction and improve the enzyme activity production)

or mixed enzyme extract (the individual enzyme extract from filamentous fungi were mixed to produced synergism). The study of synergistic co-culture fungi as well as mixed enzyme extract to improve the enzymatic hydrolysis process and ruminal digestibility of OPF have never been characterized and studied.

1.2 Hypothesis

Enzyme extract from identified filamentous fungi contains high ligninolytic enzyme activity. The synergistic effect of identified filamentous fungi can improve the enzymatic hydrolysis process and ruminal digestibility of OPF. Biological pre-treatment using enzyme extract from filamentous fungi is a promising technique to loosen up the lignocellulose structure of OPF and improve the *in vitro* and *in vivo* performance, as well as the carcass and meat quality of goats.

1.4 Objectives

This study aims to assess the effect of feeding OPF pre-treated with enzyme extract from identified filamentous fungi as to reduce the high lignin content that restrict the degradation by rumen microbes on ruminal digestibility and the overall performance of goats.

1. To identify the filamentous fungi collected from rotten oil palm frond and determine the enzyme activity of enzyme extract from identified

filamentous fungi.

2. To assess the synergistic effect on the co-culture of selected white rot fungi on remazol brilliant blue reactive (RBBR) media decolourization observation in enhancing ligninolytic enzyme activity.

3. To assess the *in vitro* ruminal degradability of oil palm frond pre-treated with enzyme extract from the selected white rot fungi.

4. To assess the *in vivo* ruminal degradability, serum biochemical profiles and performance of goats fed with oil palm frond pre-treated with enzyme extract from the selected white rot fungi.

5. To assess the carcass characteristics, volatile fatty acid and meat quality of goats fed with oil palm frond pre-treated with enzyme extract from selected white rot fungi.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of ruminant industry in Malaysia

The global food crisis is currently becoming the main concern in many parts of the world. This crisis affects many countries, and as a small country like Malaysia which is predominantly dependent on import sources, the capability to sustain sufficient food for domestic consumption has become the highlight in the twelfth Malaysian Plan that was held in 2023. For agriculture and agro-based industry, the government aims to strengthen self-sufficiency and food security through the participation of the private sector. The ruminant industry sheltered under the Ministry of Agriculture and agro-based industry also became the main concern in the issues of food security.

Other than fish and poultry, ruminants (goats, cows, buffaloes, and sheep) are also an essential source of protein that needs to be improved, not only in the production but also in the high-quality meat. According to a report by the Department of Veterinary Services (2020), the Malaysian self-sufficiency level (SSL) percentage of beef and mutton in 2018 was 22.88% and 10.95%, respectively. The percentage of beef and mutton are reducing by 0.56% and 0.91% every year since 2013, respectively. This report proved that the supply of beef and mutton was still not stimulating while the demand is escalating (Abdullah & Noor, 2020).

Ruminant farming in Malaysia needs to be more favourable and attractive enough to be run by farmers. The slow return, even with government intervention, demotivates the farmers to stay in this industry. The limited and expensive sources of ruminant feed are one of the main reasons that demotivated the farmers. Various strategies have been proposed in an effort to improve the ruminant industry. It still resulted in a small yield contribution of beef and mutton supply, and eventually, the government failed to achieve the targeted beef's SSL percentage that set up to 32.7% in 2020 (Ariff et al., 2015).

2.2 Issue and challenges in ruminant feeding

Researchers listed the factors that contributed to the small yield of beef and mutton supply in Malaysia. It includes breeding problems, limited grazing area and expensive ruminant feed (Ariff et al., 2015; Hashim, 2015). The ruminant breeding problems refer to the low farm production and inefficient quality of meat, which are mainly caused by the issues in ruminant feeding (Abdullah & Noor, 2020). The common breed of beef cattle and mutton goats in Malaysia is not the main reason that contributes to the low productivity. According to Anderson and Fader. (2004), agricultural productivity and farmer's livelihood can be improved by information transfer and knowledge sharing from researchers to farmers. In Malaysia, the knowledge transfer programs are mainly organized by the Veterinary Services Department and Agriculture Ministry.

Hence, ruminant productivity is influenced by ruminant feeding. The limited

grazing area for the ruminant is one of the main issues and challenges in ruminant feeding. The natural grazing area is very limited due to factors like land competition within other industries. Owners are more interested in investing their land for palm oil, fruits and vegetables or in manufacturing industries rather than ruminant farming (Hashim, 2015). The farming lifestyle changed due to the slow return, and the farmers decided to replace it with higher profit returns like poultry. The urbanization that is rapidly happening in developing countries like Malaysia also contributes to diminishing the grazing area. Modernization and the increase in population activated urbanization and indirectly reduced the grazing area for ruminant feeding (Devendra & Leng, 2011).

The expensive ruminant feed also burdened and discouraged the farmers from sustainably growing. This ruminant industry becomes uneconomic with the increased price of ruminant feeds (Devendra & Leng, 2011). More than 25% of total production costs are from ruminant feed. The utilization of imported feed resources, such as from Europe and Thailand, also contributed to the high cost of feed (Abdullah & Noor, 2020). The utilization of domestic feed resources needed to be implemented as Malaysia has many sources of feed available. The four categories of feeds are pastures and forages (herbaceous legumes, grasses, or multipurpose trees), agro-industrial by-products (palm kernel cake, coconut cake, soya bean meal), crop residues (rice straw, corn residues), and non-conventional feed resources (oil palm frond, palm press fiber, empty fruit bunch, sugar cane baggase). The successful utilization of these feed resources will probably

help to reduce the cost of production and increase the productivity of the overall ruminant industry (Devendra & Leng, 2011).

2.3 Utilization of oil palm frond as animal feed

In Malaysia, oil palm tree plantations become one of the most pivotal and valuable commercial crops (Lai & Idris, 2013). The total amount of 59 million tons annually (dry weight matter) of solid oil palm biomass waste reported in 2012 was contributed by palm kernel shells (PKS), empty fruit bunches (EFB) obtained from the mill, oil palm fronds (OPF) obtained during routine pruning and oil palm trunk (OPT) from the field after clearing off the farm (Chen & Danapal, 2012). In regards to the implementation of green policies by various governments worldwide to turn out a cleaner environment, many ideas have been proposed to utilize the abundant oil palm fibrous by-products, such as in the production of bio-ethanol (Goh et al., 2010), animal feed (Goh, 2017), pulp and paper industry (Pooja et al., 2013), biofuels (Roslan et al., 2014) and bio-composites (Shinoj et al., 2011). Turning the waste materials into valuable product is currently the research interest as to promote green technology by saving biodiversity and ecosystem. The application of waste materials from industries such as the oil palm plantation industry in the production of other value-added products will help in waste material management.

Oil palm has several by-products, such as OPF, OPT, PKS, EFB, palm kernel cake (PKC), palm press fiber (PPF) and palm oil mill effluent (POME)

(Zahari et al., 2003; Abdullah & Sulaiman, 2013). These by-products have been widely reported in Malaysia on their availability in the ruminant feeding system (Kum and Zahari, 2011). The OPF is a strong candidate for animal feed (Namoolnoy et al., 2011). The OPF considered as highly fibrous materials is suitable to be included in ruminant diet, even though the crude protein content is considered as low. Besides low crude protein and total fat, OPF is also low in vitamin and mineral contents, indicating its poor nutritive value limits the effective use of OPF for ruminant feeding (Wanrosli et al., 2007). However, OPF has great potential as the source of dietary energy for ruminants, as it contains about 80% of the structural carbohydrates on a dry matter basis (Tang et al. 2004). The digestibility of OPF in ruminants is affected by various physico-chemical factors. The presence of lignin in the OPF provides a structural strength of the cell wall matrix, limiting the optimal hydrolysis of structural carbohydrates (Binod et al., 2012). Lignin is chemically difficult to be degraded due to a complex three-dimensional network formed by various ether and carbon-carbon linkages among the lignin subunits and structural carbohydrates (Qi-he et al., 2011). The complete mineralization of lignin into water-soluble compounds and CO_2 is a highly oxidative process, which explains the limited degradation of lignin by the rumen microbes (Figure 2.1). Therefore, it is important to disintegrate the lignocellulose structures to improve the digestibility of OPF in ruminants. That will increase the potential inclusion amount of this poor-quality roughage in the animal feed ration. This structural complexity of the lignin stresses the importance of the pre-treatment to improve the degradation of OPF by the rumen microbes (Waghorn & McNabb, 2003).

Pre-treatment is a very important part of industrial processing procedure as it increases the subject's availability. In adjustment of the particle size as well as the chemical composition and structure of materials, pre-treatment is required. Rapid hydrolysis process and greater yields of products (carbohydrate release from lignocellulosic materials) can be achieved and increase the product quality and quantity. Eventually, the pre-treatment aims to provide the benefits such as reduced energy requirements, low cost of production, ease of handling and time savings (Madadi & Abbas, 2017). Many factors that influence the efficiency of the pre-treatment such as the physical structure of the materials, chemical composition, and the treatment requirement (Duque et al., 2016). These factors become the main challenges for the pre-treatment to be successfully done.

Previously, physical (pelletizing, steaming under high temperature and high pressure) and chemical (urea, ionic liquid technology, sequential acid-alkali pre-treatment) pre-treatment showed promising methods to improve the degradability of the lignin by increasing the interactions surfaces of the materials and altering the lignin structure or solubilizing lignin (Liu et al., 2014). The discussion on the methodological variations on physical and chemical pre-treatment compared to biological pre-treatment were discussed extensively by many other previous studies (Nyakuma et al., 2020; Rusli et al., 2019; Ismail et al., 2018; Kamarludin et al., 2014). Due to several disadvantages, such as required high energy input, expensive, and produce toxic that may be harmful to the environment, the choice of pre-treatment

method shifted to biological pre-treatment, which not only can reduce the recalcitrance of fibrous by-products but is also energy-efficient and economically friendly (Wan & Li, 2012).

Taniguchi et al. (2012) reported that the pre-treatment of rice straw by *Pleurotus ostreatus* increased the yield of sugar production compared to chemical pre-treatment. The most effective and actively used biological pre-treatment method for fibrous by-products (rice straw, wheat straw, sugar cane bagasse, OPF, and many others) conversion by lignin degradation is fungal pre-treatment by filamentous fungi (Zhao et al., 2014; Nayan et al., 2020). Especially when the pre-treatment by enzyme extract from filamentous fungi were applied and further enhanced the enzyme activity production by co-culture fungi or mixed enzyme extract to produce synergistic effect.

According to Madadi & Abbas.(2017), white rot fungi (WRF) also a group of filamentous fungi are the only organisms that can decrease lignin faster than other organisms. The growth and metabolism of WRF were influenced by factors, such as fungal species, substrate, culture and growth conditions. Different fungi have different lignin degradation abilities. That makes the selection of fungi/strains for pre-treatment needed to ensure the nutritious consistency of the fibrous by-products before further use as animal feed (Nayan et al., 2020).

The WRF is capable of secreting an extracellular lignocellulosic enzyme for

delignification of the fibrous by-product. There are four major enzymes that can be produced, which are laccase, manganese peroxidase (MnP), lignin peroxidase (LiP) and versatile peroxidase (VP). The ligninolytic accessories enzymes such as feruloyl esterase (FEA), glyoxal oxidase (GLOX), and aryl-alcohol oxidase (AAO) are also produced to help the lignin degradation (Masran et al., 2016). Each of the ligninolytic enzymes aiming to alter and degrade the phenolic and non-phenolic compounds of the lignin. For example, laccase catalyzes the oxidation of the phenolic and non-phenolic substrate, causing one oxygen molecule to be reduced to two molecules of water and aromatic compounds (Liu et al., 2020). The catalysis of laccase enzymes on lignin degradation causes the formation of ketone by oxidation of C-hydroxyl, cleavage of alkyl-aryl bonds, demoethoxylation, polymerization, and cleavage of C-C bonds in phenolic lignin structure. The non-phenolic substructure of lignin can also be oxidized by laccase in the mediators' presence (Isroi et la., 2011).

For manganese peroxidase (MnP) enzyme delignification, like other heme peroxidases enzymes (LiP and horseradish peroxidase (HRP)), the catalytic cycle of MnP is also initiated by hydrogen peroxide (H_2O_2) or an organic peroxide to the native ferric enzyme in the formation of the iron peroxide complex. The iron peroxide complexes oxidize Mn^{2+} to yield Mn^{3+} and water. The Mn^{3+} eventually diffuses into the lignified cell wall and attacks the lignin structure (Xu et al., 2013). Lignin peroxidase (LiP) is monomeric homoproteins enzyme also mentioned as diaryl propane oxygenase, which catalyzes the oxidation process in lignin degradation (Falade et al., 2017).

The oxidation is also a hydrogen peroxidase-dependent process, resulting in the breakdown of non-phenolic lignin structure, which provides highly reactive aryl cation radicals and leads to a random non-enzymatic reaction (Mäkelä et al., 2020). Those events cause lignin depolymerization whenever the cleavage of β-0-4 ether bonds and Cα–Cβ linkages occurs (Xu et al., 2013).

While for versatile peroxidase (VP), the degradation of lignin happened by VPs oxidizing Mn^{2+} to Mn^{3+} and degrading the veratrylglycerol β-guaiacyl ether the nonphenolic lignin model to veratraldehyde and oxidise veratryl alcohol. The degradation also degrades *p*-dimethoxybenzene to veratraldehyde and *p*-benzoquinone (Higuchi, 2004). This biological pre-treatment was applied in various industrial applications such as bio-ethanol production (Kamcharoen et al., 2014), bio-fuels (Wan & Li, 2012), methane production (Mustafa et al., 2016), bio-gas production (Liu et al., 2014) and animal feed production (Isroi et al., 2011) in their processing method. This review is very important to understand the research and improvement trek of the biological pre-treatment of OPF in its application as animal feeding. The optimization, issues, challenges and potential in commercialization were also discussed to show the study's run-through.

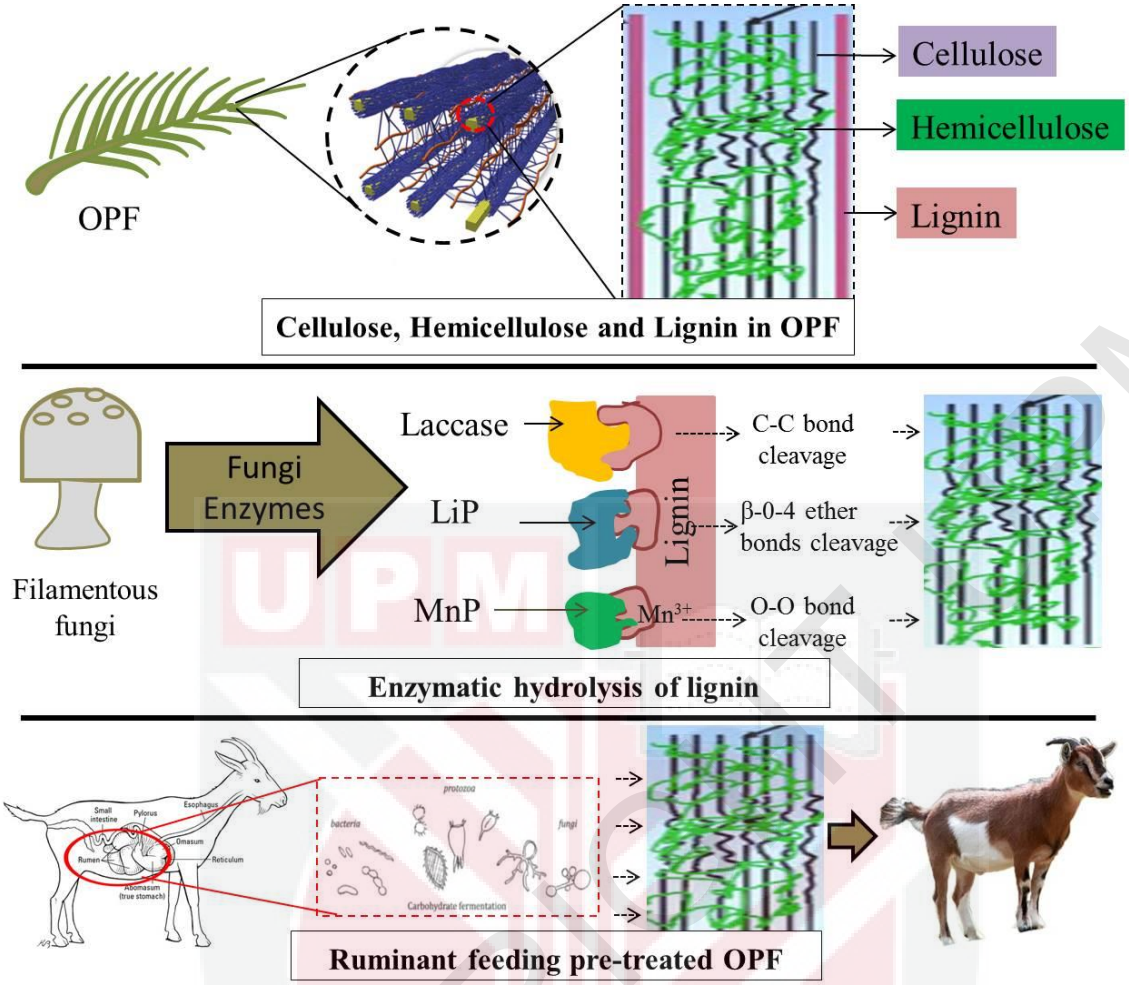


Figure 2.1: Overview of biological pre-treatment of oil palm frond for ruminant feeding

2.4 Specifications for fungal pre-treatment on fibrous by-products

Fungal pre-treatment can be influenced by various factors, especially the fungi species's nature and the fibrous by-product used (Kainthola et al., 2021). Different fungi strain degrades fibrous by-product differently, and the fibrous by-product itself has a different cell wall composition (Noonari et al., 2020). It is proven that the lignin degradation pattern differs in various fungi substrate combinations. For example, *Ceriporiopsis subvermispora* able to degrade more than 50% lignin in rice straw, OPF and sugarcane bagasse,

but only 16% in maize stover (Nayan et al., 2020). Therefore, the evaluation of the impact of fungal strains on different fibrous by-product and/or different fungal strain on the same fibrous by-product is needed (Noonari et al., 2020). Thus, the pre-treatment is also affected by many other factors, such as incubation time, temperature, acidity (pH), inoculums concentration, moisture content and aeration rate, which are other factors that also need further attention (Kainthola et al., 2021; Madadi & Abbas, 2017).

2.4.1 Fungi strains

Due to their unique ligninolytic system, white rot fungi (WRF) which are also a members of filamentous fungi are the most effective microorganism that can degrade fibrous by-products (Wan & Li, 2012). Under solid-state fermentation (SSF), WRF have been studied on their degradation ability towards biological crop residues. They prove that they degrade lignin better than other microorganisms (Shabtay et al., 2009). Many WRF species have been studied in various pre-treatment of OPF, such as *Pleurotus ostreatus* (Tuyen et al., 2013), *Lentinussajor-caju* (Chanjula et al., 2017), *Ceriporiopsis subvermispora* (Rahman et al., 2011), *Coriolus versicolor, Phelbia* sp and *Cyathus stercoreus* (Shrivastava et al., 2011) and many others. According to Tian et al. (2012), less than 20 species out of 1500 different species of WRF were applied in biological pre-treatment. There are possibilities that the unknown WRF can be found in nature, which is potentially suitable to be used in the pre-treatment (Tian et al., 2012). The efficiency of their delignification ability probably showed in different substrates; however, there

is no clear evidence to show the ability of those species in the pre-treatment of OPF.

Rahman et al. (2011) reported that *Ceriporiopsis subvermispora*, *Lentinula edodes*, and *Phlebia brevispora* were the most promising fungi for the OPF pre-treatment. Their study showed that the OPF was treated using WRF for 21 days and 63 days to see the effect of the different incubation periods. The result showed that after 21 days of pre-treatment, *Ceriporiopsis subvermispora* degraded about 45% of the lignin content of OPF, compared to the untreated OPF (9%) with low cellulose loss. While *Lentinula edodes*, and *Phlebia brevispora* reduced about 50% lignin content for 63 days incubation period. However, the cellulose degradation was ±37% and ±10% higher by *Ceriporiopsis subvermispora* for weeks 9 compared with *Lentinula edodes*, and *Phlebia brevispora,* respectively. *Ceriporiopsis subvermispora* also resulted in a lower loss of neutral detergent fiber (NDF) (Rahman et al., 2011).

Hermiati et al. (2013) studied the effects of biological pre-treatment using *Phanerochaete chrysosporium* and *Trametes versicolor* on the enzymatic saccharification of OPF. The result shows that the lignin content of the OPF pre-treated sample of *Phanerochaete chrysosporium* (24.08%) and *Trametes versicolor* (25.79%) is higher (more lignin degradation) than the non-treated sample (16.17%). That increase in lignin degradation is probably due to the decrease of ethanol-benzene extract and hemicellulose, as this result was also shown before in the pre-treatment of OPF by hot compressed water

reported by Goh et al. (2012). They concluded that *Trametes versicolor* is better fungi than that *Phanerochaete chrysosporium* to pre-treat the OPF after incubation of 28 days (Hermiati et al., 2013).

Ceriporiopsis subvermispora and *Trametes versicolor* probably showed the potential in the pre-treatment of OPF. However, there are still many fungi strains waiting in nature that probably have better pre-treatment of OPF. For example, Azmi et al. (2019) reported that the biological pre-treatment of OPF by isolated fungi from the OPF itself found that *Trichoderma harzianum* and *Fusarium solani* showed an optimal enzyme activity compared to *Trichoderma asperellum*, and *Trichoderma koningiopsis* proves that there are many other potential fungi can be found in nature for the pre-treatment of OPF. The pre-treatment of different fibrous by-products by filamentous fungi to degrade lignin have been tabulated in many different studies (Kainthola et al., 2021; Rusli et al., 2021; Rizal et al., 2018; Madadi & Abbas, 2017; Rouches et al., 2016; Vasco et al., 2016; Narayanaswamy et al., 2013; Saritha & Arora, 2012; Isroi et al., 2011). However, the study on the biological pre-treatment of OPF is minimal and needs more specification.

2.4.2 Substrate

The role of the ligninolytic enzyme of filamentous fungi in the delignification of the fibrous by-products remains unclear due to the complicated and heterogeneous nature of fibrous by-products (substrate). It is because different fibrous by-products will have different physic-chemical structures.

According to Vasco-Correa et al. (2019), the various fungal pre-treatment effectiveness on different fibrous by-products is influenced by the preference and the adaptation of the fibrous by-products itself (Vasco-Correa et al., 2019). The structural features of the biomass, including physical (crystallinity, accessible surface area, particle size and pore volume) and chemical compositions (lignin, cellulose, hemicellulose, degree of polymerization), are the limiting factors affecting the enzymatic hydrolysis (Isroi et al., 2011). The pre-treatment of five different fibrous by-products, which are corn stover, switch grass, hardwood, wheat straw, and soybean straw, by *Ceriporiopsis subvermispora* under the same conditions showed that corn stover, switch grass, and hardwood increased glucose yield, but not for wheat straw, and soybean straw (Wan & Li, 2011).

In Malaysia, the feeding of OPF by ruminants has been adopted as a substitute for tropical grasses. The intake and digestibility of OPF by ruminants can be enhanced to more than 30% of the optimum inclusion on their total diet by adding other oil palm by-products (Wan et al., 2003). There are no issues in feeding the OPF to ruminants other than the low nutritive value of OPF due to the high lignin content (Rusli et al., 2019). Rusli et al. (2021) reported on their review of the pre-treatment of OPF showed that the pre-treatment of OPF with *Ceriporiopsis subvermispora* and *Lentinula edodes* studied by Tuyen et al. (2013) had the highest *in vitro* gas production relative change compared to non-treated samples which are +68% compared to the other fungi pre-treatment studies on OPF such as *Lentinussajor-caju* (+0%) (Chanjula et al., 2017), *Ganoderma lucidum* (+9%)

(Rahman et al., 2011), and *Phanerochaete chrysosporium* (-15%) (Hermiati et al., 2013).

From this review, *Ceriporiopsis subvermispora* and *Lentinula edodes* showed the potential to be used in the pre-treatment of OPF (Rusli et al., 2021). However, when comparing the OPF pre-treatment between *Ganoderma lucidum* and *Lentinula edodes*, Rusli et al. (2019) showed that *Ganoderma lucidum* removes lignin ±14% better. Rahman et al. (2011) reported earlier that *C. subvermispora* also showed higher lignin loss than *Lentinula edodes*. They are showing that *Ganoderma lucidum* also has the potential to provide better pre-treatment of OPF. The optimization of OPF pre-treatment by different fungi strain is still needed as many other neglected filamentous fungi probably have better pre-treatment of OPF. The overview of fungi pre-treatment of OPF with the relative changes is tabulated in Table 2.1.

Table 2.1: Overview of fungi pre-treatment of OPF

Fungi Species	Substrate	Assessment of parameters	Result/Relative changes compared to untreated sample	References
Ceriporiopsis subvermispora	OPF	Lignin degradation	Lignin loss (44.72%)	(Rahman et al., 2011)
Phlebia brevispora	OPF	Lignin degradation	Lignin loss (53.77%)	(Rahman et al., 2011)
Lentinula edodes	OPF	Lignin degradation	Lignin loss (56.28%)	(Rahman et al., 2011)
Trichoderma harzianum	OPF	Enzyme activity profile	Better enzyme activity profile compared to control fungi *Lentinula edodes* and *Ganoderma lucidum*.	(Azmi et al., 2019)

Table 2.1: Continued

Ganoderma lucidum	**OPF**	**Lignin degradation**	**Lignin loss (52.63%)**	**(Rusli et al., 2019)**
Lentinula edodes	OPF	Lignin degradation	Lignin loss (37.37%)	(Rusli et al., 2019)
Phanerochaete chrysosporium	OPF	Lignin degradation	Lignin loss (14.55%)	(Hermiati et al., 2013)
Trametes versicolor	OPF	Lignin degradation	Lignin loss (8.48%)	(Hermiati et al., 2013)
Ceriporiopsis subvermispora	OPF	In vitro gas production	Increased ARDC (13%)	(Hassim et al., 2012)
Lentinula edodes	OPF	In vitro gas production	Increased ARDC (10%)	(Hassim et al., 2012)

Notes: Apparent rumen digestible carbohydrate (ARDC), oil palm frond (OPF).

2.4.3 Incubation time

One of the primary issue with large-scale applications of fungal pre-treatment is the long pre-treatment time, which usually takes several weeks to months to get the optimal lignin degradation (Wan & Li, 2012). The fungi strain will be preferable if the pre-treatment produced high lignin degradation with the lowest holocellulose (cellulose and hemicellulose) reduction and happened in the fastest time (Kainthola et al., 2021). It is essential to determine the optimal pre-treatment time of fungi as the enzymatic hydrolysis is also affected by reaction time. Those criteria needed to be found in a particular fungal strain so that their fast-growing rate and high delignification selectivity may improve the pre-treatment time (Tian et al., 2012). For example, a study by Mishra et al. (2017) showed that the pre-treatment of sweet sorghum bagasse by *Coriolus versicolor* produced a ±4.7% higher selectivity value in the pre-treatment for 20 days of SSF compared to 30 days.

The pre-treatment of wheat straw with *Pleurotus ostreatus* in 15 days of incubation improved the biogas production as 17% lignin degradation was found (Albornoz et al., 2018). Tišma et al. (2018) reported that with 7 days of incubation, the pre-treatment of corn silage by *Trametes versicolor* showed 70% of lignin degradation with pH stability and increased bio-gas productivity (Tišma et al., 2018). Mustafa et al. (2017) stated that even though the increasing incubation time may increase the degradation of lignin in rice straw, the incubation time is not impactful to selectivity value of the pre-treatment. As for OPF, previous study reported that *Ceriporiopsis subvermispora* increased apparent rumen degradability carbohydrate (ARDC) after 3 weeks of incubation while *Lentinula edodes* after 9 weeks of incubation during the pre-treatment of OPF (Hassim et al., 2012). Therefore, as the fungi pre-treatment in different incubation times showed no significant selectivity value, the optimization needed to be done depending on the fungi strain on a particular fibrous by-product to have an efficient enzymatic hydrolysis.

2.4.4 Incubation temperature

As a mesophile, fungi are dependent and influenced by temperature to grow. In the fungi pre-treatment, to get the optimum delignification, the optimum incubation temperature is needed, and it is probably different from one fungus strain to another due to their nature of growth. A study was done by Wan & Li. (2010) to test the range of temperature from 4^{o} to 37^{o}C for the pre-treatment of corn stover by *Ceriporiopsis subvermispora*. The result showed

that the optimum temperature for the pre-treatment is 28°C. The previous studies stated, in general, that the optimal temperature range for high delignification by filamentous fungi is 25° to 30°C, even though the fungi can still grow in the temperature range of 15° to 35 °C (Yoon et al., 2014; Wan & Li, 2012).

Kainthola et al. (2021) stated that the 28°C is the optimum temperature for laccase enzyme activity of *Pleurotus ostreatus* while 30°C is the optimum temperature for LiP enzyme activity for *Phanerochaete chrysosporium*. Tian et al. (2012) stated that the optimum incubated temperature for pre-treatment using filamentous fungi in solid-state fermentation (SSF) is 25° to 35°C below the temperature is for chemical and physiochemical pre-treatment. Similar to the other fibrous by-products, the optimum temperature of OPF pre-treatment also depends on the particular fungi strain used. Most of the previous studies on fungi pre-treatment showed to incubate in the range of room temperature, which is 28° to 32°C (Azmi et al., 2022; Rusli et al., 2019; Chanjula et al., 2017; Hermiati et al., 2013; Rahman et al., 2011).

However, there is also a study that incubates the OPF below room temperature, such as a study by Tuyen et al. (2013), which incubates OPF at 24°C but in an air-conditioned chamber with 75% relative humidity. The fungi, which are *Ceriporiopsis subvermispora*, *Lentinula edodes*, *Pleurotus eryngii*, and *Pleurotus ostreatus,* still managed to grow (Tuyen et al., 2013). The higher temperature possibly produces rapid delignification, gives researchers an idea to use the extremophilic fungi in their pre-treatment

(Tian et al., 2012). The incubation time will probably be reduced if the higher temperatures were applied in the OPF pre-treatment, which eventually helps in large-scale production. Therefore, to achieve the optimum incubation temperature, it is essential to consider the other effects on the other parameters such as incubation time, moisture and many others.

2.4.5 Inoculums concentration

There are many different methods in the preparation of inoculums, such as mycelium grown in liquid or agar medium, spawn grown in cereal grains or fungal pre-colonized substrate. Usually, to achieve effective colonization and subsequent delignification, a minimum level of inoculum is already enough to be applied in solid-state fermentation. The increase in inoculum concentration will not change the fungal colonization rate and subsequent growth of the fungi (Wan & Li, 2012). A pre-treatment of aspen wood by different levels of inoculums of pre-colonized wood chips was the alternative method of mechanical pulping. The result showed that increasing the inoculation level for 2-5 % to 20% did not correspondingly increase the energy savings for mechanical pulping (Akhtar et al., 1998). Most of the studies on the pre-treatment of OPF by fungi did not mention the inoculum concentration in their method study (Chanjula et al., 2017; Tuyen et al., 2013; Hassim et al., 2012; Rahman et al., 2011). Some studies, such as Azmi et al. (2022), mention that the pre-treatment used three of 10 mm agar plugs, while earlier, Rusli et al. (2019) used 7 mm of agar plugs in the OPF pre-treatment. No standard inoculation was achieved yet as to determine the

optimum inoculums concentration for the pre-treatment of OPF. A specific optimization study on the inoculum concentration of the OPF fungi pre-treatment was needed to see the effectiveness and overall performance of the pre-treatment.

2.4.6 Nutrients supplementation

Fungal pre-treatment in solid-state fermentation usually uses supplementations of inducers such as hydrogen peroxide (H_2O_2), manganese (Mn^{2+}), nitrogen (N), copper (Cu^{2+}) and aromatic compounds to trigger and stimulate the secretion of ligninolytic enzymes and lignin degradation of the substrate (Shrestha et al., 2008). The formation of fungal biomass and colonization will typically increase when there is nutrient supplementation in the pre-treatment. According to Majeau et al. (2010), the nutrient supplementations in SSF, such as carbon, nitrogen source and even inducer concentration, are needed for fungi growth, which is a crucial factor that affects enzyme production.

However, Wan & Li (2012) stated that, for WRF, the lignin degradation will be better with N starvation, which means lignin degradation will be inhibited by the addition of N supplementation as that will only stimulate the growth of the fungi and increase the consumption of holocellulose of the substrate. The limiting nutrient for fungal growth in wood and soils is nitrogen (Tian et al., 2012). In a study of fungi pre-treatment with *Pleurotus ostreatus*, supplementation of inorganic and organic nitrogen was compared and

resulted that the inorganic nitrogen reduced the ligninolytic enzyme production activity and increased the activity in organic nitrogen supplementation (Kainthola et al., 2021).

The existence of Mn^{2+} influences the production of MnP enzyme activity. The supplementation of Mn^{2+} will cause the domination of MnP enzyme activity production compared to LiP and vice versa. Isroi et al. (2011) also stated that this Mn^{2+} regulation showed in the fungi pre-treatment by *Phanerochaete sp*., *Phanerochaete chrysosporium*, *Phlebia sp*., *Lentinula edodes*, and *Phellinus pini*. However, a study by Shi et al. (2009) reported that the supplementation of Mn^{2+} does not improve the solid-state cultivation pre-treatment as the lignin content shows no significant difference in corn stalks after 14 days of pre-treatment compared to control.

The production of laccase enzyme activity can be controlled by the supplementation of Cu^{2+}. Wyman et al. (2018) reported that the laccase enzyme activity increased 7.7 times for *Pleurotus ostreatus* and 1.29 times for *Pleurotus eryngii* in the pre-treatment of corn stover with Cu^{2+} supplementation. Various studies (ligninolytic enzyme production analysis, glucose yield, Klason lignin decomposition, gas production) were done to indicate nutrient supplementation's effect on fungi pre-treatment. However, it still needs to be significant due to the variety result and effectiveness (Kainthola et al., 2021). In other words, the nutrient supplementation in the pre-treatment of OPF needs to follow the specific fungi strain necessity to ensure the pre-treatment will be successful (Tian et al., 2012).

2.4.7 Moisture content

The moisture content of the substrate is an essential parameter in fungal growth for solid-state fermentation pre-treatment. The initial moisture of substrate ranging from 70 - 80% is generally the optimal level of humidity suggested for delignification activity for WRF (Turgay, 2007). It is the nature of fungi to have better colonization in a high-moisture environment. The higher the initial moisture content of the substrate, the better the fungi growth will be. The water molecules absorbed by the substrate causing the easy access of enzyme as the substrate softens, inner cohesive forces reduction, and swelling of the crystalline matrix of cellulose (Kainthola et al., 2021). However, the over-moist condition will cause oxygen circulation problems for the fungi growth as the surface particles of the substrate were submerged in water (Deswal et al., 2011).

A study by Wan & Li. (2010), the solid-state cultivation of corn cobs by *Phanerochaete chrysosporium* resulted in the highest lignin degradation by the corn cobs cultivated with 85% moisture content with 31.33% lignin degradation percentage. While the corn cobs that were cultivated with 60% and 75% moisture content showed 19.48% and 29.54% lignin degradation percentages, respectively. The moisture content at 75% showed about 10% higher lignin degradation compared to 60%, while not significantly different compared to 85% moisture content (Wan & Li, 2010). In contrast, Shi et al. (2007) reported that the lignin degradation of cotton stalks by *Penicillium chrysosporium* is 6% higher in 75% and 80% moisture content compared to

65% which showed similar observation as Wan & Li. (2010).

Another study on the pre-treatment of rice straw by *Pleurotus ostreatus* on 20 days of incubation showed that lignin degradation is higher in 75% moisture content compared to 65 and 85% (Mustafa et al., 2016). According to Tian et al. (2012), the inchoative humidity for fungal primary growth and secondary metabolism is 60 to 90 wt%. The dry or excess moisture of biomass potentially inhibits growth and kills the fungi. According to Xu et al. (2007), having a lower solid-liquid ratio in the pre-treatment is preferable to produce better LiP and MnP enzyme activity. As for OPF pre-treatment, no specific study has yet identified the optimum moisture to achieve the optimum delignification with low holocellulose digestion.

2.4.8 Aeration rate

Aeration is one of the critical parameters in the pre-treatment of substrate by WRF. The oxidative process of lignin degradation by WRF makes oxygen availability essential to be applied by ligninolytic enzyme activity. The reasonable supply of oxygen will determine fungal growth, enzyme production and lignin degradation (Shi, 2007). Aeration is a crucial factor in fungi metabolism as to help in the processes such as oxygenation, CO_2 elimination, heat production, humidity regulation and volatile compound distribution (Turgay, 2007). Solid-state fermentation (SSF) usually uses the Erlenmeyer flask and is covered with cotton plugs, which sufficient air circulation was expected for delignification processes. According to Tian et

al. (2012), SSF treatment is a preferable pre-treatment technique as the oxygen diffusion is rapid and favourable for WRF growth.

However, active aeration is needed for reactors containing packed substrate to provide uniform air diffusion. Otherwise, the delignification will be inhibited by the insufficient oxygen needed by ligninolytic enzyme activity. The delignification rate can be increased with the high oxygen supplied, but it will not help in delignification selectivity. The higher aeration rate can sometimes increase the delignification, although the parameter is very strain-dependent (Brethauer et al., 2017). Lopez et al. (2002) reported that the increase of the aeration in the pre-treatment of fibrous by-products by *Coriolis versicolor* showed a significant increase in organic mineralization rate.

Certain fungi pre-treatment reduce their pre-treatment time with the concentrated oxygen supplied, such as *Phanerochaeta chrysosporium* (Madadi & Abbas, 2017), *Phanerochaete sordida* and *Pycnoporus cinnabarinus* (Shi et al., 2009). Eventually, the aeration of the fungal pre-treatment needed to be controlled to achieve optimal enzyme activity performance (Wan & Li, 2012). There is no specific optimization study on the aeration supplementation in the pre-treatment of OPF that were done before. The optimization study on the pre-treatment of OPF needed to consider the importance of this parameter.

2.5 Feeding pre-treated oil palm frond to ruminants

Ruminants are adapted well at digesting the carbohydrate in plant cell walls. Therefore, the fibrous by-products can be included in the ruminant diet to prevent competition with human nutrition ingredients besides reducing the production of waste materials that may become pollutants (Van et al., 2015). However, the low nutritional value of the fibrous by-products caused by the high lignin content may influence the overall performance of ruminants.

The nutritional value of fibrous by-product, such as OPF, can be improved by pre-treatment. The effectiveness of the pre-treatment to enhance the nutritional value becomes the highlight in an animal feeding study. The finding on the analysis of the effectiveness of the pre-treatment is complex and need standard information and method used in that study. The study on the efficacy of the biological pre-treatment on chemical analysis, *in vitro* and *in vivo* study needed to be optimized more in detail.

2.5.1 Nutritive value

Fibrous by-product like OPF is one of the world's waste streams with cell walls consisting of lignin, cellulose and hemicellulose. The cellulose and hemicellulose can be energy source when the polysaccharides are converted to sugar (Van et al., 2015). Cellulose and hemicellulose can be the primary feed ingredient for ruminants as the rumen microbes can degrade most of the carbohydrate in the plant. The nutritional value of the biomass is different depending on various factors such as the plant species, geographical conditions, soil chemistry, agronomic practices, and many others. Even

different pre-treatment of the OPF resulted in different nutritional values for ruminants. Those differences allowed the researchers to optimize and evaluate the effect of biological pre-treatment on the nutritional value of fibrous by-products.

Raw OPF nutrient content is enough to be offered to the ruminant. The OPF potential value for livestock feeding is supported by the average crude protein (CP) composition (11.0%) in the leaflets, which is far above the critical CP level (6.25%) required to maintain the regular intake by the animal (Dahlan, 2000). Shin et al. (1999) reported that the dry matter intake (DMI) of OPF (14.57 kg/ head/ day) by lactating dairy cows is higher compared to rice straw (RS) (13.63 %), Chinese wildrye hay (CWH) (14.02 kg/ head/ day) and sugarcane leaf hay (SLH) (12.54 kg/ head/ day). Besides that, the digestibility of OPF is better than SLH and RS when it resulted in higher carboxymethyl cellulase (CMCase) and xylanase activity, ammonia production, total volatile fatty acid (VFA) concentration, and acetate and propionate ratio (Shin et al., 1999). The metabolizable energy intake of OPF-fed goat (311.3 kg ME/kg) is in the range of goat maintenance requirement (238.7 – 585.13 kg ME/kg). The nutrient composition of OPF is sufficient for feed maintenance and the production of quality meat from goats (Dahlan, 2000).

Including OPF into the ruminant diet increased the meat quality of the animal. Ebrahimi et al. (2012) reported that the inclusion of OPF in the goat diet reduces the saturated fatty acid (SFA), primarily palmitic acid (16:0) and

stearic acid (18:0), levels in the longissimus dorsi (LD) and biceps femoris (BF) muscles. High levels of SFA in human diets can cause heart disease when the low-density lipoprotein (LDL) cholesterol levels increase. The increased OPF feeding intake by the goat increased the fiber feeding and directly increased the polyunsaturated fatty acid (PUFA) (arachidonic acid, C20:0) in the animal tissues (Ebrahimi et al., 2012).

Some studies show no improvement in metabolizable energy in animal feeding the OPF diets (Chanjula et al., 2017; Rahman et al., 2015; Ebrahimi et al., 2013). However, the digestibility of OPF increases the animal's fiber intake improves the animal's carcass characteristics. The inclusion of OPF in goat-feeding diets showed better carcass characteristics such as lower dressing percentage, lower warm carcass weight, and lower back fat thickness compared to control diets (without the inclusion of OPF) (Ebrahimi et al., 2013). Using OPF in animal diets could reduce the fat content in the carcass. Feeding high OPF to sheep significantly decreased the total amount of subcutaneous fat (Rajion et al., 2001).

In Malaysia, the feeding of freshly chopped OPF to ruminants has widely used by farmers. Zahari et al. (2012) suggested that 40% of newly chopped OPF in a palm kernel cake (PKC) based diet for cattle are the most economical ratio. However, the study showed that the cattle's average daily gain (ADG) decreases as the OPF inclusion percentage increased (Kum & Zahari, 2011). The reduction explained that the freshly chopped OPF is not a good source of nutrition for the animal to consume solely. The OPF contains

poor nutrient composition due to the high amount of lignin (15-56% w/w) limits the effective use of OPF (Wanrosli et al., 2007). The degradation of the lignin structure allows the rumen microbes' penetration to digest the cellulose and hemicellulose of the OPF.

2.5.2 *In vitro* digestibility

Various reports on *in vitro* rumen fermentation studies were done on the digestibility and effect of pre-treatment to improve the quality of the animal feed. The *in vitro* gas production technique is usually preferable to evaluate the ruminal degradability of meal as it has a good correlation with *in vivo* digestibility (Rusli et al., 2019). This method is used to analyse the effect of the digestion of feed formulation by rumen fluid or fecal inoculum from ruminants on the gas production or weight loss methods (Van et al., 2015). In common research practice, *in vitro* will be conducted first before *in vivo* evaluation as the advantage of being less expensive, time-saving and allowing maintenance of more precise experimental conditions. The benefit of studying fermentation kinetics of roughage in rumen animals increases the usage of *in vitro*, especially when the interest in the efficient utilization of roughage diets is up surging (Huang et al., 2021).

The possibility to evaluate the digestive, fermentative and microbial parameters simultaneously shows a close correlation with the *in vivo* method. The *in vitro* studies can predict the ruminal digestion of the roughage before any further supplementation in higher risk. Despite the

accurate approach of *in vivo* digestive evaluation, the evaluation of intake and digestibility of fibrous by-products is time-consuming, laborious, expensive, requires a large quantity of feed and is unsuitable for large-scale feed evaluation (Sallam et al., 2007). Several factors can affect the *in vitro* evaluation of fibrous by-products nutrient content. The anaerobiosis, pH, temperature, buffer used, feed quantity, the animal and batch of inoculum are the factors affecting fermentation activities by rumen microorganisms that influence the *in vitro* gas production. Hence, specific protocols and skills are needed to perform the method to achieve a standard with known gas production (Amanzougarene & Fondevila, 2020).

The *in vitro* study on biological pre-treated OPF reported in various studies to assess the effect of the fungi pre-treatment in increasing the *in vitro* ruminal degradability (Table 2.2). For example, Tuyen et al. (2013) reported that the OPF pre-treated with *Ceriporiopsis subvermispora*, *Lentinula edodes*, *Pleurotus eryngii*, and *Pleurotus ostreatus* showed an increase in gas production by 68-132% with *Ceriporiopsis subvermispora* showed the highest volume when fermentation was done in the rumen fluid from two non-lactating Holstein–Friesian cows. This study was supported by Rahman et al. (2011), which also reported that the OPF pre-treated with *Ceriporiopsis subvermispora* showed the highest in vitro gas production when compared to the other fungi pre-treatment (*Pleurotus eryngii*, *Bjerkandera adusta*, *Lentinula edodes*, *Phanerochaeta chrysosporium*, *Phlebia brevispora*, *Schizophyllum commune*, *Ganoderma lucidum*, *Pleurotus ostreatus*, and *Trametes versicolor*) after 96 h incubation in lamb rumen fluid.

The *in vitro* method performed by Rahman et al. (2011) was then applied in another study to assess the effect of fungi pre-treatment of OPF with *Ganoderma lucidum* and *Lentinula edodes* which was reported by Rusli et al. (2019). The result showed that the OPF pre-treated by *Ganoderma lucidum* showed the highest gas production (±27% higher than control) and apparent rumen degradable carbohydrate (ARDC) value compared to the other pre-treatment method. This study successfully proved that the *in vitro* method followed Rahman et al. (2011) is an effective way to assess the effect of the pre-treatment by evaluate the gas and short-chain fatty acid production resulting from the digestion of pre-treated OPF by rumen fluid (Rusli et al., 2019). The value of the gas production studied by Rusli et al. (2019) is lower (48 ml) when compared to Rahman et al. (2011) (330 ml) due to the different fermentation conditions factors such as the source of rumen fluid, different fungi strains of pre-treatment and many others.

Table 2.2: Overview of the *in vitro* assessment of biological pre-treated OPF

Fungi Species	Substrate	Rumen fluid animal	Fermentation period and temperature	Assessment of parameters	Result	References
Ceriporiopsis subvermispora	Pre-treated OPF for 3 and 9 weeks	Lamb rumen fluid adapted to OPF	39°C, 96 h	Gas and ARDC production	±40% higher gas and ARDC (3 weeks) production compared to control	(Rahman et al., 2011)
Phlebia brevispora	Pre-treated OPF for 3 and 9 weeks	Lamb rumen fluid adapted to OPF	39°C, 96 h	Gas and ARDC production	±39% higher gas and ARDC (3 weeks production compared to control	(Rahman et al., 2011)

Table 2.2: Continued

Fungi Species	Substrate	Rumen fluid animal	Fermentation period and temperature	Assessment of parameters	Result	References
Lentinula edodes	Pre-treated OPF for 3 and 9 weeks	Lamb rumen fluid adapted to OPF	39°C, 96 h	Gas and ARDC production	±32% higher gas and ARDC (9weeks) production compared to control	(Rahman et al., 2011)
Pleurotus eryngii	Pre-treated OPF for 3 and 9 weeks	Lamb rumen fluid adapted to OPF	39°C, 96 h	Gas and ARDC production	±34% higher gas and ARDC (9 weeks) production compared to control	(Rahman et al., 2011)
Bjerkandera adusta	Pre-treated OPF for 3 and 9 weeks	Lamb rumen fluid adapted to OPF	39°C, 96 h	Gas and ARDC production	±3% higher gas production compared to control for 3 weeks only	(Rahman et al., 2011)
Phanerochaete chrysosporium	Pre-treated OPF for 3 and 9 weeks	Lamb rumen fluid adapted to OPF	39°C, 96 h	Gas and ARDC production	±25% higher gas and ARDC (3 weeks) production compared to control	(Rahman et al., 2011)
Schizophyllum commune	Pre-treated OPF for 3 and 9 weeks	Lamb rumen fluid adapted to OPF	39°C, 96 h	Gas and ARDC production	Lower gas and higher ARDC (9 weeks) production compared to control	(Rahman et al., 2011)
Ganoderma lucidum	Pre-treated OPF for 3 and 9 weeks	Lamb rumen fluid adapted to OPF	39°C, 96 h	Gas and ARDC production	±25% higher gas and ARDC (9 weeks) production compared to control	(Rahman et al., 2011)
Pleurotus ostreatus	Pre-treated OPF for 3 and 9 weeks	Lamb rumen fluid adapted to OPF	39°C, 96 h	Gas and ARDC production	±24% higher gas production compared to control	(Rahman et al., 2011)
Trametes versicolor	Pre-treated OPF for 3 and 9 weeks	Lamb rumen fluid adapted to OPF	39°C, 96 h	Gas and ARDC production	±1% Higher gas production compared to control for 3 weeks only	(Rahman et al., 2011)

Table 2.2: Continued

Fungi Species	Substrate	Rumen fluid animal	Fermentation period and temperature	Assessment of parameters	Result	References
Ganoderma lucidum	Enzyme pre-treated OPF for 5 days incubation	Katjang goats adapted to OPF	39°C, 24 h	Gas production and ARDC	±27% higher gas and ARDC production compared to control	(Rusli et al., 2019)
Lentinula edodes	Enzyme pre-treated OPF for 5 days incubation	Katjang goats adapted to OPF	39°C, 24 h	Gas production and ARDC	±18% higher gas and ARDC production compared to control	(Rusli et al., 2019)
Ceriporiopsis subvermispora	OPF	non-lactating Holstein–Friesian cows	Not mention temp, 72 h	Gas production	±41% higher gas production compared to control for 6 weeks incubation	(Tuyen et al., 2013)
Lentinula edodes	OPF	non-lactating Holstein–Friesian cows	Not mention temp, 72 h	Gas production	±33% higher gas production compared to control for 6 weeks incubation	(Tuyen et al., 2013)
Pleurotus eryngii	OPF	non-lactating Holstein–Friesian cows	Not mention temp, 72 h	Gas production	±29% higher gas production compared to control for 6 weeks incubation	(Tuyen et al., 2013)
Pleurotus ostreatus	OPF	non-lactating Holstein–Friesian cows	Not mention temp, 72 h	Gas production	±25% higher gas production compared to control for 6 weeks incubation	(Tuyen et al., 2013)
Trichoderma harzianum	Enzyme pre-treated OPF	Four brahman bulls	39°C, 72 h	Gas production and ARDC	±81% higher gas and ARDC production compared to raw OPF	(Azmi et al., 2022)
Fusarium solani	Enzyme pre-treated OPF	Four brahman bulls	39°C, 72 h	Gas production and ARDC	±80% higher gas and ARDC production compared to raw OPF.	(Azmi et al., 2022)

Notes: Apparent rumen degradable carbohydrate (ARDC), oil palm frond (OPF).

2.5.3 *In vivo* study

In vivo or animal trial, study is the final phase in evaluating the effectiveness of the formulated animal feed. This study is the most accurate and precise approach to determine the digestibility of fibrous by-products, while the *in vitro* study is the alternative method to achieve the *in vivo* standard (Butts et al., 2012). The rumen degradability *in vitro* cannot directly extrapolate to *in vivo* applications. This is the biggest concern as the palatability issue aroused whether the feed offered to the animal is acceptable or rejected (Van et al., 2015). For example, the poorer palatability of the treated OPF reduces feed intake by the animal and could alter the OPF degradability *in vivo*. This is proved by some studies where animals fed the control diet with non-treated rice straw compared with animals fed the diet containing fungal-treated straw showed that control performs better. The high mineral content of fungal-treated straw caused by the addition of minerals modified the fed structure conditions and ruined the palatability of the straw (Walli, 2010).

With enzyme pre-treatment, no additional mineral needs to be added to cause palatability problems, however, the presence of lignin derivatives during lignin degradation can lower the palatability (Salama et al., 2019). Several *in vivo* studies showed satisfying results with the pre-treated feed diet, especially in small ruminants. The study on the effect of pre-treated

OPF feeding on meat quality and animal performance is minimal. Musnandar et al. (2011) studied the effect of fermented OPF (FOPF) feeding on body weight gain and goat meat quality compared to grass feeding. The study showed that the FOPF-feeding goat increases in growth performance by incrementing 36% the average daily gain (ADG) of the goat. That improvement directly increases the carcass weight, and yield ranged from 38.41 to 41.38% and increased the value of rib-eye area ranged from 7.76 to 9.73 cm^2.

In this study, the CP value of FOPF feeding goat is 6% higher than the control diet. However, it does not improve the protein of the meat. No improvement in feed intake proved that the animals have no problems consuming the FOPF (Musnandar et al., 2011). Indeed, this study is insufficient to show the efficient effects of OPF pre-treatment on meat quality and growth performance as the lack of optimization parameters. Hence, more parameters need to study on to understand further the effectiveness of pre-treated OPF in livestock feeding.

2.6 Issues and challenges for up-scaling

Up-scaling is one of the critical parts of any pre-treatment generally. For biological pre-treatment, the slow processing of delignification becomes the most significant barrier for up-scaling in terms of quality control. Besides that, the additional cost of processing becomes the main concern in up-scaling for the industrial level. Even though biological pre-treatment is less expensive

and eco-friendly compared to physical and chemical pre-treatment, the effectiveness and cost of the pre-treatment when it's come to up-scaling at the industrial level still need to be optimized. The biological pre-treatment effectiveness must be improved to achieve the quality requirement in industry (Zheng et al., 2014). There are many challenges in the application of biological pre-treatment of OPF, even though the research with different fungi strains indicates that the successful improvement of rumen degradability depends on the choice of WRF (Azmi et al., 2016; Rouches et al., 2016; Lee et al., 2007). The challenges are not restricted to the choice of filamentous fungi with the highest degradability and effective enzyme.

Another parameter, such as the length of pre-treatment time, also contributes to the overall enzyme production system. The incubation or pre-treatment period of fungi is time-consuming, which leads to the reduction of the nutritional value of the fibrous by-products. The highest ligninolytic enzyme activity could be observed after 25 days of the pre-treatment (Namoolnoy et al., 2011). While Hassim et al. (2012) reported that *L. edodes* laccasse activity showed the highest enzyme activity after 45 days of treatment, which gradually developed over the treatment period.

Another aspect that needs to focus on is the action of the fungi and enzyme, which degrading not only OPF lignin but also cellulose and hemicellulose simultaneously. Rahman et al. (2011) reported the pre-treatment of OPF by *C. subvermispora* reduced the cellulose content by 9.12%. Therefore, more research on the characterization of enzyme activities of filamentous fungi needed to further understand its potential benefits on plant cell wall

degradation. Otherwise, another option must be considered, such as the mix culture of WRF proven to enhance the enzymatic activities by synergistic effect (Chi et al., 2007). To further improve the rumen degradability, better characterization of the substrate before pre-treatment with WRF enzyme extracts could also help to choose the best WRF species and length of treatment needed.

The prosperous research study of the pre-treatment to be applied to farm practice becomes a significant challenge in improving rumen degradation as specific knowledge and technical skills are required in the field. A workshop, training, or application guidelines need to be established by the nutritionist to show the right way on how the pre-treatment works on a farm. However, another challenge arises when the well-trained farmers leave (Owen et al., 2012). Walli (2010) recorded these struggles in India when the trained people disappeared, and the farmer stopped treating their straw with urea in the post-project stage. After all, the farmer's interest in the technology and pre-treatment will depend on how effective the pre-treatment is to improve the animal performance. In India, for example, after the observation and satisfaction with the increase in the growth rate of calves of 100 to 150 g/d and increased milk yield of cows of 0.5 to 1.5 L/d, the farmers willing to accept urea pre-treatment to cereal straw (Roy & Rangnekar, 2006). The effect of OPF pre-treated with filamentous fungi or enzyme extracts in animals needed further examination to detect the possible limiting factor affecting the animal performance.

In the commercial view of the pre-treatment, the advantage will be on the application in an industrial environment compared to individual farmers. The industry not only can avoid extra work for the individual farmers but also can standardize the pre-treatment condition on a larger scale with better quality. However, a problem arises when the additional cost for the pre-treatment, such as the transportation costs, could burden the industry. Excellent facilities are needed to transport the large volume of fibrous by-products for further processing. Indeed, in achieving the optimal improvement of degradability, specific technologies, and skills to perform the pre-treatment are crucial for further research.

2.7 Optimization and improvement of biological pre-treatment

Several problems in the direct application of WRF in industrial level directed researchers to implement the pre-treatment by using purified enzyme extract alone (Masran et al., 2016). However, enzyme extract application resulted in less effectiveness in lignin degradation. Then, studies found that the implementation of enzyme extract can be improved by applying an enzyme cocktail/mixed to maximize the delignification and increase energy. Combining ligninolytic enzyme extract from different fungi will trigger the various active isoenzymes to degrade lignin. For example, the purified mixed enzymes of *P. florida*, *P. sajor-ceju* and *P. eryngii* used to pre-treat a corn cob resulted in 5.2% lignin loss compared to single *P.florida* with 2.8% lignin loss (Naraian et al., 2010). Hence, the fungi pre-treatment enhancement optimization in delignification and preserving cellulose were further studied

with gene modification technology.

2.7.1 Co-culture of fungi

The attention switched from mono-culture to co-culture fungi pre-treatment when a study reported the ability of WRF to degrade lignin increased in co-culture as the competition of space and nutrients elevated the production of lignin modifying enzymes (LMEs) such as laccase and MnP. The term fungal co-culture is described as the simultaneous cultivation of two or more in the same medium, either as part of a natural community or artificial (Sperandio & Ferreira, 2019). The co-culture of two or more WRF may result in a synergistic effect and improve the secretion of degrading lignin enzymes (Sperandio & Ferreira, 2019). Many co-culture studies show more efficient enzyme activity production due to their synergistic action than a single culture (Parani & Eyini, 2010). The co-culture of *T. reesei* and *Coprinus comatus* to enhance the corn stover hydrolyzation increased laccase activity. This study managed to improve the digestion of corn stover with almost 49% weight loss, higher than mono-cultures with 14% weight loss after over 72 h of hydrolyzation (Ma & Ruan, 2015).

2.7.2 Enzyme extract of filamentous fungi

Several limitation in the direct application of filamentous fungi such as time-consuming, difficulties in satisfying the growth requirements on a large scale and penetration of mycelia into the substrate, directed researchers to

implement the pre-treatment using purified enzyme extract alone (Masran et al., 2016). Other than that, it is essential to prevent the loss of nutrients in fibrous by-products that might be consumed by the inoculated fungi in direct fungal pre-treatment (Adaskaveg et al., 1990). Hence, study on the application of crude enzyme extract in fibrous by-products degradation is warrant. In addition, the study on the applications of enzyme extract in OPF pre-treatment is minimal as most of the studies were extensively studied in other fibrous by-products.

The pre-treatment of OPF with enzyme extract from *Ganoderma lucidum* and *Lentinula edodes* reduced the lignin by 11% and hemicellulose by 13% compared to untreated. Besides that, the pre-treatment also increased the CP (80 g/kg DM) and total *in vitro* gas production compared to untreated OPF. This result proves that the pre-treatment of OPF with enzyme extract increased the lignin degradation and rumen digestibility of OPF (Rusli et al., 2019).

Lu et al. (2010) looked at the efficiency of the laccase enzyme extracted from *Pycenoporus sanguineus* in the pre-treatment of corn stover and wheat straw to be potential biofuel feedstock. The study showed that from 1 g wheat straw, over 97% of the klason lignin was removed without loss of Somogyi carbohydrate. In this study, crude enzyme pre-treatment might represent a more straightforward and more effective method besides can minimize the limitations encountered in direct fungal pre-treatment
(Lu et al., 2010).

Naraian et al. (2010) reported that the delignification of fibrous by-products by a single type of purified ligninolytic enzyme showed low lignin loss. Therefore, a purified enzyme mixture is used to maximize the delignification and increase the effectiveness. The combination will set off the active isoenzymes to degrade lignin in the fibrous by-products cell wall matrix. For example, the purified enzymes cocktail of *Pleurotus florida*, *Pleurotus sajor-ceju* and *Pleurotus eryngii* used to pre-treat a corn cob resulted in 5.2% lignin loss compared to single *Pleurotus florida* with 2.8% lignin loss (Naraian et al., 2010). In the production of enzymes, SSF has become the top choice by industries (Figure 2.2**).** SSF provides low capital costs, high volumetric productivity, energy savings and easy to applied (Patel & Gupte, 2016; Tian et al., 2012). Due to better ligninolytic enzyme activity and near to nature conditions, SSF is a better solution than liquid-state cultivation (Kainthola et al., 2021).

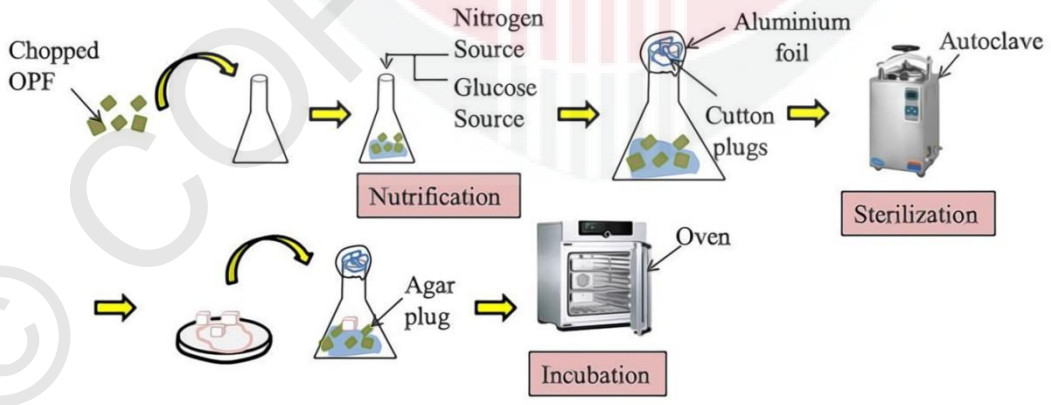


Figure 2.2: Solid-state fermentation method flow (Rahman et al., 2011).

2.7.3 Gene editing

Gene editing technology is currently applied in various research fields. Researchers were interested in exploring and developing their studies by gene editing technologies (Zhang et al., 2022). It has been proposed to genetically engineer the fungi strain to only use the hemicellulose as their source of growth and preserve the cellulose. The mutation breeding and cross-breeding technologies proposed to improve the WRF strain years ago were finally unsuccessful. However, the idea managed to give a better understanding of gene editing technology on fungi. According to Zhang et al. (2022), the successful bio-engineering through gene editing technology of fungi could improve and better understanding the complex gene system, which can be used for further improvement. Another idea in genetic engineering of fungi is to fabricate fungi that can survive and degrade lignin at higher temperatures to produce rapid pre-treatment, consequently reducing production costs (Tian et al., 2012).

The latest gene editing technology called clustered regularly interspaced short palindromic repeats (CRISPR) has been used to genetically modify various microorganisms. The most popular CRISPR system, the type II CRISPR associated with endonuclease Cas9 protein, was used to cleavage the target DNA at the specific desired site (Zhang et al., 2022). This CRISPR-Cas9 gene-editing tool has been widely used in many filamentous fungi such as *Ganoderma lucidum* (Wang et al., 2020), *Coprinopsis cinerea* (Sugano et al., 2017), *Agaricus bisporus* (Waltz, 2016), *Cordyceps militaris* (Chen et al., 2018) and also *Pleurotus Sp* (Boontawon et al., 2021). The

CRISPR-Cas9 gene editing tool applications in different filamentous fungi were tabulated in Table 2.3.

Table 2.3: Overview of CRISPR-Cas9 gene editing tool applications in different filamentous fungi

Fungi Species	Application	Outcome	References
Ganoderma lucidum	Edit the functional genes of ganoderic acid (GA) biosynthesis in *G. lucidum* which have unique triterpenoid-anti-tumor ganodric acids.	The mutant showed a significant decrease in the titer of four identified GAs compared to wild-type *G. lucidum*.	(Wang et al., 2020)
Coprinopsis cinerea	To express Cas9 and a *U6-snRNA* promoter from *C. cinerea* to express gRNA or $CcDED1_{pro}$ promoters were applied to develop more efficient genome editing of CRISPR/Cas9 in C. cinerea.	$CcDED1_{pro}$ novel promoter was identified to have seven times more potent activity than the conventional promoter *GPD2* in a high-throughput reporter assay with cryopreserved protoplasts.	(Sugano et al., 2017)
Agaricus bisporus	To resist the browning of *Agaricus bisporus* (white button mushroom) by knocking out one of six polyphenol oxidase (PPO) enzyme genes.	Reduced the enzyme activity of PPO, an enzyme that causes browning by 30%.	(Waltz 2016)
Cordyceps militaris	To generate efficient genome editing of *Cordyceps militaris* by using Pcmlsm3 promoter and Tcmura3 terminator that involved the negative selection marker *ura3,* a CRISPR-Cas9 system that included the Cas9 DNA endonuclease, RNA presynthesized *in vitro* and a single-strand DNA template.	Efficiently generated site-specific deletion and insertion.	(Chen et al., 2018)
Pleurotus Sp	To observe and analysed the phenotype *P.*	The pseudo-clamp cell was observed in monocaryotic	(Boontawon et al., 2021)

	ostreatus pcc1 or clp1 (Popcc1 or Poclp1) mutants generated using CRISPR/Cas9.	Popcc1 mutants after crossing two compatible strains with Popcc1 mutations.

Notes: Ganoderic acid (GA), Clustered regularly interspaced short palindromic repeat (CRISPR), CRISPR-associated protein-9 nuclease (CAS-9), glycerol-3-phosphate dehydrogenase 2 Promoter (GPD2), *Coprinopsis cinerea* DED1 promoter (CcDED1pro), Vertebrate U6-small nuclear RNA (U6-snRNA), pseudo clamp cell 1 (pcc1), clampless 1 (clp1).

2.8 Potential commercialization of pre-treated OPF

In commercialization, the essential attribute needed to be considered first in applying biological pre-treatment is the processing cost (Tian et al., 2012). The cost can be very cheap as fibrous by-products are available due to the abundant by-products waste production. However, the management part of the production, such as transport, processing and treatment, will be the main cost contributing to inefficient production for commercialization as animal feed. According to Malenica et al. (2022), due to prevent the microbial deterioration and spoilage of fruit vegetable wastes (FVWs), it has to be transported immediately with water reduction strategies which are very costly, especially if the location is far away. This problem triggered to dry the FVWs and producing the feed immediately at the collection area. However, the drying method reduces the quality of the FVWs as it decreases the level of bio-active compounds (Silva et al., 2019). Several methods can potentially improve and solve the problem, which make sure the quality of the feed is still good to be offered to the animal. The methods are freeze-drying, pelletizing, and ensiling.

2.8.1 Freeze-drying

Freeze-drying is a dehydration technique uses a low temperature based on the sublimation mechanism of water resulting in a reduction of water activity. The sublimation and desorption of water causes the freezing of the product (fibrous by-product), which eventually maintains the quality by preserving the taste, colour, appearance, aromas and nutritional value (Oyinloye & Yoon, 2020; Silva et al., 2019). However, due to the lengthy process time and energy cost related to the vacuum stage, its produces a high cost which is economically inefficient to be used in the commercialization of the animal feed. The freeze-drying method is not suitable for a high-volume production product such as animal feed. It has only been widely used in the product with high value, such as in pharmaceutical industries and some specific food industry (Silva et al., 2019).

2.8.2 Pelletizing

Pelletizing has been known for more than two decades as animal feed consisting of mixed food material and supplements. It evolved after they found out that animal is not attract to the powder forms of food supplements produced by industry (Belanger & Pelletier, 2006). The densification gives benefit as it has uniformity in shape and size, low humidity content, less dust and ashes, reduced the cost of transport and storage and greater energy density (Valdés et al., 2018). Pelletizing of fibrous by-products feed material will increase the particle density, allowing for a greater rate of ruminal

turnover, which directly increases the DM intake. Besides that, pelletizing provides a greater surface area for enzyme activity to increase the energy content of the feedstuff.

Terrill et al. (2007) reported that *sericea lespedeza* hay's pelleting reduced the gastrointestinal nematodes infection in goats. Dahlan et al. (2000) reported that the OPF mixed pellet increased the live weight gain of goats as the increased digestible nutrient intake. Pi et al. (2005) studied that the pre-treated rice straw pellet with chemical pre-treatment enhanced its *in vitro* organic matter digestibility and metabolizable energy content being more favourable by the Boar goats. The process of pelletizing involved five steps; drying, milling, conditioning, actual pelletizing and cooling of the biomass (Stelte et al., 2012). The process has problems if the pellet produced is not in a proper configuration. The types of fibrous by-products and the quantity of water added become the research challenges. The Van der Waals forces during contact between particles will increase depending on the by-products moisture content (Valdés et al., 2018).

However, researchers found several disadvantages in pelletizing. Some pellets were mixed with feed materials with binders which gave an additional cost of production. Another method to bind the feed materials together is with high temperatures, which, in result, destroy some of the nutrients needed by animals. Studies also tried to adhere the material in the pellet by mixing the nutrients with different additives. However, the pellet produced has low nutrient concentration with relatively high volume which is somewhat

cumbersome to handle (Belanger & Pelletier, 2006). Palletizing also reduced its adequate neutral detergent fiber (aNDF), which decreased saliva production and ruminal buffering for chewing stimulation, regurgitation and rumination of animals (Ware & Zinn, 2005). According to Wan et al. (2003), due to the smaller particle size of the pellet based on ground OPF, it is less suitable to be utilized as ruminant feeding.

2.8.3 Ensiling

Ensiling is a fermentation process of a crop, forage or agricultural by-product in a container called a silo**.** It has many advantages, such as being less weather dependent, easily mechanized, better large-scale livestock production and suitable for many different crops (Balehegn et al., 2022). Ensiling is considered the best preservation technique to deal with the rainy season crop harvest and storage problem, especially for protein-rich foliage (Sahoo, 2018). The preservation of silage pre-treatment method's main purpose has always been to magnify the conversion of water-soluble carbohydrate into lactate. The method evolved when some of the water-soluble carbohydrate was retained to increase the rumen's microbial efficiency (Merry et al. 2006).

The ensiling processes have to be considered on two main features; the maturity of the crops and the knowledge on the management of the silage by the maker. Ensiling can be divided into four phases; aerobic phase, followed by fermentation, stable and feed-out phase (Figure 2.3). The silage maker

needs to have a significant understanding of each stage to make accurate decisions to maintain the silage quality throughout the periods (Ferraretto et al., 2018). Lactic acid bacteria (LAB) play an important role in fighting for fermentable carbohydrates with other microorganisms such as clostridia spores, yeast and molds. The low-quality silage will produced if low fermentable carbohydrates ferment by LAB (Borreani et al., 2018). Permeability of the silo to air is also an essential factor affecting the silage quality. Several other issues that need to be considered in the preservation of silage, such as nutritional imbalance, palatability of the silage (for particular biomass), preservation time and moisture content (Xu et al., 2007). According to Ishida & Abu (1997), the nutritional value of OPF silage is as high as rice straw. Compared to pelleted OPF, the total digestible nutrient of OPF silage is 30 % higher on a dry matter basis. The availability of silage to be added with additives allowed the biological pre-treatment to be practiced by using silage. As the silage can process in large-scale production, this method is the most suitable and has the potential to be applied in animal feed commercialization.

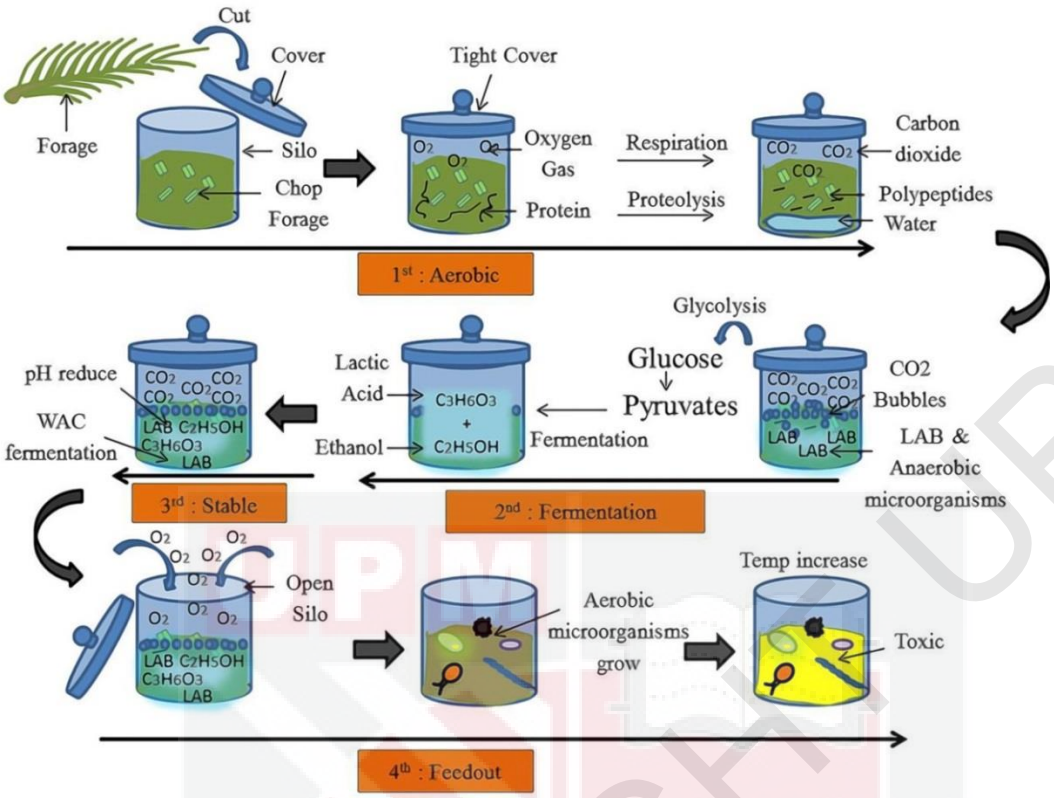


Figure 2.3: Ensiling phases and method flow (Borreani et al., 2018).

CHAPTER 3

ISOLATION AND SCREENING OF INDIGENOUS FILAMENTOUS FUNGI COLLECTED FROM OIL PALM FROND

3.1 Introduction

The abundant fibrous by-products from either agriculture or forestry triggered various applications to utilize and turn scraps into valuable products (Manavalan et al., 2015). In Malaysia, oil palm plantations represent 60% of total agricultural land, contributing to high fibrous by-products (Ghani et al., 2017). The planted area of palm oil tree in Malaysia for year 2022 is 5.67 million hectares which is 1.1 % lower compared to 5.74 million hectares in 2021 (Parveez et al., 2022). However, the reducing planted area is caused by the replanting of the trees and the numbers will be increased and directly increased the number of the oil palm frond (OPF) production in Malaysia. The valorization of fibrous by-product waste materials becomes the subject of intense study since they are potentially harmful to the environment.

The fibrous by-product like OPF is a carbohydrate-rich residue containing significant cellulose, hemicellulose and lignin (Sukri et al., 2014). OPF contains of 22 % soluble carbohydrate and 70 % fiber on a dry matter (DM) basis (Saminathan et al., 2012). According to Islam et al. (2000), the nutritional composition of OPF are 439 g/kg of dry matter (DM), 926 g/kg organic matter (OM), 698 g/kg neutral detergent fiber (NDF), 501 g/kg acid detergent fiber (ADF), 168 g/kg cellulose, 196 g/kg hemicellulose, 748 g/kg total carbohydrate (TC), and 52 g/kg non-fiber carbohydrate (NFC) for leaflet

parts of the OPF. While the petiole contain of lower (p<0.01) DM, CP and EE than leaflet (Islam et al., 2000).

As a substitute for grasses or roughages, OPF is commonly utilized as ruminant feed, especially during the short supply of feed (Rusli et al., 2019). The potential of OPF to be livestock feed is hindered by the high neutral detergent fiber (NDF) and lignin contents of the OPF itself (Rahman et al., 2011), making it difficult to be degraded by ruminant digestive systems. To overcome this problem, various pre-treatment methods such as enzymatic treatment have been used to improve the digestibility of fibrous by-products in ruminant feed.

The white rot fungi (WRF) which are the members of filamentous fungi are decaying wood fungus found in agricultural residues. It can leave a remarkable reaction by leaving a bleached on the attacked wood by lignin degradation (Asgher et al., 2008). The valuable enzyme system of WRF and its effectiveness in degrading fibrous by-products attracted considerable research interest in different fields of study (Manavalan et al., 2015). The lignin-degrading capabilities of WRF inspired studies to deepen the knowledge and explore more fungi strain and its effectiveness on lignin degrading ability. Many species have been studied including *Ceriporiopsis subvermispora* (Rahman et al., 2011), *Pleurotus ostreatus* (Tuyen et al., 2013), and *Lentinussajor-caju* (Chanjula et al., 2017). However, less than 20 species out of 1500 different species of WRF were applied in biological pre-treatment. There are possibilities that the potential WRF can be found in

nature (Tian et al., 2012). This study aims to identify and determine the species of filamentous fungi cultured from the collected OPF from oil palm trees plantation in Pusat Pertanian Putra, Universiti Putra Malaysia (PPP, UPM).

3.2 Materials and Methods

Since there are less than 20 species out of 1500 species of WRF were applied in biological pre-treatment and high possibilities for the new WRF can be found and use (Tian et al., 2012), the isolation of the filamentous fungi was done by direct culture from the OPF on the potato dextrose agar (PDA) media. The microscopic observation after the molecular identification in this study was done to validate the fungi species identified.

3.2.1 Filamentous fungi culture and isolation

The fungi were collected at the oil palm tree plantations in Pusat Pertanian Putra, Universiti Putra Malaysia (PPP, UPM). The fungi were collected from rotten oil palm fronds, placed in paper bag, and taken to FAMTEC laboratory in the Faculty of Biotechnology UPM (Azmi et al., 2019). The collected samples were cultured on potato dextrose agar (PDA) (Bacton, Dickinson and Company, USA) containing streptomycin and penicillin antibiotics. The sub-culturing process was repeated until the pure culture was obtained. Stocks of isolated fungi were prepared using the filter paper method and agar slant.

3.2.2 Morphological identification of filamentous fungi

The isolated fungi were cultured on PDA agar for seven days before being used for microscopic observation and identification. The morphology of the fungi on the agar plate was first observed. The texture, the structure, the edge of the culture, and the elevation of the culture were observed (Galal et al., 2017).

3.2.3 Molecular identification of filamentous fungi

The isolated fungi were cultured on agar plates layered with cellophane before undergoing DNA extraction (Azmi et al., 2019). The DNA extracted from the isolated fungi underwent a polymerase chain reaction (PCR) using Bio-Rad T100 PCR Thermal Cycler with primer internal transcribed spacer, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). ITS1 and ITS4 acted as forward and reversed primers, respectively. The amplified DNA was run on gel electrophoresis and viewed under UV light with the range size of 600 and 700 base pairs (bp). The sequencing was done, and the Apical Scientific (1st Base) result was processed with Chromas and MEGA7 software. The aligned sequence was compared with the sequence in GenBank using Basic Local Alignment Search Tool (BLAST), NCBI, and the fungal species was determined. The phylogenetic tree was constructed using NCBI taxonomy and MEGA7 software.

3.2.4 Microscopic observation of filamentous fungi

The fungi were placed onto a glass slide and covered with a cover slip, and pressed to get a good separation of the hyphae and methylene blue solution was used for staining (Azmi et al., 2019). The staining was done right before the observation to avoid delayed and caused any possible changes in fungi structure as well as the colour intensity. The fungi characteristics were then observed using a compound microscope (Olympus BX53M, Japan) with a digital camera. The morphological structure of the fungi was compared with the previous studies and determined based on fungi characteristics stated by Galal et al. (2017). The characteristics involve branching, hyphae, conidiospores and spores (Galal et al., 2017).

3.3 Results

3.3.1 Morphological identification and selection of filamentous fungi

In this experiment, 79 fungi isolates were identified and labelled as F01 until F79. The isolates were classified into eight groups based on their morphology on the PDA agar. One isolate was selected from each group, which is F14, F03, F32, F39, F49, F65, F77, and F02 (Table 3.1). All fungi were selected from the filamentous structures on the PDA agar. The fungi colony showed in white concentric rings for F14, F39, F49, and F02. The colony for F03 and F32 were green in color. The F65 was brownish peach cottony, and F77 showed multiple scattered colonies with grey-green color

rings. The fungi were also are in augmented raised cottony structure with abundant aerial mycelium grown on PDA agar (Galal et al., 2017).

Table 3.1: Selected filamentous fungi culture from eight different groups (1) F49, (2) F02, (3) F77, (4) F32, (5) F14, (6) F39, (7) F03, and (8) F65

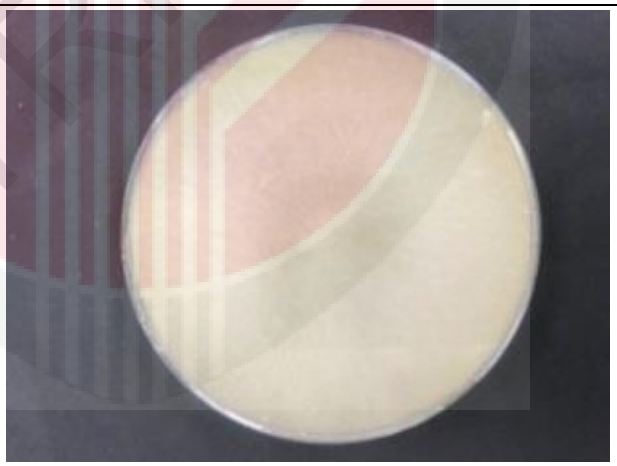
No	Fungi ID	Morphology characteristics	Image
1	F49	Colour: White Structure: Cottony Formation: Concentric rings	
2	F02	Colour: White Structure:Hair-like Formation: Concentric rings	

Table 3.1: Continued

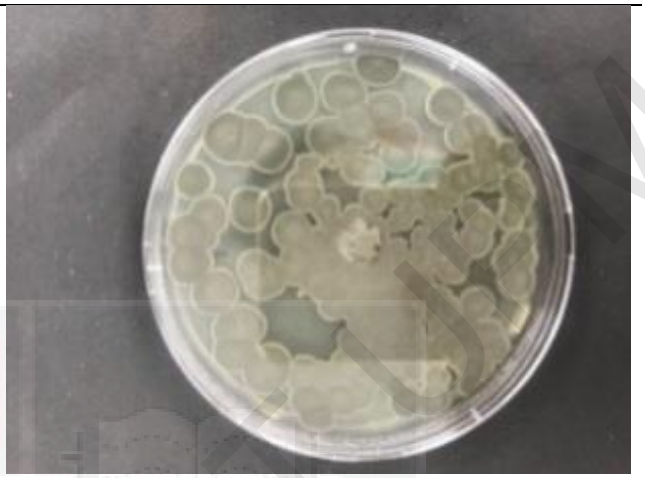
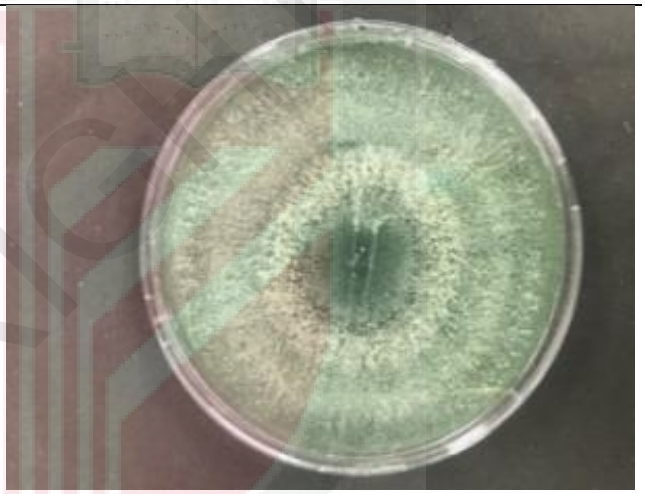
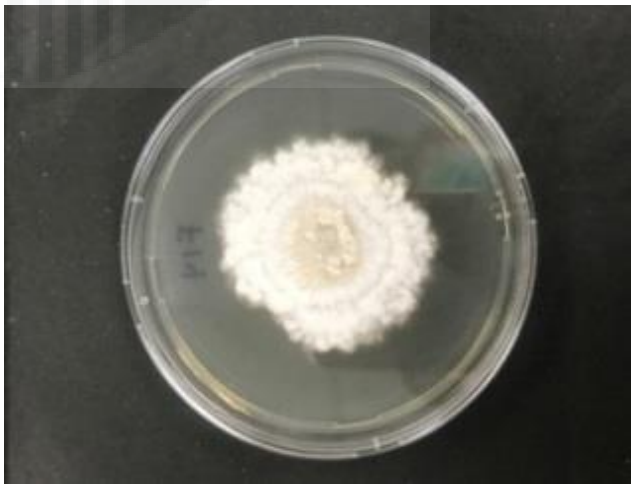
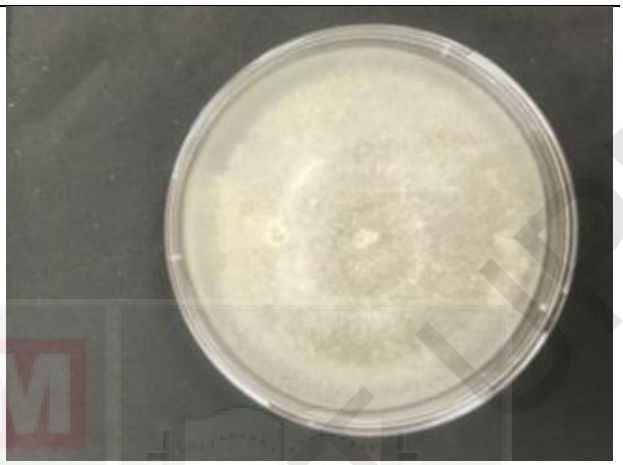
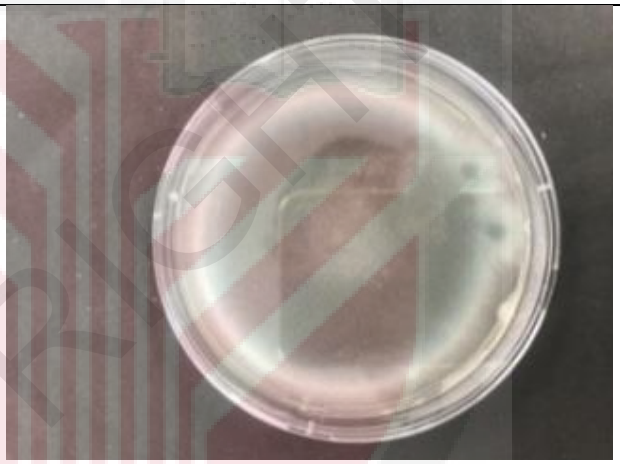
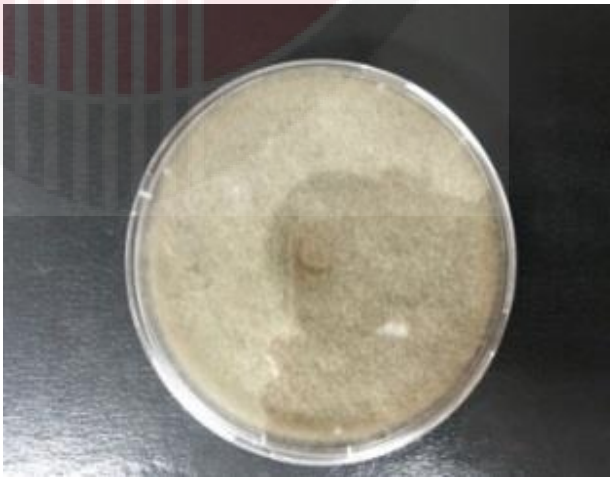
No	Fungi ID	Morphology characteristics	Image
3	F77	Colour: Gray-green Structure: Cottony Formation: Multiple scattered	
4	F32	Colour: Whitish-green Structure: Cottony Formation: Concentric rings	
5	F14	Colour: White Structure: Cloud-cottony Formation: Concentric rings	

Table 3.1: Continued

No	Fungi ID	Morphology characteristics	Image
6	F39	Colour: White Structure: Cottony Formation: Concentric rings	
7	F03	Colour: Whitish-green Structure: Cottony Formation: Concentric rings	
8	F65	Colour: Brownish-peach Structure: Cottony Formation: Flat ground	

3.3.2 Molecular observation of filamentous fungi

After series of DNA extraction, amplification, and sequencing, the phylogenetic tree showed that the fungi were identified as F49 (*Marasmius palmivorus* MN871732), F02 (*Mucor fusiformis* KY560315), F77 (*Penicillium citrinum* MK312421), F32 (*Trichoderma asperellum* MK928414), F14 (*Schizophyllum commune* MN856258), F39 (*Phanerina mellea* or Ceriporia mellea MK432982), F03 (*Aspergillus neoellipticus* MG596660) and F65 (*Syncephalastrum racemosum* MH857909) (Appendix A1).

3.3.3 Microscopic observation of filamentous fungi

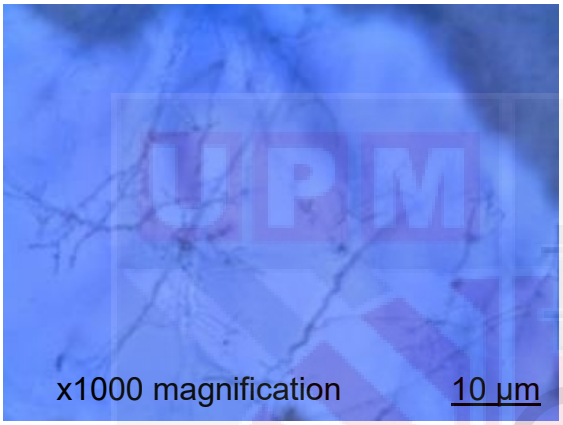
Figure 3.1 shows the morphology of each selected fungus under the microscopic view. The microscopic observation was based on their branching, hyphae, conidiospores and spores followed Galal et al. (2017) and the structures were compared with the previous studies as shown in Appendix A2-10. The *Schizophyllum commune* showed the appearance of hyphae, and these fungi formed a clamp connections at the septa (Sigler et al., 1999). The hyphae also formed a thread-like branching and septate. However, the conidiophores and spores could not be seen (Figure 3.1e). For *Aspergillus neoellipticus*, it was confusing to differentiate to other *Aspergillus* sp. as the shapes were almost the same. However, *Aspergillus neoellipticus* conidia were elliptical in shape (Figuure 3.1g) (Zafar et al., 2017). *Aspergillus neoellipticus* cannot be seen clearly under the compound microscope, but the multiple conidia was showing that the fungi were *Aspergillus* sp.

The *Trichoderma asperellum* showed in branch structure with septate hypha. The conidia could be seen near the conidiophores at the tip of the hyphae (Figure 3.1d). The structure was similar to *Trichoderma asperellum* observed by Poddee & Ghosh. (2019). *Phanerina mellea* hyphae structure was monomitic, brunching with sharp angle and simple septa (Figure 3.1f) (Miettinen et al., 2016). The conidiophores could not be seen, and no clamp formed by the fungi.

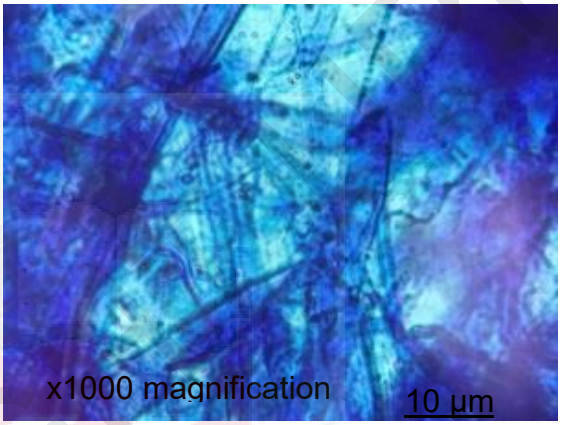
The *Marasmius palmivorus* showed hypha with a thread-like structure under x1000 magnification of a compound microscope (Figure 3.1a). The image by Tamur et al. (2019) showed the septal wall of fungal hyphae with the clamp connection between hyphae which was also shown by the microscopic observation of *Marasmius palmivorus.* The branched rough hypha structure was absent of conidiophores. The *Syncephalastrum racemosum* could be seen, and the structure was very similar to the image shown by Raju et al. (2020). The conidiophores were shown in sporangiophores, and the hyphae were shown in broad aseptate (Figure 3.1h).

The *Penicillium citrinum* showed a microscopic structure similar to the image shown by Saif et al. (2020). Like other *Penicillium sp*., *Penicillium citrinum* contained highly branched septate hyphae (Figure 3.1c). At the tip of the hyphae, there were branches conidiophores, the main dispersal route for the fungi. The sporangiospores structure of *Mucor fusiformis* could be seen under the compound microscope (Figure 3.1b). However, the conidiophores

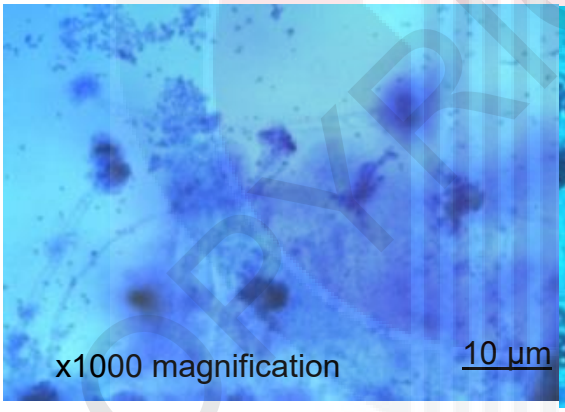
could not be seen clearly as the image shown by Walther et al. (2013), which was viewed using a Scanning electron microscope (SEM). The *Mucor fusiformis* also known as *Zygorhynchus psychrophilus* is in a *Mucor* group with sporangiopores structure that is poorly sympodially branched tall with small sporangia (Walther et al., 2013).



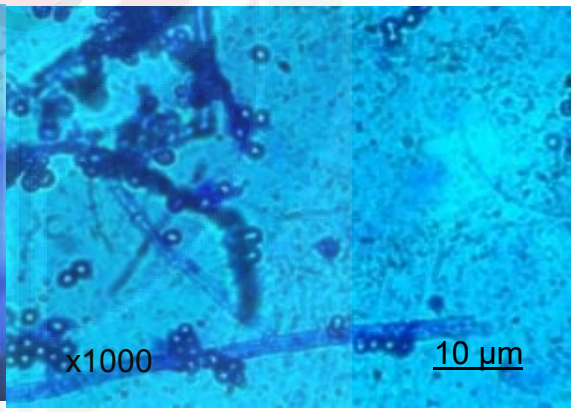
(a) MP



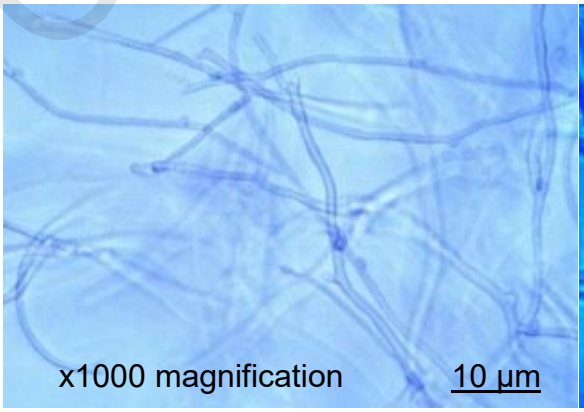
(b) MF

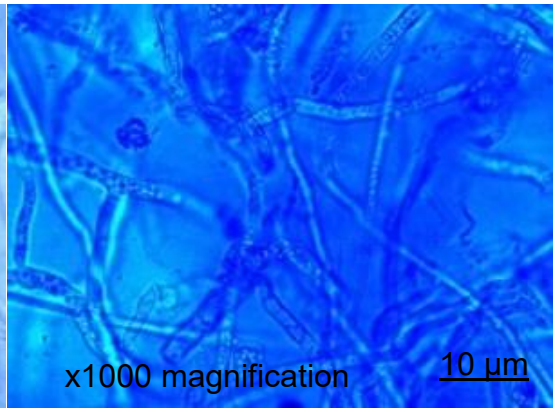


(c) PC

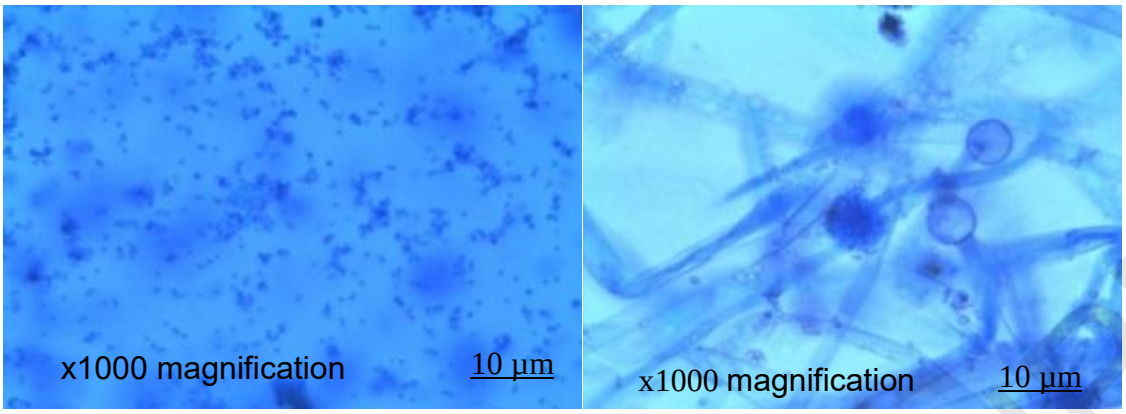


(d) TA





(e) SC (f) PM



(g) AN (h) SR

Figure 3.1: Microscopic morphology of eight different fungi identified: (a) *Marasmius palmivorus* (MP), (b) *Mucor fusiformis* (MF), (c) *Penicillium citrinum* (PC), (d) *Trichoderma asperellum* (TA), (e) *Schizophyllum commune* (SC), (f) *Phanerina mellea* (PM), (g) *Aspergillus neoellipticus* (AN), and (h) *Syncephalastrum racemosum* (SR).

3.4 Discussion

The different coloration and colony structure indicated the differences between each fungus. Some fungi can easily be noticed based on their morphological structure in nature. For example, the fungi filamentous structure showed in the cottony fibrous structure of hyphae with cellulose-enriched materials (Mäkelä et al., 2020). The white color concentric rings of F14, F39, and F49, are very synonymous with white rot fungi (Abdel et al., 2013). While fungi F03, F32, F77, F65, and F02 are brown or soft rot fungi.

Each species identified in this study has its own special characteristics.

Some of the fungi species used in commercial industries, and some are still under investigation. For example, *Marasmius palmivorus* pre-treatment on empty fruit bunches (EFB) and palm kernel meal (PKM) did not improve the growth of larvae of the Black soldier fly (BSF) when need as feedstock (Colombatto e al., 2003). The *Mucor fusiformis* or *Zygorhynchus psychrophilus* is a fungus usually used in food manufacturing, such as cheese and tofu. However, it is infectious and dangerous to human health (Walther et al., 2013). The Penicillium citrinum used in β-mannanase enzyme production, applied in industrial applications including animal feed production (Lima et al., 2021) whereas Schizophyllum commune is used in rice straw fermentation for the production of high level of xylanase enzyme under solid-state fermentation method for the pre-bleaching of ethanol-soda pulp (Gautam et al., 2018).

The *Phanerina mellea* is also a WRF commonly called *Ceriporia mellea* (Miettinen et al., 2016). The information on this fungus is limited and the enzyme activity production and application have never been investigated. *Aspergillus neoellipticus* is a well-known wood-decaying fungus as various studies used this fungus in cellulose production study. This fungus is usually used in pharmaceuticals, food ingredients and enzyme productions (Abdel-Azeem et al., 2020). The fungi identified species with the characteristics are tabulated in Table 3.2.

Table 3.2: Filamentous fungi identified species with the characteristics

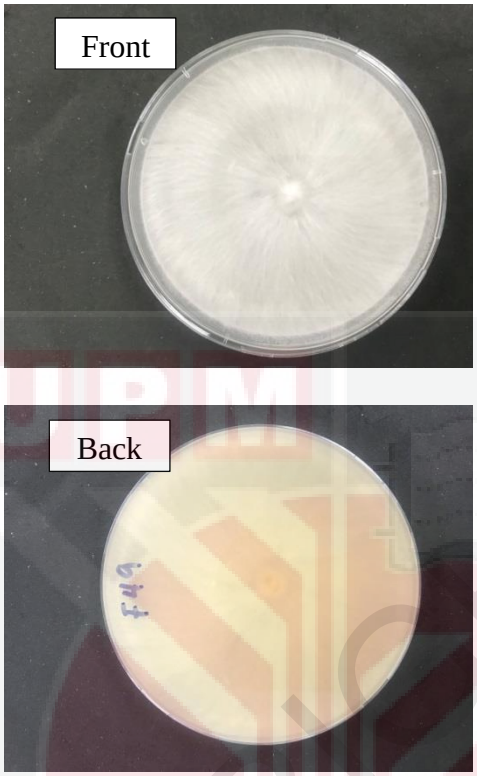
No	Sample ID	Scientific Name	Characteristics
1	F49	*Marasmius palmivorus* 	• Mushroom-forming fungi • Division: Basidiomycota • Causing white rot • Mainly in monocots and particularly oil and coconut palms • Produced disease symptoms and death of non-wounded Formosa palm tree. (Pham et al., 2019)
2	F02	*Mucor fusiformis*	• Epithet: Fusiformis • Other name: Zygorhynchus psychrophilus. • Division: Zygomycota. • Filamentous fungi.

Table 3.2: Continued

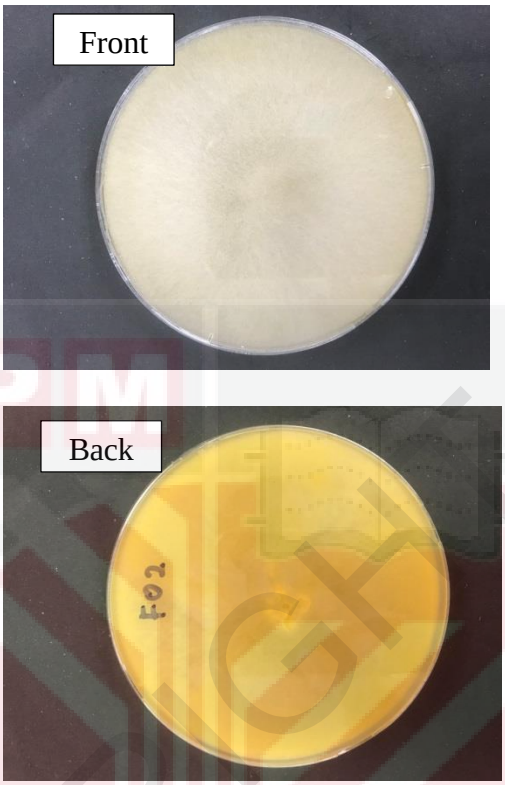
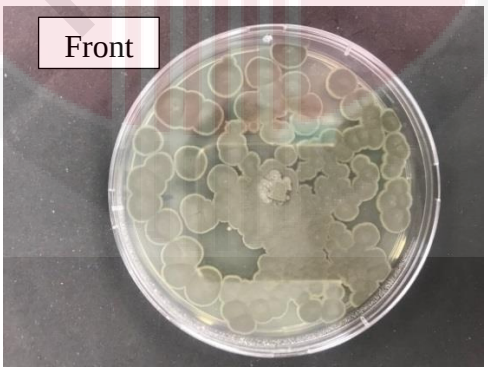
No	Sample ID	Scientific Name	Characteristics
			• Saprotroph organisms inhabiting dead plant material, soil and dung. • Used in the • Fermentation of tempeh or tofu. • Used in the production of several cheese. • Responsible for the spoilage of fresh and manufactured food. • Involved in human infection. • (Walther et al., 2013)
3	F77	*Penicillium citrinum* 	• Division: Ascomycota • Common Endophytic fungus of cereal plants. • Gives benefit to the host plant such as increase tolerance to herbivory, disease, heat, salt, and drought and increase below and above ground biomass. • (Khan et al., 2008).

Table 3.2: Continued

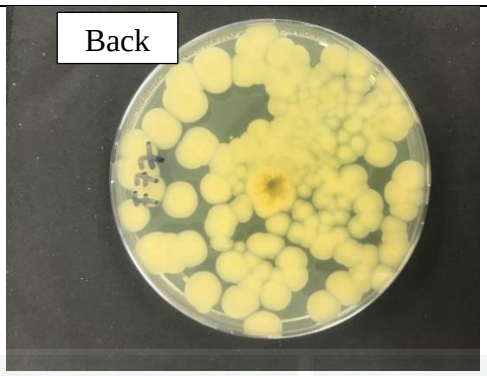
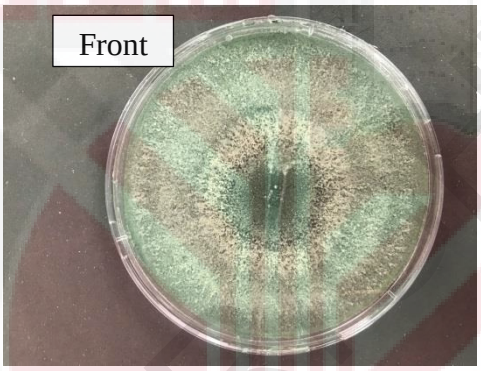
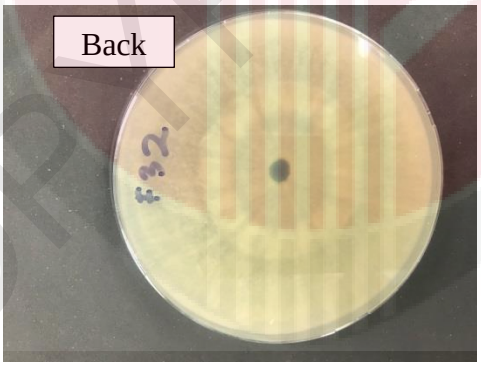
No	Sample ID	Scientific Name	Characteristics
			
4	F32	*Trichoderma asperellum*  	• Division: Ascomycota. • Causing soft-rot. • Used commercially and experimentally as a biopesticide and biocontrol agent. • Well known as rivalry for several soil phytopathogens including other fungi, bacteria and invertebrates. • Help on other plant growth (domestic and green house plants and agricultural crops) (Verma et al. 2007).
5	F14	*Schizophyllum commune*	• Other Name: Split gill Fungus • Division: Basidiomycota • Causing white rot. • Found on every continent except Antarctica.

Table 3.2: Continued

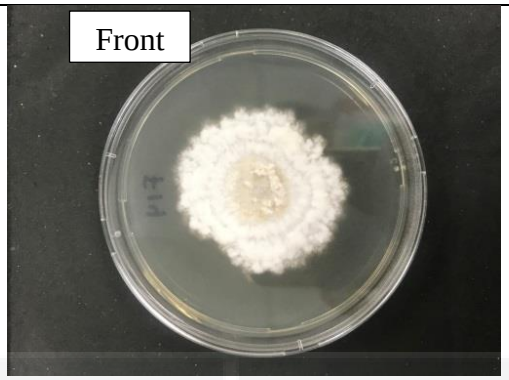
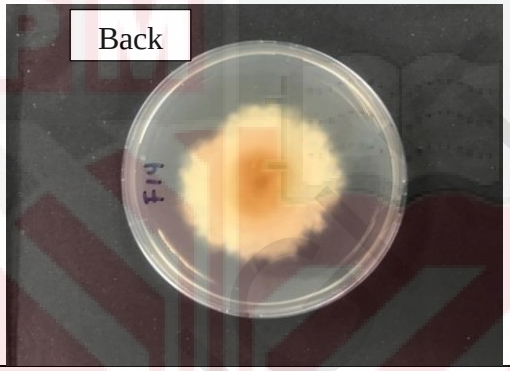
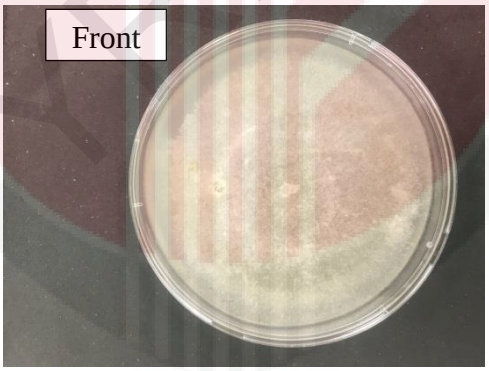
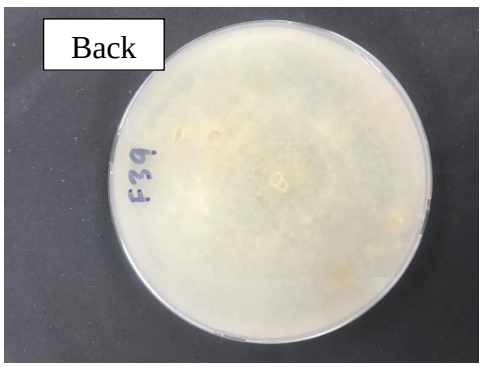
No	Sample ID	Scientific Name	Characteristics
		 	• The gills function to produce basidiospores. • Can be found on fallen branches and timber of deciduous trees. • (Ohm et al. 2010)
6	F39	*Phanerina mellea*  	• Other Name: *Polyporus melleus*, *Poria mellea*, *Ceriporia mellea*, *Gloeoporus melleus*. • Division: Basidiomycota • Causing white rot • Genus: *Phanerochaete* (Miettinen et al., 2016)

Table 3.2: Continued

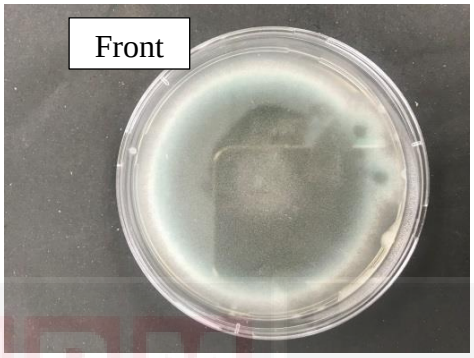
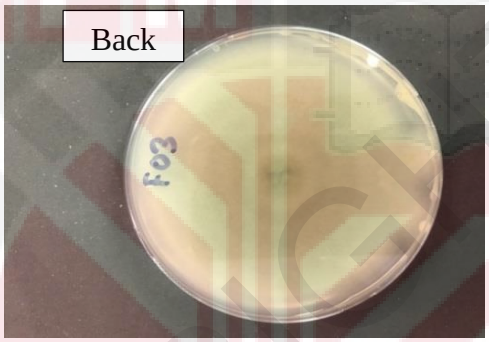
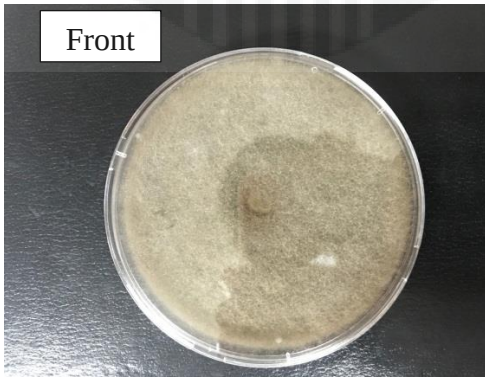
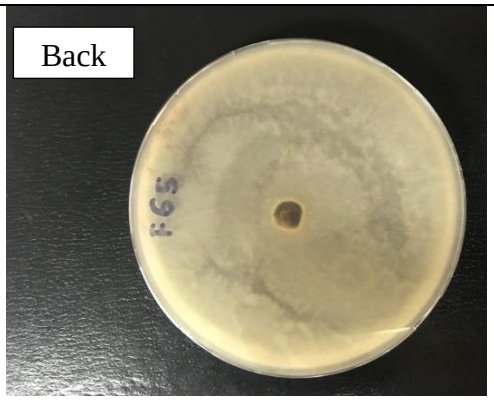
No	Sample ID	Scientific Name	Characteristics
7	F03	*Aspergillus neoellipticus*  	• Divisions: Ascomycota • Other name: Aspergillus helicothrix. • Used for pharmaceuticals, food ingredients and enzymes (Hu et al. 2011). • Show high degree of β-glycosidase activity. • Wood decaying fungi • Synergism between *Aspergillus neoellipticus* and *Aspergillus fumigatus* improves hydrolytic and β-glycosidase activity. • (Abdel-Azeem et al., 2020)
8	F65	*Syncephalastrum racemosum* 	• Other name: Syncephalastraceae • Filamentous fungi • Can cause nail disease • Isolated fungal species of soil in tropical and subtropical region. • Produce the highest DNase activity.

Table 3.2: Continued

		• Used in microbial transformation for hydroxylation of steroids, olivetol, milbemycins, and cinobufagin. (Buayairaksa et al., 2011)

3.5 Conclusion

This study managed to identify eight fungi isolated from OPF at PPP, UPM. After gone through series of fungi culture and morphological, molecular, and microscopic observation, the fungi were identified and characterized as *Marasmius palmivorus, Mucor fusiformis*, *Penicillium citrinum*, *Trichoderma asperellum*, *Schizophyllum commune*, *Phanerina mellea*, *Aspergillus neoellipticus*, and *Syncephalastrum racemosum*. Only three fungi were classified as WRF which are *Marasmius palmivorus, Schizophyllum commune*, and *Phanerina mellea* which potentially used in the pre-treatment of OPF.

CHAPTER 4

DETERMINATION OF ENZYME ACTIVITY FROM IDENTIFIED WHITE ROT FUNGI AND THEIR CO-CULTURE INTERACTION

4.1 Introduction

The pre-treatment of oil palm frond (OPF) by white rot fungi (WRF) is promising to improve the low nutritional value of OPF. Three major enzymes which are working in the degradation of lignin produced by WRF are laccase, lignin peroxidase (LiP), and manganese peroxidase (MnP) (Robinson et al., 2001). These ligninolytic enzymes are responsible to mineralize the lignin into carbon dioxide (CO_2) and water. These enzymes were used in various applications for example, bleaching and wastewater treatment for laccase enzyme (Madhavi & Lele, 2009), coal depolymerization and skin lightening by melanin oxidation for LiP enzyme (Falade et al., 2017), and juice extract clarification and biofuel production for MnP enzyme (Kumar & Arora, 2022). The delignification of the fibrous by-products will allow more cellulose and hemicellulose release for rumen microbial activity. The access of rumen microbes to cellulose and hemicellulose are the basic key to improve the rumen degradability (Rusli et al., 2021).

A study reported by Azmi et al. (2022) showed that the nutritional value of OPF can be improved by pre-treating it with ligninolytic enzyme extract of WRF. Another study reported by Hamchara et al. (2018) showed that the feeding of OPF pre-treated with *L. sajor*-ceju to 16 month-old crossbred male goats improved the estimated energy intakes (ME Mcal/DM/d) up to 53 %. The co-culture or mixed fungal cultures have been the nature of the fungi

that typically live and grow close to each other. Therefore, the fungi co-culture may result in an antagonistic interaction as one fungus cannot cooperate with the other fungi forming either parasitism (competition on nutrient exploitation) or deadlock (the other species cannot enter the territory owned by the other) interactions. The co-culture may also form a synergistic interaction if there is a mutual interaction between fungi to degrade the same substrate (Wang et al., 2014).

In the pre-treatment of OPF as the animal feed, the ligninolytic enzyme produced by some WRF degrade the lignin content of OPF that restrict the degradation of cellulose and hemicellulose by rumen microbes. Indeed WRF are the most efficient lignin degrader in nature. However, the pre-treatment by mono-culture fungus resulted in low efficiency. Researchers came out with fungal co-culture, the interactions were said will increase the efficiency of enzyme production (Ma et al., 2011). The competition of space and nutrients increased the ligninolytic enzyme activity and enhanced the lignin degradation of fibrous by-products (Qi-he et al., 2011).

According to studied by Lakovelev & Stenlid. (2000), the interactions between two WRF increase laccase production. The co-culture of *Pleurotus ostreatus* and *Ceriporiopsis subvermispora* effectively stimulates wood decay (Chi et al., 2007). Whereas another study stated the pre-treatment of coffee pulp waste by co-culture fungi showed enhancement in reducing sugar content and accelerating the cellulose and hemicellulose degradation (Parani & Eyini, 2010).

In the current study, the compatibility of identified WRF (*Phanerina mellea*, *Schizophyllum commune,* and *Marasmius palmivorus*) co-culture was investigated based on the decolorization of Remazol brilliant blue R (RBBR) dye, media and their enzyme activity were also used in the assessment by comparing mono-and co-culture fungi. After that, one selected co-culture of WRF was used in the pre-treatment of OPF (silage and overnight pre-treatment) and further used in *in vitro* rumen fermentation analysis. The main objective of this study is to assess the enzyme activity of the identified WRF and their synergistic effect.

4.2 Materials and Methods

4.2.1 Solid-state fermentation for extraction of enzyme from identified filamentous fungi

The OPF were collected from Pusat Pertanian Putra, Universiti Putra Malaysia (PPP, UPM) (2.983592895423529, 101.71000476197045). Next, the OPF were chopped into a smaller size (Azmi et al., 2019). In the Erlenmeyer flask, 15 g of chopped OPF were added. Then, 0.05% of glucose and 2.4 mM of nitrogen source, ammonium sulfate ($(NH_4)_2SO_4$) were added to 45 mL of distilled water. The flasks were then covered with cotton plugs and aluminium foil. Each flask was autoclaved to remove any existing microorganisms before was added with three 3-10 mm agar plugs from each fungi culture. The cotton plugs was removed and the agar plugs were inserted immediately to avoid contamination. The flasks were incubated at

28°C for 10, 20, and 30 days (Azmi et al., 2019).

4.2.2 Enzyme extraction of identified filamentous fungi

Enzyme extraction begins once the fermentation period (10, 20, and 30 days) ends. Each flask received 150 mL of distilled water, and the flasks were shaken for 3 hours. The mixture was then filtered using six layers of gauze. The filtrate was mix with polyvinylpolypyramidone (PVPP) to remove tennins (Dagadkhair et al., 2018). The amount of PVPP added is 0.5% of the total filtrate. The mix of the filtrate and PVPP was centrifuged (Avanti J-26S XP1, High-Performance Centrifuge, Beckman Coulter, USA) with the temperature of 4°C at 12,000 g for 10 minutes. The supernatant was then collected and stored for enzyme activity determination (Azmi et al., 2019).

4.2.3 Ligninolytic enzyme activity determination of enzyme extract from identified filamentous fungi

4.2.3.1 Laccase

Laccase enzyme activity determination used citrate-phosphate buffer and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) substrate in the experiment (Azmi et al., 2019). Each experimental setup was replicated three times to ensure statistical reliability. To prepare citrate-phosphate buffer, 1.921 g of citric acid, $C_6H_8O_7$, was added into 100 mL distilled water, and the pH was calibrated to pH 5.0, and labelled as solution A. Then, 3.581 g of sodium phosphate dibasic, Na_2HPO_4, was added into 100 mL distilled

water and labelled solution B. The substrate was prepared with 0.165 g of ABTS added into 10 mL of distilled water. The substrate was then transferred to an Eppendorf tube. The blank solution was prepared before reading at 420 nm light absorbance of spectrophotometer nano drop (Tecan Infinite M200 Pro^{TM}, Switzerland) against blank. All enzyme activity values were represented in unit U/mL (unit/milliliter) with three replicates. The calculation for laccase enzyme activity determination was done as below.

$$\text{Concentration (U/ml)} = \frac{A}{\Sigma\ mM^{-1}\ cm^{-1}} \times \frac{1500\ ul}{100\ ul}$$

A = Absorbance
Total volume in cuvette = 1500 ul
Total volume of enzyme in cuvette = 100 ul
Light absorbance at 420 nm = $\Sigma\ mM^{-1}\ cm^{-1}$

4.2.3.2 Lignin peroxidase

Lignin peroxidase enzyme activity was determined with acid tartrate buffer, substrate, and hydrogen peroxide, H_2O_2 used in the experiment (Azmi et al., 2019). Each experimental setup was replicated three times to ensure statistical reliability. The buffer was prepared with 3.752 g of acid tartrate, $C_4H_6O_6$ was added to 250 mL of distilled water, and the pH was calibrated to pH 3.0 with HCL before being kept at 4^{o}C. Substrate preparation was done with 0.105 mL of veratryl alcohol (3, 4-Dimethoxybenzyl alcohol) added to 25 mL of distilled water and stored at 4^{o}C in an Eppendorf tube. Then, the H_2O_2, was prepared according to the manufacturer specifications. After that, the mixture tube was prepared by adding 2570 µL of acid tartrate buffer, 200 µL

veratryl alcohols, 30 µL hydrogen peroxide, and 200 µL of enzyme extract. The blank prepared with 2570 µL of acid tartrate buffer was added to 200 µL veratryl alcohol and 30 µL hydrogen peroxide. Finally, the mixture tubes were read at 310 nm light absorbance of spectrophotometer nano drop (Tecan Infinite M200 Pro^{TM}) against blank. The calculation for LiP enzyme activity determination was done as below.

$$\text{Concentration (U/ml)} = \frac{A}{\Sigma\ mM^{-1}\ cm^{-1}} \times \frac{3000\ uL}{200\ uL}$$

A = Absorbance
Total volume in cuvette = 3000 uL
Total volume of enzyme in cuvette = 200 uL
Light absorbance at 310 nm = $\Sigma\ mM^{-1}\ cm^{-1}$

4.2.3.3 Manganese peroxidase

Manganese peroxidase was determined by preparing sodium tartrate buffer with 5.752 g of sodium tartrate added to 250 mL distilled water (Azmi et al., 2019). Each experimental setup was replicated three times to ensure statistical reliability. The solution was adjusted to pH 5.0 using HCL or NaOH and kept at 4^{o}C. The substrate was prepared by adding 0.254 g of manganese sulphate to 50 mL of distilled water and stored at 4^{o}C. The H_2O_2 was prepared according to the manufacturer's specifications. After that, the mixture tube was prepared by adding 2570 µL of sodium tartrate buffer mixed with 200 µL manganese sulfate, 30 µL H_2O_2, and 200 µL of enzyme extract. The blank was prepared by 2570 µL of sodium tartrate buffer added to 200 µL manganese sulfates and 30 µL H_2O_2. The mixture tubes were read

at 238 nm light absorbance of spectrophotometer nano drop (Tecan Infinite M200 Pro^{TM}) against blank. The calculation for MnP enzyme activity determination was done as below.

$$\text{Concentration (U/ml)} = \frac{A}{\Sigma\ mM^{-1}\ cm^{-1}} \times \frac{3000\ uL}{200\ uL}$$

A = Absorbance
Total volume in cuvette = 3000 uL
Total volume of enzyme in cuvette = 200 uL
Light absorbance at 238 nm = Σ mM^{-1} cm^{-1}

4.2.4 Cellulolytic enzyme activity determination of enzyme extracted from identified filamentous fungi

4.2.4.1 Carboxymethyl cellulase

Substrate and citrate buffers were prepared to screen carboxymethyl cellulase (CMCase) enzyme activity (Azmi et al., 2019). Each experimental setup was replicated three times to ensure statistical reliability. The substrate was prepared with 1 g of carboxymethylcellulose and added to 100 mL of distilled water in a 250 mL Erlenmeyer flask. The solution was then stirred until homogeneous and stored at 4^{o}C. For the citrate buffer, 0.1 M citrate buffer was prepared by adding 0.1 mol of citric acid to 0.1 mol of sodium citrate adjusted to pH 4.8 using HCL or NaOH. After that, the mixture tube was prepared by adding 0.5 mL substrate to 0.5 mL enzyme. Three control tubes were prepared: buffer control tube, enzyme control tube, and substrate control tube. For the buffer control tube, 1 mL citrate buffer in a tube was prepared. For the enzyme control tube, 0.5 mL of enzyme was added to 0.5

mL citrate buffer and 0.5 mL of substrate was added to 0.5 mL citrate buffer to prepare substrate control tube. The tubes were then incubated in a water bath at 50^{o}C for 30 min (Dinis et al. 2009). The reaction was stopped by adding 3 mL of dinitrosalicylic (DNS) acid to each tube. The mixture, buffer control, enzyme control and substrate control tubes were then placed in boiling water for 10 min. The tubes were then read at 575 nm light absorbance of spectrophotometer nano drop (Tecan Infinite M200 Pro^{TM}) against blank. The glucose solution was prepared in 6 different concentrations (100 mg, 300 mg, 600 mg, 900 mg, 1200 mg, and 1500 mg) with distilled water dilution as the standard. The enzyme activity was eventually measured in unit U/mL (unit/milliliter).

4.2.4.2 Avicelase

Avicelase enzyme activity determination was done with avicel microcrystalline substrate and citrate buffer (Azmi et al., 2019). Each experimental setup was replicated three times to ensure statistical reliability. Substrate of 1 g of avicel microcrystalline was used and added to 100 mL distilled water in a 250 mL erlenmeyer flask. The solution was then stirred until homogeneous. The citrate buffer was prepared by adding 0.1 mol of citric acid with 0.1 mol of sodium citrate to get 0.1 M of citrate buffer at pH adjusted to pH 4.8 either using HCL or NaOH. The mixture tube was prepared by adding 0.5 mL enzyme to 1.0 mL substrate. For the buffer control tube, only 1.5 mL of citrate buffer was prepared in the tube. For the enzyme control tube, 0.5 mL of the enzyme was added to 1.0 mL citrate

buffer in a tube, and for the substrate control tube, 1.0 mL substrate was added to 0.5 mL citrate buffer. The tubes were then incubated in a water bath at 50^{o}C for 30 min (Dinis et al. 2009). The reaction was stopped by adding 3 mL of dinitrosalicylic (DNS) acid to each tube. All tubes were then placed in boiling water for 10 min. The tubes were then read at 575 nm light absorbance of spectrophotometer nano drop (Tecan Infinite M200 Pro^{TM}) against blank. The glucose solution was prepared in six different concentrations (100 mg, 300 mg, 600 mg, 900 mg, 1200 mg, and 1500 mg) with distilled water dilution as the standard. The enzyme activities were eventually measured in unit U/mL (unit/milliliter).

4.2.5 Hemicellulolytic enzymes activity determination of enzyme extracted from identified filamentous fungi

4.2.5.1 Xylanase

In this experiment, 0.25 g xylan from beechwood was added to 10 mL distilled water as the substrate (Azmi et al., 2019). Each experimental setup was replicated three times to ensure statistical reliability. The solution was then heated at 70^{o}C with continuous shaking. The mixture tube was prepared by adding 0.5 mL enzyme to 0.5 mL substrate. The buffer control tube was prepared with only 1.0 mL citrate buffer. For the enzyme control tube, 0.5 mL enzyme was added to 0.5 mL citrate buffer and the substrate control tube was prepared by adding 0.5 mL substrate to 0.5 mL citrate buffer. The tubes were then incubated in a water bath at 50^{o}C for 30 min (Dinis et al. 2009). The reaction was stopped by adding 3 mL of

dinitrosalicylic (DNS) acid to each tube. All tubes were then placed in boiling water for 10 min. The tubes were then read at 575 nm light absorbance of spectrophotometer nano drop (Tecan Infinite M200 Pro^{TM}) against blank. The xylose solution was prepared in six different concentrations (100 mg, 300 mg, 600 mg, 900 mg, 1200 mg and 1500 mg) with citrate buffer dilution as the standard.

4.2.6 Optimization of ligninolytic enzyme production time and fungi selection

The optimum pre-treatment time of OPF was determined based on the average enzyme activity result on day 10, 20, and 30 of solid-state fermentation (SSF). The pre-treatment time (day 10, 20, or 30) with high average ligninolytic (laccase, LiP, and MnP) enzyme activity production and minimum cellulolytic (CMCase and avicelase) and hemicellulolytic (xylanase) enzyme activity production was selected and used in fungi selection and OPF pre-treatment. For fungi selection, the findings from enzyme activity analysis were used to select fungi with the most optimum ligninolytic enzyme activity at the optimum pre-treatment time. The method of fungi selection followed Azmi et al. (2022), which a scatterplot the average of ligninolytic enzyme activity against the average of cellulolytic + hemicellulolytic enzyme activity was constructed. Fungi that produced higher ratio of average ligninolytic enzyme activity against the average cellulolytic + hemicellulolytic enzyme activity were selected (Azmi et al., 2022). The fungi that produced the high ligninolytic enzyme activity with low cellulolytic and hemicellulolytic enzyme activity were selected.

4.2.7 Assessment on co-culture of white rot fungi

In this experiment, a polymeric dye RBBR was used for screening the lignin-degrading peroxidases. Inoculum plugs 3 to 10 mm in diameter was placed 30 mm apart on petri dishes containing 20 mL potato dextrose agar (PDA) media with 50 ppm RBBR (Sumandono et al., 2015). Three WRF (*Phanerina mellea* (PM), *Schizophyllum commune* (SC), and *Marasmius palmivorus* (MP)) were selected due to its potential as resulted in highest enzyme activity determination from 4.2.6. The *Marasmius palmivorus* was selected due to its profile as WRF. Three possible co-culture combinations of WRF, i: (PM + SC), ii: (SC + MP), and iii: (PM + MP) were assessed. The co-culture was done in 3 replicates with mono-culture as the control. The interspecies interactions between three WRF were investigated visually on the agar plates. Based on the dye decolorization rate on mono-and co-culture of the fungi, the comparison was done by measuring the diameter (cm) of the decolorization area and all possible combinations were observed and analysed.

4.2.8 Solid-state fermentation for the extraction of enzyme from co-culture of white rot fungi

The SSF was done to prepare the enzyme extract for the enzyme activity determination on co-culture of WRF (Azmi et al., 2019). The Erlenmeyer flask, 15 g of chopped OPF was added. Then, glucose and nitrogen source was added before being covered with cotton plugs and aluminum foil. The flask was then autoclaved at 121^{o}C for 15 minutes. After that, two inoculum

plugs 3 - 10 mm in diameter were added for the mono-culture experiment. For co-culture fungi, two inoculum plugs each from different selected WRF (eg: one from *P. mellea*, and another one from *S. commune*), 3 to 10 mm in diameter, which finally incubated at 28^{o}C for ten days.

4.2.9 Enzyme activity determination on co-culture of white rot fungi

After 10 days of SSF, the mixture was filtrated and mixed with polyvinylpolypyramidone (PVPP) before centrifuged (Avanti J-26S XP1, High-Performance Centrifuge, Beckman Coulter, USA) at 12,000 g for 10 minutes in 4^{o}C (Azmi et al., 2019). The supernatant was collected and stored in 4^{o}C for enzyme activity determination. Enzyme activity determination was done for the six enzymes namely laccase, lignin peroxidase (LiP), manganese peroxidase (MnP), carboxymethyl cellulase (CMCase), avicelase and xylanase followed the method mentioned before in ligninolytic enzyme activity determination. Each experimental setup was replicated three times to ensure statistical reliability. The method referred to 4.2.3, 4.2.4 and 4.2.5.

4.2.10 Statistical analysis

IBM SPSS statistics 27 was used in this study and presented as mean ± SEM (standard error of mean) for enzyme activity determination of mono- and co-culture analysis. Analysis of variance (ANOVA) was applied to compare the significant difference with $p<0.05$, followed by Tukey's test.

4.3 Results

4.3.1 Enzyme activity determination of enzyme extract from filamentous fungi

The enzyme activity analysis showed that all eight species of identified filamentous fungi were successfully producing the enzyme in 4.2.3, 4.2.4, and 4.2.5. Only *Mucor fusiformis* (MF) and *Aspergillus neoellipticus* (AN) showed no laccase enzyme activity production (Figure 4.1). The enzyme activity analysis showed that all eight species of identified filamentous fungi were successfully producing the enzyme in 4.2.3, 4.2.4, and 4.2.5. Only *Mucor fusiformis* (MF) and *Aspergillus neoellipticus* (AN) showed no laccase enzyme activity production (Figure 4.1).

The laccase enzyme activity of *Phanerina mellea* (PM) showed the highest average enzyme activity compared to the other filamentous fungi (Figure 4.1). The laccase enzyme activity of *Phanerina mellea* increased from 0.28 U/mL on day 10 to 0.53 U/mL on day 20 of SSF. On day 30, the laccase enzyme activity of *Phanerina mellea* decreased to 0.24 U/mL. It is followed by *Marasmius palmivorus* (MP), *Schizophyllum commune* (SC), *Penicillum citrinum* (PC), *Trichoderma asperellum* (TA), and *Syncephalastrum racemosum* (SR). In general, most fungi, such as PM, MP, SC, and TA, showed the highest laccase enzyme activity on day 20 of SSF.

All identified filamentous fungi produced lignin peroxidase (LiP) enzyme activity (Figure 4.2). The LiP enzyme activity of PM showed the highest

value, which is 0.90 U/mL, on day 10 of SSF, compared to the other fungi. The LiP enzyme activity was then reduced to 0.34 U/mL and 0.08 U/mL on days 20 and 30 of SSF, respectively. The SC, SR, AN, PC, MF, TA, and MP follow the same trend. Most of the fungi showed the highest LiP enzyme activity on day 10 of SSF including PM, SC, SR, AN, and PC. The MF, TA, and MP showed the highest LiP enzyme activity on day 20 of SSF.

All identified filamentous fungi showed to produce manganese peroxidase (MnP) enzyme activity (Figure 4.3). The MnP enzyme activity of SC is 0.58 U/mL on day 10 of SSF, showing the highest value, followed by PM, MF, PC, TA, AN, SR and MP. The SC MnP enzyme activity shows a decrease of 0.03 U/mL on day 20 with a value of 0.55 U/mL and decreased again to 0.39 U/mL on day 30 of SSF. The majority of the fungi showed to produce the highest MnP enzyme activity on day 10 of SSF except for AN and SR, which produced the highest on day 20 of SSF.

The carboxymethylcellulose (CMCase) enzyme activity for all identified filamentous fungi were showing a similar pattern as the activity increased from day 10 to 20, setting the highest activity before reducing on day 30 of SSF (Figure 4.4). The CMCase enzyme activities for all fungi are the highest on day 20 of SSF compared to days 10 and 30. The highest value is shown by MF with the value of 0.20 U/mL and, the lowest value is shown by PC on day 30 of SSF with a value of 0.02 U/mL.

The avicelase enzyme activity was produced by all identified filamentous

fungi, with the majority of the fungi had the highest activity on day 20 of SSF (Figure 4.5). A similar pattern to CMCase enzyme activity, no obvious comparison can be seen by each fungus, and the highest avicelase enzyme activity is shown by TA with a value of 0.38 U/mL on day 20 of SSF, and the lowest is by AN with a value of 0.15 U/mL on day 10 of SSF. Xylanase enzyme activity is shown to be similar for each fungus where the highest activity is shown on day 10 before it reduces on days 20 and 30 of SSF (Figure 4.6). All fungi are showing to produce a similar pattern of xylanase enzyme activity, where TA shows the highest value on day 10 with the value of 0.27 U/mL.

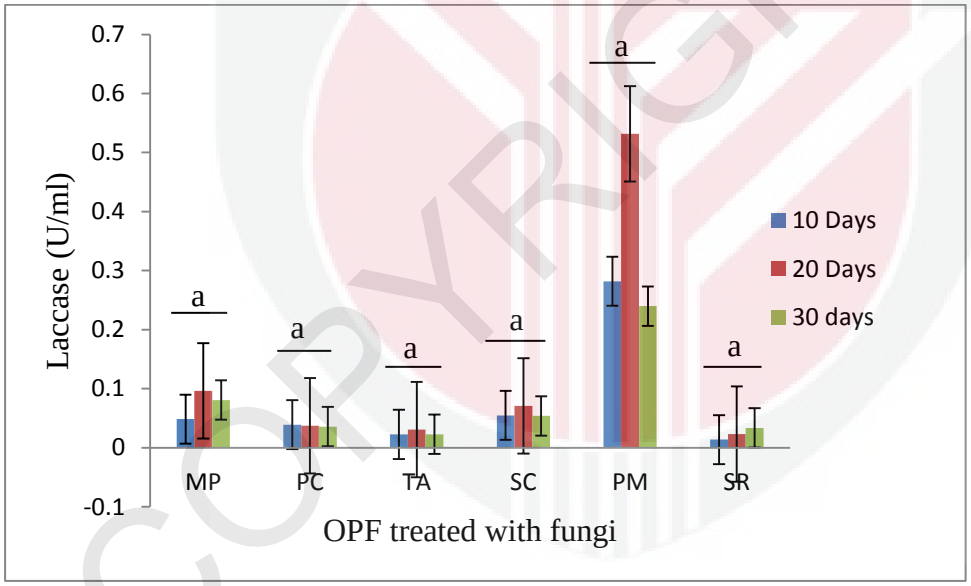


Figure 4.1: Laccase activity of enzyme extract after oil palm frond pre-treatment with different filamentous fungi at day 10, 20, and 30 of solid-state fermentation. *Note: Marasmius palmivorus* (MP), *Penicillum citrinum* (PC), *Trichoderma asperellum* (TA), *Schizophyllum commune* (SC), *Phanerina mellea* (PM), *Syncephalastrum racemosum* (SR).

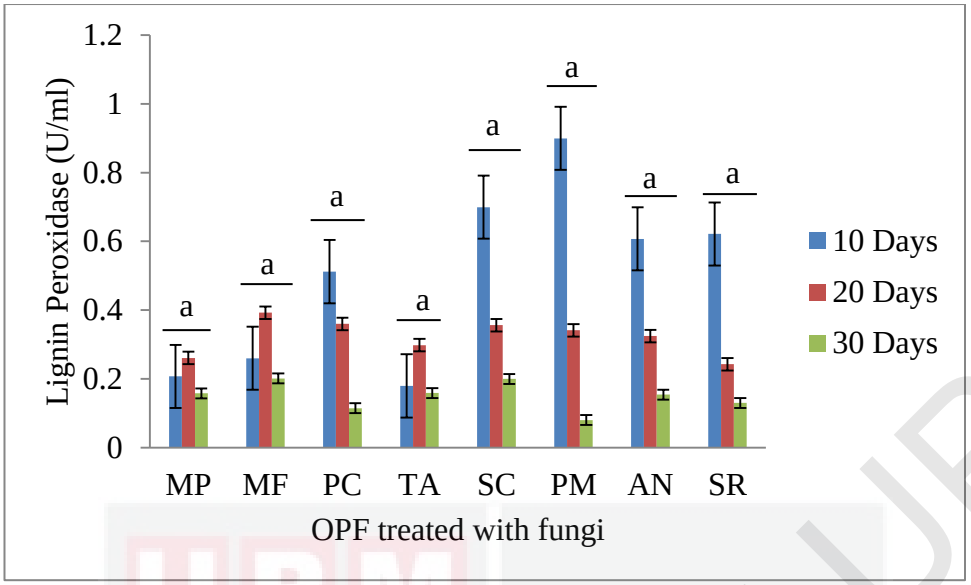


Figure 4.2: Lignin peroxidase activity of enzyme extract after OPF pre-treatment with different filamentous fungi at day 10, 20, and 30 of solid-state fermentation. *Note: Marasmius palmivorus* (MP), *Mucor fusiformis* (MF), *Penicillum citrinum* (PC), *Trichoderma asperellum* (TA), *Schizophyllum commune* (SC), *Phanerina mellea* (PM), *Aspergillus neoellipticus* (AN), *Syncephalastrum racemosum* (SR).

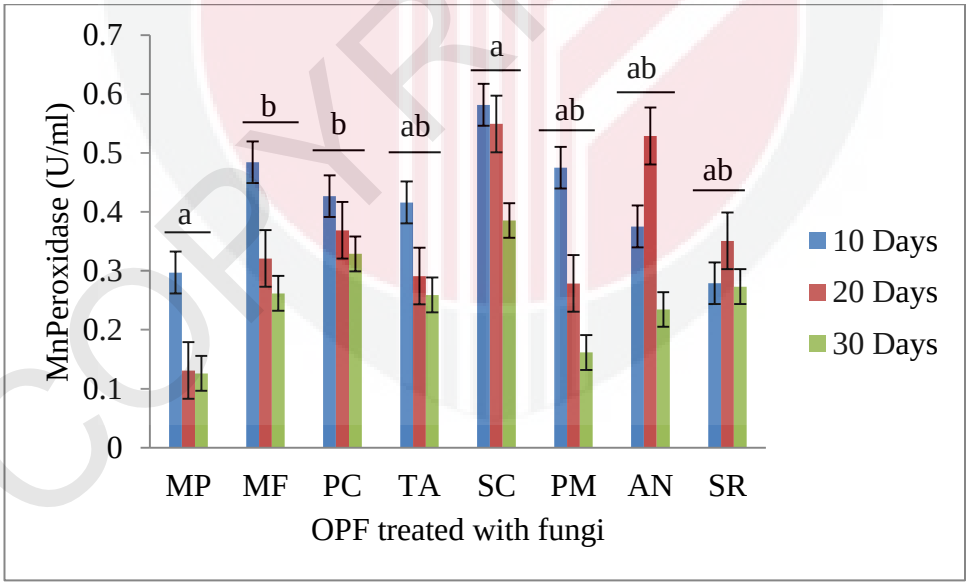


Figure 4.3: Manganese peroxidase activity of enzyme extract after oil palm frond pre-treatment with different filamentous fungi at day 10, 20, and 30 of solid-state fermentation. *Note: Marasmius palmivorus* (MP), *Mucor fusiformis* (MF), *Penicillum citrinum* (PC), *Trichoderma asperellum* (TA), *Schizophyllum commune* (SC), *Phanerina mellea* (PM), *Aspergillus neoellipticus* (AN), *Syncephalastrum racemosum* (SR).

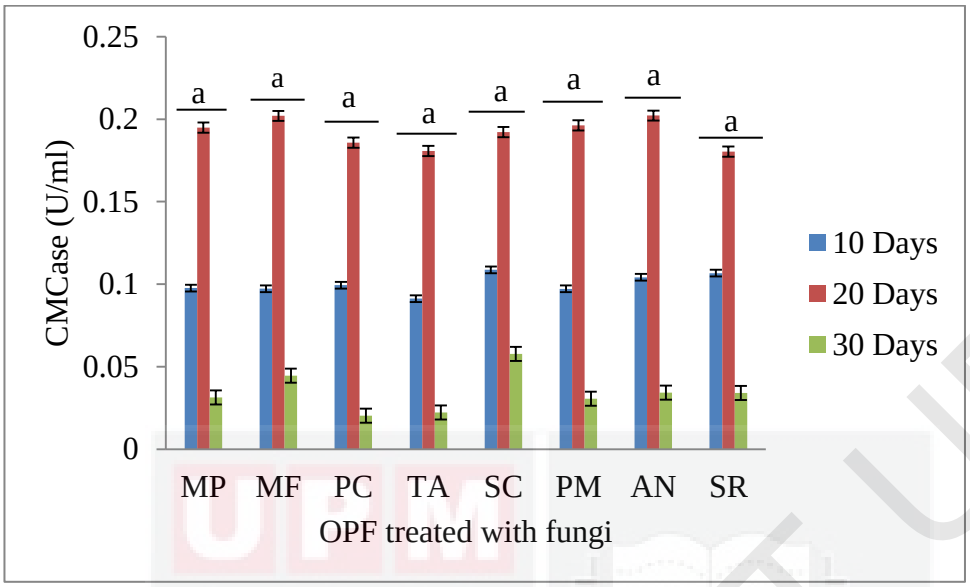


Figure 4.4: CMCase activity of enzyme extract after oil palm frond pre-treatment with different filamentous fungi at day 10, 20, and 30 of solid-state fermentation. *Note: Marasmius palmivorus* (MP), *Mucor fusiformis* (MF), *Penicillum citrinum* (PC), *Trichoderma asperellum* (TA), *Schizophyllum commune* (SC), *Phanerina mellea* (PM), *Aspergillus neoellipticus* (AN), *Syncephalastrum racemosum* (SR).

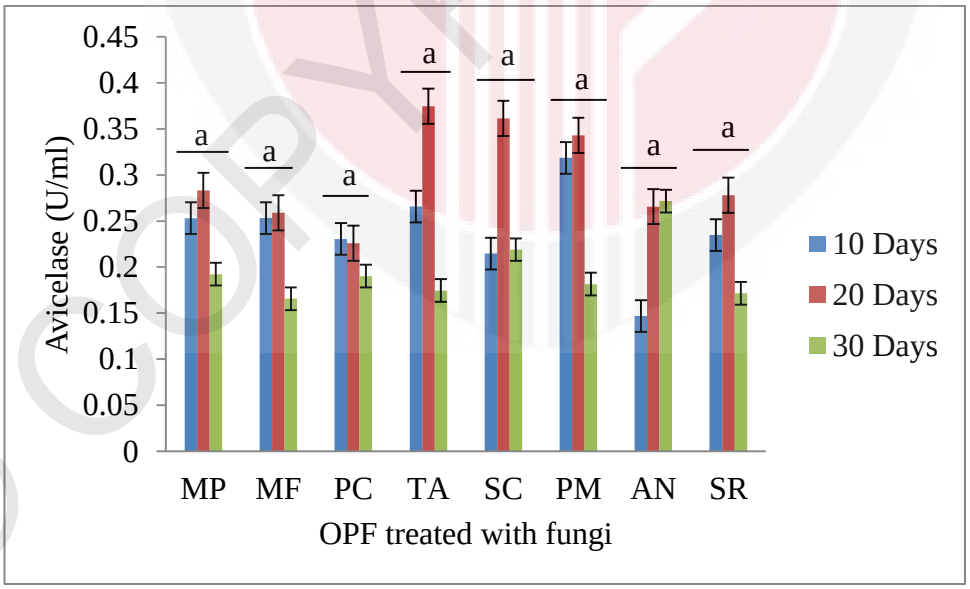


Figure 4.5: Avicelase activity of enzyme extract after oil palm frond pre-treatment with different filamentous fungi at day 10, 20, and 30 of solid-state fermentation. *Note: Marasmius palmivorus* (MP), *Mucor fusiformis* (MF), *Penicillum citrinum* (PC), *Trichoderma asperellum* (TA), *Schizophyllum commune* (SC), *Phanerina mellea* (PM), *Aspergillus neoellipticus* (AN), *Syncephalastrum racemosum* (SR).

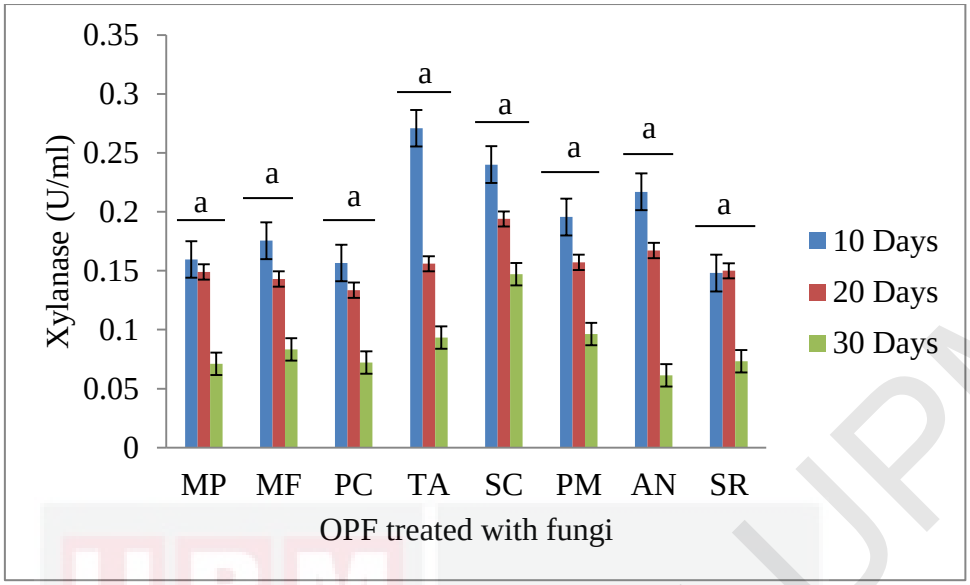


Figure 4.6: Xylanese activity of enzyme extract after oil palm frond pre-treatment with different filamentous fungi at day 10, 20, and 30 of solid-state fermentation. *Note: Marasmius palmivorus* (MP), *Mucor fusiformis* (MF), *Penicillum citrinum* (PC), *Trichoderma asperellum* (TA), *Schizophyllum commune* (SC), *Phanerina mellea* (PM), *Aspergillus neoellipticus* (AN), *Syncephalastrum racemosum* (SR).

4.3.2 Selection of the optimal ligninolytic enzyme production time of filamentous fungi

The variance and Tukey's analysis data of days 10, 20, and 30 of SSF of each enzyme were determined. Table 4.1 shows the average enzyme activity of fungi at different treatment time based on the type of enzyme that was experimented. The average laccase enzyme activity shows that day 20 has the highest enzyme activity (0.10 U/mL) than day 10 (0.06 U/mL), and 30 (0.06 U/mL) of SSF. However, there is no significant difference (p>0.05) between days 10, 20, and 30 of SSF. For average LiP and MnP enzyme activity, day 10 shows the highest activity 0.50 U/mL and 0.42 U/mL, respectively, followed by day 20 and 30. However, no significant difference (p>0.05) exists between day 10 and 20. It also showed no significant

difference (p>0.05) between day 20 and 30 of average LiP and MnP enzyme activity. However, day 10 is significantly different (p<0.05) from day 30 of SSF.

The mean CMCase enzyme activity of day 20 shows the highest activity (0.19 U/mL), followed by day 10 (0.10 U/mL) and 30 (0.03 U/mL). Day 10, 20, and 30 significantly differ (p<0.05) between each treatment time. Like CMCase, average avicelase enzyme activity also shows that day 20 has the highest enzyme activity (0.30 U/mL) compared to day 10 (0.24 U/mL) and 30 (0.20 U/mL). There is no significant difference (p>0.05) between day 20 and 30 of SSF. However, day 10 significantly differ (p<0.05) from day 20 and 30. The mean xylanase enzyme activity of day 10 shows the highest activity (0.20 U/mL), followed by day 20 (0.16 U/mL), and 30 (0.09 U/mL). The variance analysis shows that day 10 has no significant difference (p>0.05) from day 20. However, day 30 significantly differ (p<0.05) from day 10 and 20 of SSF.

On average, ligninolytic and hemicellulolytic enzyme activity showed the highest activity on day 10, while cellulolytic enzyme activity showed the highest activity on day 20 of SSF. In this study, ligninolytic enzymes were needed for lignin degradation with minimum cellulolytic and hemicellulolytic enzyme (Azmi et al., 2022). Therefore, the optimum OPF pre-treatment time for fungi selection is day 10 of SSF.

Table 4.1: Average enzyme activity of identified filamentous fungi on day 10, 20 and 30 of solid-state fermentation

Treatment time (Days)	Average enzyme activity (U/ml)					
	Laccase	LiP	MnP	CMCase	Avicelase	Xylanase
10	0.06^{a}	0.50^{b}	0.42^{b}	0.10^{b}	0.24^{a}	0.20^{b}
20	0.10^{a}	0.32^{ab}	0.35^{ab}	0.19^{c}	0.30^{b}	0.16^{b}
30	0.06^{a}	0.15^{a}	0.25^{a}	0.03^{a}	0.20^{a}	0.09^{a}
SEM	0.01	0.04	0.03	0.03	0.02	0.02
***p*-Value**	0.75	<0.001	0.23	<0.001	<0.001	<0.001

Note: Means that do not share a letter vertically are significantly different, where (p< 0.05) is considered as significant value.

4.3.3 Selection of fungi based on enzyme activity determination on day 10 of pre-treatment

The selection of the most favorable filamentous fungi for pre-treatment of OPF was based on the average enzyme activity determination results on day 10 of SSF. Table 4.2 shows the average ligninolytic (A), cellulolytic + hemicellulolytic (B) enzyme activity and the ratio between A and B. The ration between A and B shows positive value indicated that the average enzyme production A is higher compared to B. The PM has the highest value of 2.75, followed by SC (2.37), PC (2.06), AN (2.06), SR (1.94), MF (1.39), MP (1.06), and TA (1.00). Figure 4.7 shows a scatterplot graph of the average ligninolytic enzyme activity against the total average of cellulolytic + hemicellulolytic enzyme activity. From the graph, *P. mellea* (PM) is plotted at the top of the graph (red circle), which shows that the fungus has the highest average ligninolytic enzyme activity (0.55 U/mL) with low average cellulolytic + hemicellulolytic enzyme activity (0.20 U/mL). It is followed by SC which is

located a bit lower than PM with average ligninolytic enzyme activity of 0.45 U/mL, and 0.19 U/mL of average cellulolytic + hemicellulolytic enzyme activity. In this experiment, *P. mellea* was the most desired filamentous fungi for the pre-treatment of OPF.

Table 4.2: Average ligninolytic and cellulolytic + hemicellulolytic enzyme activity of extracted enzyme from filamentous fungi on day 10 of solid-state fermentation.

Fungi	Average enzyme activity (U/mL)		Ratio A/B
	(A) ligninolytic	(B) Cellulolytic + hemicellulolytic	
MP	0.18[a]	0.17[a]	1.06
MF	0.25[a]	0.18[a]	1.39
PC	0.33[a]	0.16[a]	2.06
TA	0.21[a]	0.21[a]	1.00
SC	0.45[a]	0.19[a]	2.37
PM	0.55[a]	0.20[a]	2.75
AN	0.33[a]	0.16[a]	2.06
SR	0.31[a]	0.16[a]	1.94
SEM	0.02	0.003	0.08
***p*-Value**	0.73	0.99	-

Note: Means that do not share a letter are significantly different, where (p<0.05) is considered as significant value. *Marasmius palmivorus* (MP), *Mucor fusiformis* (MF), *Penicillum citrinum* (PC), *Trichoderma asperellum* (TA), *Schizophyllum commune* (SC), *Phanerina mellea* (PM), *Aspergillus neoellipticus* (AN), *Syncephalastrum racemosum* (SR).

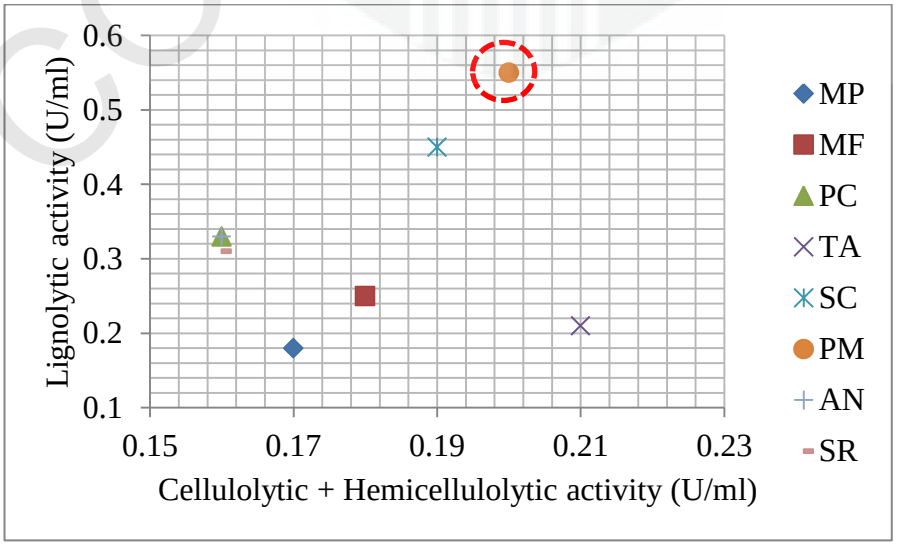


Figure 4.7: Graph of average ligninolytic enzyme activity against total average of cellulolytic and hemicellulolytic enzyme activity of filamentous fungi on day 10 of solid-state fermentation. Notes: red circle is where *P. mellea* is plotted at the top of the graph. *Marasmius palmivorus* (MP), *Mucor fusiformis* (MF), *Penicillum citrinum* (PC), *Trichoderma asperellum* (TA), *Schizophyllum commune* (SC), *Phanerina mellea* (PM), *Aspergillus neoellipticus* (AN), *Syncephalastrum racemosum* (SR).

4.3.4 Remazol Brilliant Blue R media analysis of mono- and co-culture of selected white rot fungi

All fungi culture on PDA media containing Remazol Brilliant Blue R (RBBR) dye showed substantial decolourization of RBBR after seven days except for mono-cultured *M. palmivorus* (MP). Figure 4.8 (a), (b), and (c) showed the dye decolourization of RBBR media by mono-cultured WRF. The mono-cultured of *Phanerina mellea* (PM) and *Schizophyllum commune* (SC) showed dye decolourization while *Marasmius palmivorus* (MP) cannot decolourize the RBBR dye media. The different degrees of dye decolourization for each WRF showed that they produced different ligninolytic enzyme activity. The bleached area was observed within five days. The PM showed a higher degree of decolourization with 6.2 cm diameter of decolorization than SC, 1.5 cm while MP has the weakest decolorization ability.

In co-culture, the interactions between the different WRF are more critical as the growth may produce mutual or antagonist interactions. Figure 4.8 (d), (e) and (f) showed the dye decolourization of RBBR media by co-cultivation of PM + SC, SC + MP and PM + MP, respectively. The PM + SC showed no dye decolourization at the confrontation between mycelia. However, the

bleached area was further enhanced in PM with the presence of SC. The dye decolourization only happened on the individual zone of WRF. The growth of SC was also inhibited by PM that dominated the agar plate area. As a result, SC's dye decolorization area is smaller than PM's.

There is also dye decolourization for SC + MP. However, there is no dye decolourization at the confrontation area between mycelia. The growth of both WRF seems to be slower than their mono-cultured. The co-cultivation of PM + MP showed similar interaction to PM + SC which the antagonist interaction happened. The PM showed dye decolourization, while the growth of MP showed no dye decolourization. The agar plate area, also dominated by PM inhibits the growth of MP. However, weak decolourization showed by each pair of WRF co-culture. None of the pair's co-culture combinations is producing synergistic effects, and the combinations seem to produce antagonistic interactions between each WRF.

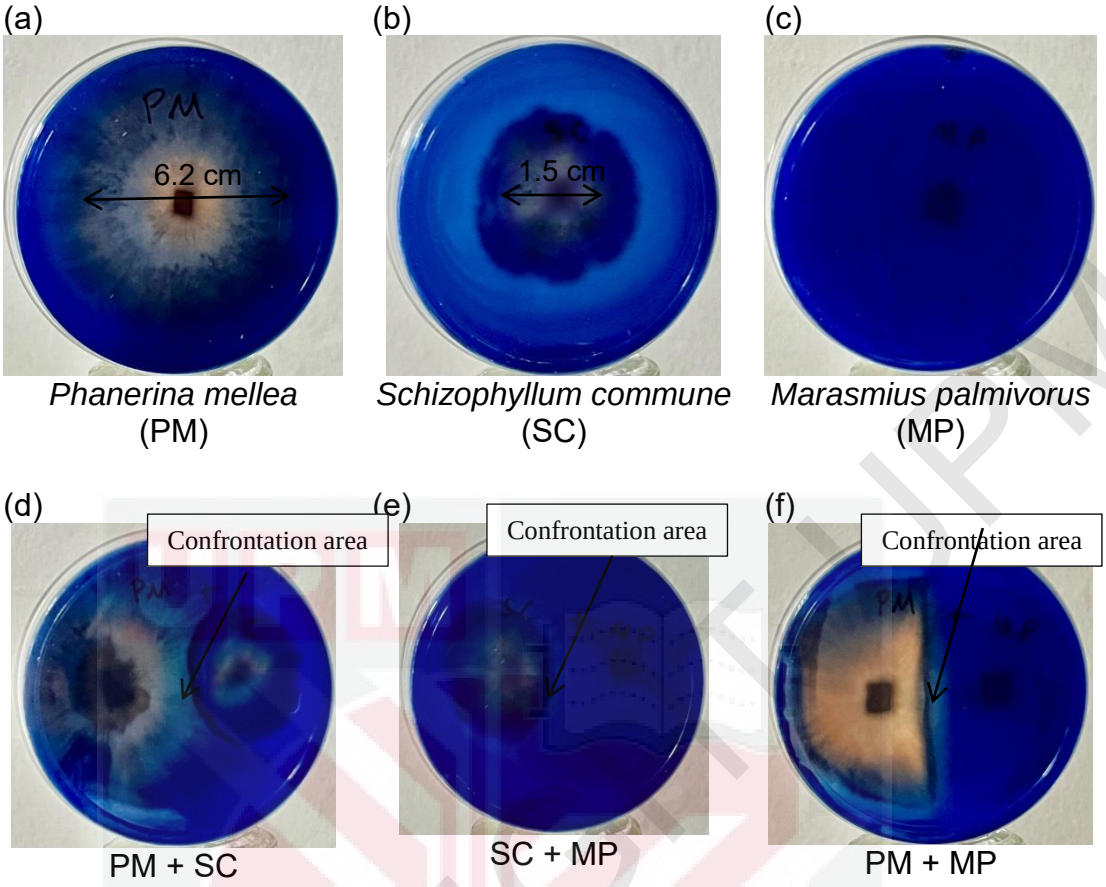


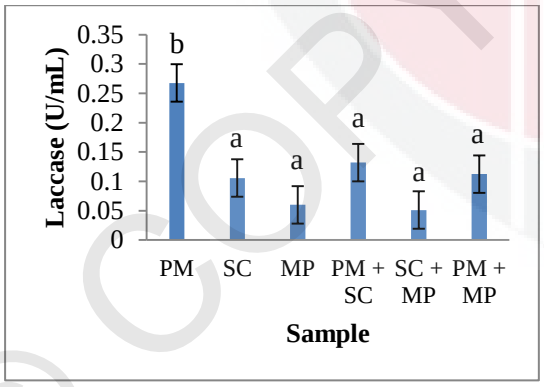
Figure 4.8: The decolorization of RBBR media with 500 ppm concentration for monoculture of white rot fungi (a) PM, (b) SC, (c) MP and co-culture of WRF (d) PM + SC, (e) SC + MP and (f) PM + MP.

4.3.5 Enzyme activity determination for mono- and co-culture of white rot fungi

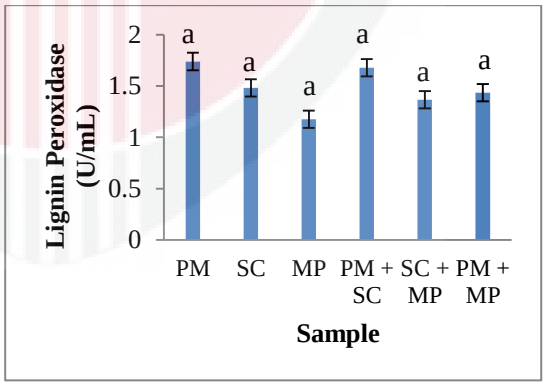
The enzyme activity determination for all mono- and co-culture of WRF in this experiment showed to produce enzyme activity of laccase, LiP, MnP, CMCase, avicelase, and xylanase for 10 days incubation of SSF. The PM showed the highest laccase and LiP enzyme activity with the value of 0.27 U/mL and 1.74 U/mL, respectively, as shown in the graphs of Figure 4.9a & b. The SC showed the highest MnP enzyme activity with a value of 0.56 U/mL (Figure 4.9c). However, the co-culture of PM + SC showed the lowest CMCase and avicelase enzyme activity, which is good for reducing cellulose

digestion in OPF (Figure 4.9d and Figure 4.9e). The SC showed the highest enzyme activity for xylanase enzyme followed by SC + MP (Figure 4.9f).

The average of ligninolytic (laccase + LiP + MnP) against cellulolytic + hemicellulolytic enzyme activity production for mono-and co-culture of WRF after 10 days of SSF was plotted in Figure 4.10. It showed that PM is located at the top of the graph which indicates that PM produced the highest ligninolytic enzyme activity. The co-culture of PM + SC showed promising combinations as it is located below PM with lower cellulolytic + hemicellulolytic enzyme activity production. It is indicated that the combination performed better at preserving cellulose. Lower ligninolytic enzyme activity produced by co-culture compared with mono-cultured of WRF determined that no synergism effect that happened by all possible combinations of individual selected WRF.



(a)



(b)

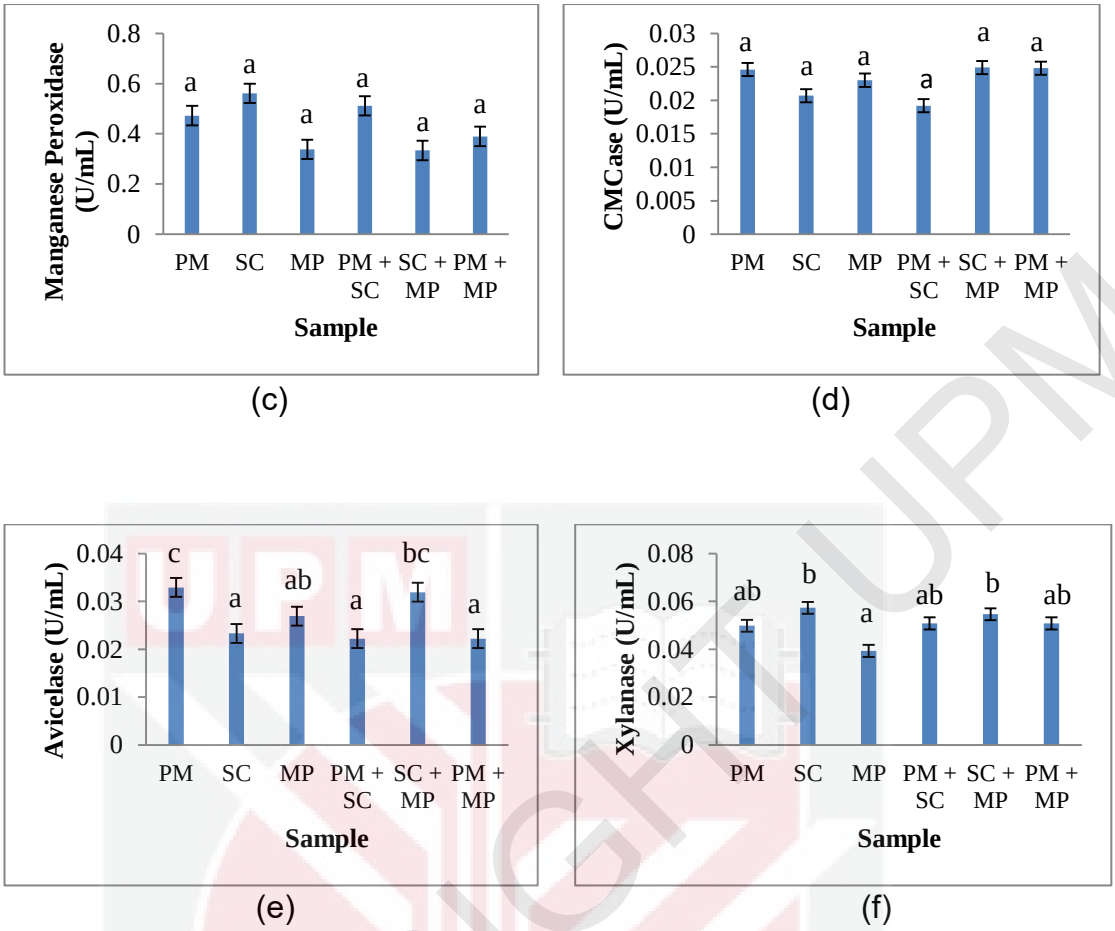


Figure 4.9: Comparison of enzyme activity of mono- and co-culture of white rot fungi for day 10 of solid-state fermentation for different enzyme (a) laccase, (b) lignin peroxidase, (c) manganese peroxidase, (d) CMCase, (e) avicelase, (f) xylanase. *Note:* Means that do not share a letter are significantly different, where (p<0.05) is considered as significant value. *Phanerina mellea* (PM), *Schizophyllum commune* (SC), *Marasmius palmivorus* (MP), *Phanerina mellea* + *Schizophyllum commune* (PM + SC), *Schizophyllum commune* + *Marasmius palmivorus* (SC + MP), *Phanerina mellea* + *Marasmius palmivorus* (PM + MP).

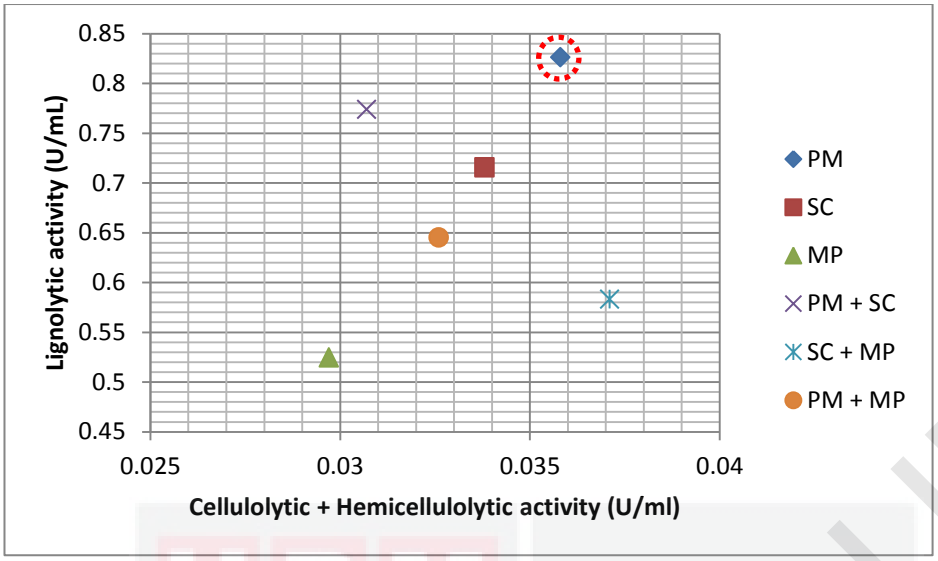


Figure 4.10: Graph of average ligninolytic against average cellulolytic + hemicellulolytic enzyme activity production for mono-and co-culture of WRF after day 10 of solid-state fermentation. *Notes: Phanerina mellea* (PM), *Schizophyllum commune* (SC), *Marasmius palmivorus* (MP), *Phanerina mellea* + *Schizophyllum commune* (PM + SC), *Schizophyllum commune* + *Marasmius palmivorus* (SC + MP), *Phanerina mellea* + *Marasmius palmivorus* (PM + MP).

4.4 Discussion

4.4.1 Ligninolytic enzyme activity determination of enzyme extract from identified filamentous fungi

In this enzyme activity determination study, some fungi were lacking of laccase enzyme activities as some physiological fungi groups were never reported to produce laccase enzyme, such as *zygomycetes,* which is the division of *Mucor fusiformis* (Mäkelä et al., 2020). According to Gupte et al. (2007), molasses distillery wastewater decolourization study via fungi pre-treatment under SSF by Pant & Adholeya. (2017) reported that no laccase activity production shown by *Phanerochaete chrysosporium*, *Fusarium verticillioides* and *Aspergillus niger*. Enzyme production by fungi can be classified into three categories which are fungi that secret laccase and two

peroxidases (MnP, LiP), laccase and one peroxidase, and laccase or peroxidase only (Wang et al., 2019). The *Mucor fusiformis* and *Aspergillus neoelepticus* are classified into fungi that secret peroxidase only.

Lignin peroxidase (LiP) resulted in the highest enzyme activity in the range of 0.08 – 0.90 U/mL with the highest recorded by *Phanerina mellea* (0.90 U/mL) on day 10 of SSF. It is lower when compared to LiP enzyme activity produced by *Phanerochaete chrysosporium* with 14.25 U/mL on day 10 of SSF with wheat straw as the substrate reported by Gupte et al. (2007). The difference in LiP enzyme activity production between these two studies could be caused by the different substrates used and nutrient supplementation.

In general, most of the fungi identified in this study produced a higher range of MnP enzyme activity average (0.2 – 0.5 U/mL) than laccase enzyme activity average (0.02 – 0.3 U/mL), and an almost similar range was shown with LiP enzyme activity average (0.2 – 0.4 U/mL). No manganese (II) ion supplementation was involved in the pre-treatment of OPF under SSF in this study. This study was trying to avoid the high supplementation of manganese (II) ion, which will potentially hinder the production of LiP enzyme activity due to its highly reactive action (Hofrichter, 2002). Previous study on the regulation of manganese (II) ion on the production of LiP and MnP enzymes of WRF showed that the low concentration of manganese (II) ion (0 – 1.6 ppm) supplementation produced higher LiP enzyme activity (300 – 450 nmol/min.mL), compared to higher concentration (8.0 – 40.0 ppm) of manganese (II) ion supplementation which resulted in lower (0 - 150

nmol/min.mL) LiP enzyme activity production (Silva et al., 2008).

Cellulolytic enzyme activity showed a similar value and trend for all fungi identified, ranged from 0.09 – 0.10 U/mL for day 10, 0.18 – 0.20 U/mL for day 20, and 0.02 – 0.06 U/mL for day 30. Day 20 showed the highest activity compared to day 10 and 30 of SSF. The peak time production of cellulolytic enzyme was delayed compared to ligninolytic enzyme. The structure of the OPF, which is covered with the cell wall components, induced the production of ligninolytic enzyme first before cellulolytic and hemicellulolytic enzyme by WRF. The WRF colonized the cell lumina of the OPF before the fungal mycelia propagated the cell wall structure by the Fenton reaction. This event caused the fungi to produce ligninolytic enzyme first before cellulolytic enzyme (Rouches et al., 2016; Ji et al., 2014; Dong et al., 2013). This in line with findings by Rahman et al. (2020), which reported *Phanerochaete chrysosporium* to secrete ligninolytic enzyme before cellulolytic enzyme that resulted in lower sugar production compared to *Aspergillus terreus*. The activity was decreased on day 30 as the cellulolytic enzyme activity was suppressed caused by the formation of aromatic water-soluble products from earlier delignification process. While the hemicellulolytic enzyme activity were decreased on day 30 due to the depletion of resources (Azmi et al., 2022).

4.4.2 Optimization of ligninolytic enzyme production time

The optimal ligninolytic enzyme production time was determined based on

the average enzyme activity of fungi identified on day 10, 20 and 30 of SSF. The current study showed that the optimal ligninolytic enzyme production time is on day 10 of SSF. The production of laccase enzyme activity is different for different fungi strains. One of the factors that influenced the production of ligninolytic enzyme production was fungi strain (Isroi et al., 2011). Study by Gupte et al. (2007) reported that the production of ligninolytic enzyme activity by *Trichoderma versicolor*, *Phanerochaete chrysosporium*, *Pleurotus ostreatus,* and *I. lateus* on wheat straw showed the highest on day 10 of SSF. Whereas other study also reported that the highest laccase enzyme production time by *Pleurotus eryngii* was observed on day 10 of SSF (Akpinar & Urek, 2012).

4.4.3 Fungi selection

In the selection of fungi, filamentous fungi that produced high ligninolytic enzymes with low cellulolytic + hemicellulolytic enzyme activity at 10 days of SSF were selected for further study. Ligninolytic enzymes are the enzyme responsible for delignification of fibrous by-products (Saha et al., 2016). Narayanazwamy et al. (2013) reported that lignin degradation involves extracellular enzymes (LiP, MnP and laccase). The lignin degradation ability of filamentous fungi is the critical factor in the pre-treatment, however, the recovery of cellulose to conserved the total availability of glucose to be used by animals will determine the fungi actual potential ability (Tian et al., 2012). In the current study, PM has the highest average ligninolytic enzyme activity (0.55 U/mL) with low average cellulolytic + hemicellulolytic enzyme activity

(0.20 U/mL).

4.4.4 Assessment on co-culture of white rot fungi

The RBBR dye decolorization method has been used as a rapid screening method to detect the ability of fungi to produce ligninolytic enzymes. The critical factor in the decolorization of RBBR dye is laccase enzyme, while MnP enzyme production is not involved in RBBR's decolorization (Sumandono et al., 2015). In this study, all WRF's co-culture developed no decolorization at the confrontation area. A confrontation area was developed due to the deadlock interaction between two different fungi. It happens when the hyphae of one species cannot enter another species' hyphae territory in the co-culture. That interaction resulted in low nutrition exploitation and delignification (Wang et al.,2014) . In contrast, the synergism happens when there is no confrontation area developed. The degree of decolorization is different between fungi as it is influenced by the ligninolytic enzyme produced, dependent on the species. Besides that, the available nutrients in the culture medium also influenced fungi's growth and enzyme productions (Qi-he et al., 2011).

The enzyme pre-treatment by the co-culture fungi was aimed to have higher lignin loss and preserve more holo-cellulose (hemicellulose and cellulose). However, it is contradicts to several previous studies as the co-culture of fungi produced an antagonistic interaction (inhibitory effect) instead of synergism (Ma et al. 2011; Parani & Eyini. 2010; Chi et al. 2007). The

synergistic interaction between fungi may result in higher enzyme activity production. For example, a study by Jimenez-Barrera et al. (2018) reported that the co-culture between *Pycnoporus sanguineus* and *Beauveria brongniartii* increased the laccase and MnP enzyme activity for 6.0-fold and 2.3-fold, respectively compared to *Pycnoporus sanguineus* mono-culture measured by using Plackett-Burman experimental design (PBED) and a central composite design (CCD), which the H_2O_2 content was analyzed. In the current study, the co-culture of the three possible combinations (PM + SC, SC + MP, and PM + MP) resulted in inhibitory effect as no decolourization of the RBBR media at the confrontation area between co-culture besides the enzyme activity determination of co-culture fungi also showed no significant (p>0.05) improvement compared to mono-culture. However, the result eventually depends on the interaction between particular species combination and culture conditions (Wang et al., 2014).

4.5 Conclusion

The enzyme activity of the identified filamentous fungi were successfully detected and analyzed on their ability to degrade lignin in OPF. The average ligninolytic enzyme activity was higher than the average cellulolytic + hemicellulolytic enzyme activity as positive value of ratio between enzymes produced (1.00 to 2.75) were identified. The PM best showed the enzyme activity production of the desired enzyme. The optimal pre-treatment time of OPF was also successfully analyzed in which day 10 of SSF showed the best pre-treatment time for producing high ligninolytic enzyme activity. The

PM showed an average ligninolytic enzyme activity value of 0.55 U/mL, and the average cellulolytic + hemicellulolytic of 0.20 U/mL. The PM and SC were selected for the co-culture of WRF study. The co-culture of selected WRF produced antagonistic interactions between each other, and no synergistic effect was identified. The enzyme activity also supports that no synergism happened on the co-culture of WRF. The enzyme extract from PM and the co-cultured enzyme extract of PM + SC is promising enzymes to improve the nutritional value of OPF.

CHAPTER 5

EFFECTS OF OIL PALM FROND PRE-TREATED WITH ENZYME EXTRACT FROM WHITE ROT FUNGI ON *IN VITRO* RUMEN FERMENTATION

5.1 Introduction

Malaysia is one of the countries with the highest production of palm oil in the world. This is associated with the production of its agricultural by-product called oil palm frond (OPF) which is currently becomes a very essential fibrous by-product due to its potential to be used in many industries including the livestock industries. The application of raw OPF as livestock feed has been implemented by many small holder farmers as the substitute to grasses. However, the low nutritive value of OPF caused by the lignin that encapsulates the cellulose and hemicellulose hinders the digestion of OPF by the ruminant. This is consistent with the poor degradation of OPF in an *in vitro* study by Hassim et al. (2010) and low OPF digestibility in cattle as reported by Kawamoto et al. (2001).

The pre-treatment of OPF by biological pre-treatment is currently the most promising pre-treatment method to effectively degrade lignin with low cost and environmentally friendly compared to physical and chemical pre-treatment. In addition, the pre-treatment of OPF by biological pre-treatment like enzyme extract from white rot fungi (WRF) gives better results as compared to using direct WRF pre-treatment. The enzyme extract pre-treatment offered minimum dry matter loss as the method is able to control the temperature and pH, which stimulate an effective pre-treatment without

stimulating the growth of the fungi (Figure 5.1) (Lu et al., 2010).

Various studies reported that pre-treatment method using enzyme extract has lower lignin degradation as compared to direct fungi due to the absence of the fungi itself which is responsible in causing Fenton reaction (induced chemical reagent, iron and H_2O_2), other than secrete a compelling mixture of components not only the oxidative enzymes but also the mediators (for example low molecular compound of hydroxyl-benzotriazole) (Rouches et al., 2016; Jung et al., 2015). However, the enzyme extract pre-treatment is still the preferable method due to its advantages such as milder process condition, higher reaction rates, higher reaction specificity, and shorter incubation processes over direct WRF pre-treatment (Masran et al., 2016).

Then, studies found that the implementation of enzyme extract can be improved by applying mixed enzyme to maximize the delignification and increase effectiveness. Other study also reported that the combination of ligninolytic enzyme extract from different fungi (mix enzyme extract) will trigger various active iso-enzymes to degrade lignin in the fibrous by-product cell wall matrix. For example, the purified enzymes mixture of *Pleurotus florida*, *Pleurotus sajor-ceju* and *Pleurotus eryngii* used to pre-treat a corn cob resulted in 5.2% lignin loss compared to single *Pleutorus florida* with 2.8% lignin loss (Naraian et al., 2010).

Ensiling is known to be the best preservation technique to deal with the rainy season, floods or insufficient feed. It also has great commercialized potential

as it can be prepared on a large scale, is easy to handle, and is less weather-dependent (Sahoo, 2018). Besides that, ensiling also allowed the enzyme extract pre-treatment to be applied as the silage preparation method has the availability to be added with additives. However, the demand for minimum permeability of the air to get a high quality in silage preparation is the main issue as the pre-treatment by enzyme extract from WRF is oxygen dependent (Xu et al., 2007). The delignification process is an oxidative process in which ligninolytic enzymes use oxygen and reduce it to exhibit a variety of different reactions on lignin (Masran et al., 2016). Therefore, a sufficient oxidizing agent, such as air, ozone, hydrogen peroxide, and oxygen, is needed in the OPF pre-treatment by enzyme extract from WRF. In the current study, the application of enzyme extracts pre-treatment in silage method was assessed by comparing it with the overnight pre-treatment method.

The *in vitro* study of pre-treated OPF has been done by various previous studies. For example, Rusli et al. (2019) reported that the biological pre-treatment resulted in higher *in vitro* gas production due to higher lignin degradation when compared to chemical and physical pre-treatment. Azmi et al. (2022) reported that the pre-treatment of OPF by enzyme extract of *F. solani* MK027309 increased the apparent rumen degradable carbohydrate (ARDC) by 35.29% compared to un-treated OPF in their *in vitro* study. Thus, the current study assessed the OPF pre-treated with enzyme extract from WRF and compared the effectiveness between silage and overnight pre-treatment method by the analysis of nutrient composition, *in vitro* gas

production, volatile fatty acid (VFA) productions, and ARDC value.

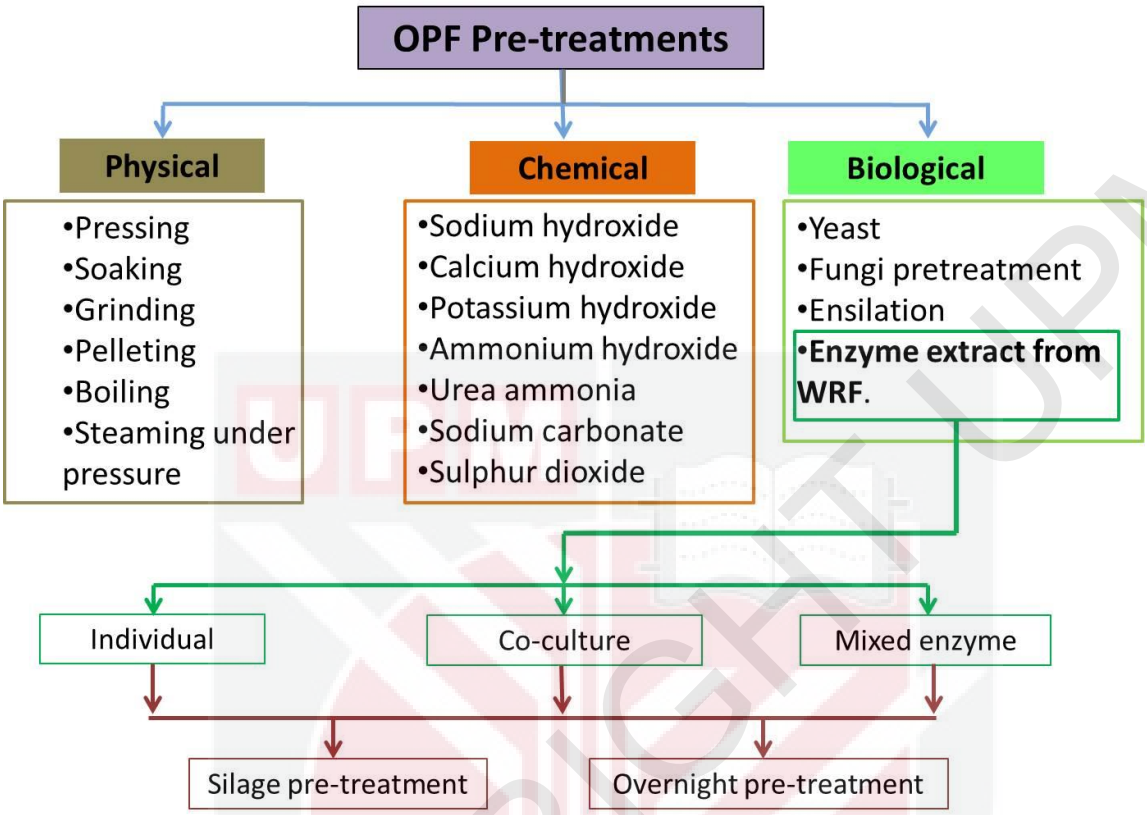


Figure 5.1: Flow chart of oil palm frond pre-treatment

5.2 Materials and Methods

5.2.1 Pre-treatment of oil palm frond

The OPF pre-treatment with enzyme extract was done in two different methods; silage preparation (Colombatto et al., 2004) and overnight pre-treatment (Wang et al., 2013) with a slight modification. In silage preparation, the chopped OPF was added into 1 L mini silo with supplementations of 5%/kg of molasses (Yunus et al., 2000), 3.1 ml/kg enzyme extract and water up to 20.4 ml/kg. Five group silages of pre-treatment were prepared based

on the type of enzyme extract used from WRF for pre-treatment, which are: (i) individual enzyme extract from *Phanerina mellea,* PM (STA), (ii) individual enzyme extract from *Schizophyllum commune,* SC (STB), (iii) selected co-culture (PM + SC) fungi enzyme extract (STC), (iv) mixed enzyme extract (combination of PM and SC) (STD), and control silage (water substitute the enzyme extract) (CST). The silages were labelled and incubated for 21 days at an ambient temperature.

As for overnight pre-treatment, the chopped OPF was mixed well with 5%/kg of molasses (Yunus et al., 2000), 1 L/kg of enzyme extract and 1 L/kg of water in a rectangular plastic basin with length of 45 cm, width of 34 cm, and height of 12 cm with rotation of 135rpm for 24 h. Like silage pre-treatment, five groups of mixtures were prepared based on type of enzyme extract used from WRF for pre-treatment which labelled as, (i) individual enzyme extract from *Phanerina mellea* (OTA), (ii) individual enzyme extract from *Schizophyllum commune* (OTB), (iii) selected co-culture (PM + SC) enzyme extract of WRF (OTC), (iv) mixed enzyme extract (combination of PM and SC) (OTD), and control (water substitute the enzyme extract) overnight pre-treatment (COT). All mixtures were left in the aerobic environment overnight (24 h). After the pre-treatment period, all pre-treated samples were dried in the oven at 60^{o}C before grounded into powder form using a stable arm (SHEN LIAN, SL-1000A, China) grinder and used for *in vitro* rumen fermentation study. All samples were tested on their pH and physical observations, including mould growth, aroma, texture and colour followed method by Ofori & Nartey. (2018).

5.2.2 Nutritional composition

All samples prepared for silage pre-treatment (STA, STB, STC, STD, CST), and overnight pre-treatment (OTA, OTB, OTC, OTD, and COT) were analyzed for nutritional composition. The nutritional composition involved were dry matter (DM) content, ash content, crude protein (CP), crude fiber (CF), ether extract, acid detergent fiber (ADF), acid detergent lignin (ADL) and neutral detergent fiber (NDF). The analysis were determined according to the method of the Association of Official Analytical Chemist (AOAC) (Chemists & Cunniff 1990).

The DM content was determined by oven-drying for 12–24 h/overnight at 105^{o}C. Ash content was determined by combustion in a muffle furnace for 4 h at 550^{o}C. CP content was determined by three method stages; digestion, distillation and titration. Digestion was done by the addition of sulphuric acid (H_2SO_4) into the Kjeldahl's flask before distillation with sodium hydroxide (NaOH) by a semi-auto Vapodest distillator. The distilled solution was titrated with 0.1 M hydrochloric acid (HCl). Crude fiber was determined by the digestion of samples with 0.13 mol/L of H_2SO_4 followed by 0.313 mol/L of NaOH in fiber bag. The digested samples were finally incinerated in the muffle furnace for 4 h at 600^{o}C. Ether extract was determined by ether extract measurement, which extracted by petroleum benzene in a round bottom flask (RBF). The RBF was attached to the Soxhlet apparatus, and the digestion was done for 4 h. All of the experiments were done in three replicates.

The ADF analysis was done by digestion of approximately 1 g of sample in ADF solution. Fiber bags were dried at 150^{o}C for 1 hour before cooling down in a desiccator for 30 min. The ADF solution was prepared by diluting N-cetyl-N, N, N-trimethyl-ammoniumbromide in 0.5 M sulphuric acid (H_2SO_4). The ADL analysis was done with ADF procedure as a preparatory step. The ADF procedure will not remove the cellulose and lignin. The cellulose was dissolved with 72% H_2SO_4 to get the crude lignin. NDF solution was prepared by using ethylenediamine tetra acetic acid-disodium salt (EDTA) and disodium tetra borate-decahydrate, dodecylsulphate-sodium salt, 2-ethoxyethanol, sodium dihydrogenphosphate and heat-stable α-amylase (Rusli et al., 2019). The protocol of NDF was similar to the ADF analysis. The ADF, ADL and NDF (%) were determined as shown in the equation below:

$$(\%) = \frac{(m_3\text{-}m_1\text{-}m_4) - (B_3\text{-}B_1\text{-}B_4) \times 100}{m_2}$$

Where, m_1- weight of the fiber bag (g), m_2- the initial sample weight (g), m_3- the weight of the crucible with dried fiber bag and sample residue after digestion (g), m_4- the weight of the crucible with ash (g), m_5- blank value of empty fiber bag (g). Cellulose was calculated as ADF-ADL, and hemicellulose was calculated as NDF-ADF (Soest, 1963).

5.2.3 *In vitro* rumen fermentation analysis

In vitro assessment of ruminal degradability of OPF pre-treated with enzyme extract from WRF was done by incubating ruminal fluid in 100 mL syringes following the method by Hassim et al. (2010), which derived from Tilley & Terry (1963). The pre-treated OPF samples were oven dried at 60^{o}C and grinded into powder form using a stable arm (SHEN LIAN, SL-1000A, China) grinder. The rumen fluid was collected by stomach tube technique (less invasive method compared to fistula) from Ladang 15, Pusat Pertanian Putra (PPP) Universiti Putra Malaysia. The rumen fluid collected was making sure to contain the solid part (cud) (ventral side of rumen was targeted) where fibrolytic bacteria (*Ruminococcus flavefaciens, Ruminococcus albus, Fibrobacter succinogene, Butyrifibrio fibrisolvens*) located which is essential for plant fiber digestion to be used in this pre-treated OPF digestion analysis (Muizelaar et al., 2020). The rumen fluids were mixed and then filtered through four layers of cheesecloth before being flushed with carbon dioxide (CO_2). Strained rumen fluid (5 mL), 20 mL of 50:50 bicarbonate and phosphate buffer, and 0.25 g of ground-formulated feed (30% pre-treated OPF + 30% concentrate + 40% Napier grass) was added to each syringe. The syringes were incubated at 39^{o}C for 24 hours.

The gas production was recorded every 2 hours until 24 hours (0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24) and compared with blank sample (the rumen fluid without substrate). Initial volatile fatty acid (VFA) was measured by acidifying 10 mL rumen fluid with 2.5 mL metaphosphoric acid (HPO_3) 1:4 ratios with rumen fluids for preservation. After 24 hours of incubation, 10 mL of incubation content was also recovered and acidified with 2.5 mL

metaphosphoric acid (HPO_3) 1:4 ratios with rumen fluids for preservation. The apparent methane gas (CH_4) production was determined by preservation with 5 mL sodium hydroxide (NaOH) before further incubation for another 1 hour. Adding NaOH will raise the pH of the medium and halt the fermentation process. The amount of the methane produced, indicated by the volume in the syringe, was recorded 1 hour after the NaOH was added (Azmi et al., 2022). The pH of the rumen fluid after incubation for 24 h was also recorded. The acidified rumen fluids from 0 hours incubation and 24 hours were centrifuged for 10 minutes at 15,000X g, and the supernatant was recovered for VFA analysis by gas chromatography (Agilent 7890N). Net production of VFA was calculated by subtracting the initial value from the final value. To have a better understanding of the degradability of oil palm fronds, the amount of apparently rumen degradable carbohydrates (ARDC) was calculated as

ARDC (mg) = (Acetate/2 + Propionate/2 + Butyrate) X 162/1000.

With 162 was the estimated molecular weight of 1 mol fermented carbohydrate. Acetate, propionate and butyrate are expressed as net micro molar productions (Rusli et al., 2019).

5.2.4 Statistical analysis

IBM SPSS statistics 27 was used in this study and presented as mean ± SEM (standard error of mean). Analysis of variance (ANOVA) was applied to

compare the significant difference with $p<0.05$, followed by Tukey's test.

5.3 Results

5.3.1 Assessment of pre-treated oil palm frond

The pH, mold growth, aroma, texture, and color of the pre-treated OPF were tabulated in Table 5.1. The pH of the silage is more acidic, which is in the range of 4.22 – 4.33, compared to overnight pre-treatment, which is in the range of 5.90 – 6.08. The pH is similar for all silages group, which are around pH 4. It decreased throughout the fermentation period, causing the pH for silage pre-treatment was recorded 16% lower than overnight pre-treatment in average. No growth of mold was observed on the pre-treated OPF for both silage and overnight pre-treatment. All silages have a fresh aroma and firm texture with light brown color. The similar characteristics of silage are the indication of good silage. The fresh aroma of the silages will increase the animal's palatability (Ofori & Nartey, 2018).

Table 5.1: Pre-treated OPF physical characteristics

Sample	pH	Mold growth	Aroma	Texture	Colour
Silage pre-treatment					
STA	4.23^{ab}	No mold	Fresh	Firm	Light brown
STB	4.22^{a}	No mold	Fresh	Firm	Light brown
STC	4.25^{ab}	No mold	Fresh	Firm	Light brown
STD	4.22^{ab}	No mold	Fresh	Firm	Light brown
CST	4.33^{b}	No mold	Fresh	Firm	Light brown
Overnight pre-treatment					
OTA	5.90^{c}	No mold	Less odor	Rough	Greenish
OTB	5.90^{c}	No mold	Less odor	Rough	Greenish
OTC	5.97^{c}	No mold	Less odor	Rough	Greenish
OTD	5.91^{c}	No mold	Less odor	Rough	Greenish
COT	6.08^{d}	No mold	Less odor	Rough	Greenish
SEM	0.32	-	-	-	-
***P* value**	<0.001	-	-	-	-

Note: Means that do not share a letter are significantly different, where (p<0.05) is considered as significant value. STA: individual enzyme extract from *Phanerina mellea,* STB: individual enzyme extract from *Schizophyllum commune*, STC: selected co-culture (PM + SC) fungi enzyme extract, STD: mixed enzyme extract (combination of PM and SC), CST: control silage (water substitute the enzyme extract), OTA: individual enzyme extract from *Phanerina mellea*, OTB: individual enzyme extract from *Schizophyllum commune*, OTC: selected co-culture (PM + SC) enzyme extract of WRF, OTD: mixed enzyme extract (combination of PM and SC), COT: control (water substitute the enzyme extract) overnight pre-treatment.

5.3.2 Nutritional composition

The nutritional compositions of pre-treated OPF were determined to see the effectiveness of each pre-treatment method. The nutritional composition of OPF pre-treated, namely STA, STB, STC, STD, CST, OTA, OTB, OTC, OTD, and COT with different enzyme extracts is tabulated in Table 5.2. The CP showed 8% higher in silage pre-treatment compared to overnight pre-treatment in overall, with STD showed the highest value of 43.47 g/kg DM compared to the other EE pre-treatment. However, there is no significant difference between STA, STB and STD. The CP content of silage pre-

treatment is significantly higher (p<0.05) compared to overnight pre-treatment and control (Figure 5.2a). The CF content of STB and STD pre-treatment is significantly lower as (p<0.05) compared to overnight pre-treatment and control (Figure 5.2b). The lignin content of silage pre-treated OPF is 14% decreased significantly (p<0.05) than overnight pre-treatment and control, but no significant differences (p>0.05) were observed among the silage pre-treatment (Figure 5.2c). Cellulose content showed 2.77% higher in silage pre-treated OPF compared to overnight pre-treatment as shown in Figure 5.2d. However, there are no significant difference (p>0.05) shown between the treatments. Similarly, the hemicellulose content of silage pre-treated OPF is higher as compared to overnight pre-treatment, however, there is no significant different (p>0.05) shown between the treatments (Figure 5.2e).

Table 5.2: Nutritional composition of OPF pre-treated with different enzyme extract from white rot fungi

Samples	DM (g/kg DM)	Ash (g/kg DM)	CP (g/kg DM)	CF (g/kg DM)	Ether Extract (g/kg DM)	ADF (g/kg DM)	ADL (g/kg DM)	NDF (g/kg DM)	Cellulose (g/kg DM)	Hemicellulose (g/kg DM)
Silage pre-treatment										
STA	531.14^{e}	120.02^{ab}	40.97^{d}	453.73^{abc}	33.68^{a}	388.56^{a}	154.19^{a}	591.18^{a}	234.37^{a}	202.62^{a}
STB	526.32^{d}	98.54^{ab}	42.80^{d}	394.98^{a}	48.65^{j}	378.53^{a}	141.42^{a}	590.56^{a}	237.11^{a}	212.03^{a}
STC	531.13^{e}	94.54^{a}	40.33^{cd}	418.45^{ab}	36.08^{d}	390.01^{a}	157.28^{a}	585.28^{a}	232.73^{a}	195.27^{a}
STD	539.28^{f}	101.76^{ab}	43.27^{d}	397.19^{a}	35.22^{c}	388.09^{a}	150.56^{a}	590.06^{a}	237.53^{a}	201.97^{a}
CST	532.46^{e}	82.13^{ab}	37.20^{bc}	463.33^{abc}	41.32^{e}	391.55^{a}	169.42^{ab}	610.21^{ab}	222.13^{a}	218.66^{a}
Overnight Pre-treatment										
OTA	498.27^{b}	111.93^{ab}	33.57^{a}	482.02^{bcd}	43.52^{f}	433.46^{b}	208.15^{c}	627.12^{ab}	225.31^{a}	193.66^{a}
OTB	498.03^{b}	118.63^{ab}	35.20^{ab}	501.75^{cd}	47.35^{i}	429.20^{b}	200.44^{bc}	627.35^{ab}	228.76^{a}	198.16^{a}
OTC	496.85^{ab}	135.84^{b}	33.80^{a}	522.53^{cd}	44.61^{h}	434.24^{b}	208.08^{c}	624.52^{ab}	226.16^{a}	190.28^{a}
OTD	508.24^{c}	105.26^{ab}	40.37^{cd}	498.37^{cd}	44.35^{g}	432.83^{b}	204.07^{bc}	623.82^{ab}	228.76^{a}	190.99^{a}
COT	496.27^{a}	111.09^{b}	32.13^{a}	550.43^{d}	33.92^{b}	432.62^{b}	209.93^{c}	645.21^{b}	222.69^{a}	212.59^{a}
SEM	5.61	4.77	1.23	16.68	1.80	7.61	8.90	6.63	1.79	3.12
***p*-value**	<0.001	0.009	<0.001	<0.001	<0.001	<0.001	<0.001	0.002	0.066	0.826

Notes: Means that do not share a superscripts letter are significantly different, where (p<0.05) is considered as significant value. DM: Dry matter, CP: Crude protein, CF: Crude fiber, ADF: Acid detergent fiber, ADL: Acid detergent lignin, NDF: Neutral detergent fiber, STA: Silage treatment A (individual fungi *S. commune*), STB: Silage treatment B (individual fungi *P.mellea*), STC: Silage treatment C (co-culture fungi), STD: Silage treatment D (mixed enzyme extract), CST: Control silage treatment, OTA: Overnight treatment A (individual fungi *S. commune*), OTB: Overnight treatment B (individual fungi *P.mellea*), OTC: Overnight treatment C (co-culture fungi), OTD: Overnight treatment D (mixed enzyme extract), COT: Control overnight treatment.

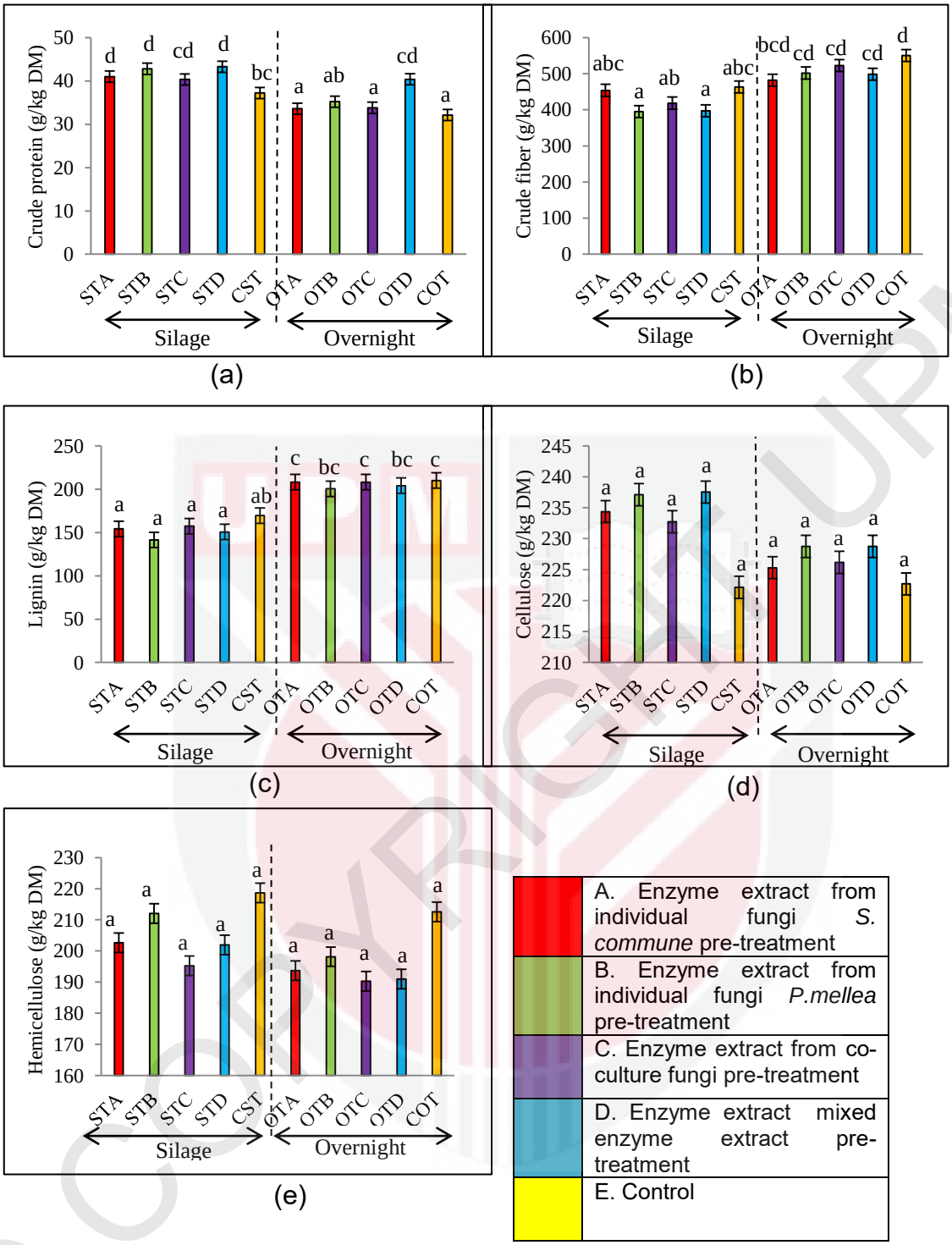


Figure 5.2: Nutritional composition (a) crude protein (CP), (b) crude fiber (CF), (c) lignin (ADL), (d) cellulose, (e) hemicellulose comparisons between different oil palm fronds pre-treatment. Notes: Means that do not share a superscripts letter are significantly different, where (p<0.05) is considered as significant value.

5.3.3 *In-vitro* analysis

The digestibility of animals on the particular feed formulation can be evaluated through *in vitro* gas production. *In vitro* rumen fermentation study always is the alternative method to *in vivo* trial study as it provides an excellent correlation to achieve the standard (Rusli et al., 2019).

5.3.3.1 Total gas production

Figure 5.3 and Table 5.3(C), shows the *in vitro* total gas production (mL/ 250 mg DM) against incubation time (h) of 10 samples throughout 24 h. The total gas production for all samples is increasing steadily with the increment of incubation time for 24 h. Silage pre-treatment of OPF (STA, STB, STC, STD) showed 5% higher total gas production than overnight pre-treatment (OTA, OTB, OTC, OTD), and STB showed the highest gas production with 15.25 mL/250mg DM followed by STD (14.73 mL/250mg DM), STC (12.72 mL/250mg DM), and STA (11.76mL/250mg DM). However, there is no significant difference (p>0.05) of total gas production between silage pre-treatment. All silage pre-treatment showed higher gas production compared to overnight pre-treatment and CST. In overnight pre-treatment of OPF, OTB shows the highest gas production with 12.53 mL/250mg DM, followed by OTC (12.09 mL/250mg DM), OTA (12.00 mL/250mg DM), OTD (11.90 mL/250mg DM), and COT (11.84 mL/250mg DM).

The apparent methane gas production was also determined after 24 h

incubation, as presented in Table 5.3(B). Silage pre-treated OPF produced for about 38% significantly ($p<0.05$) higher apparent methane than overnight pre-treatment with standard rumen fluid at pH7.

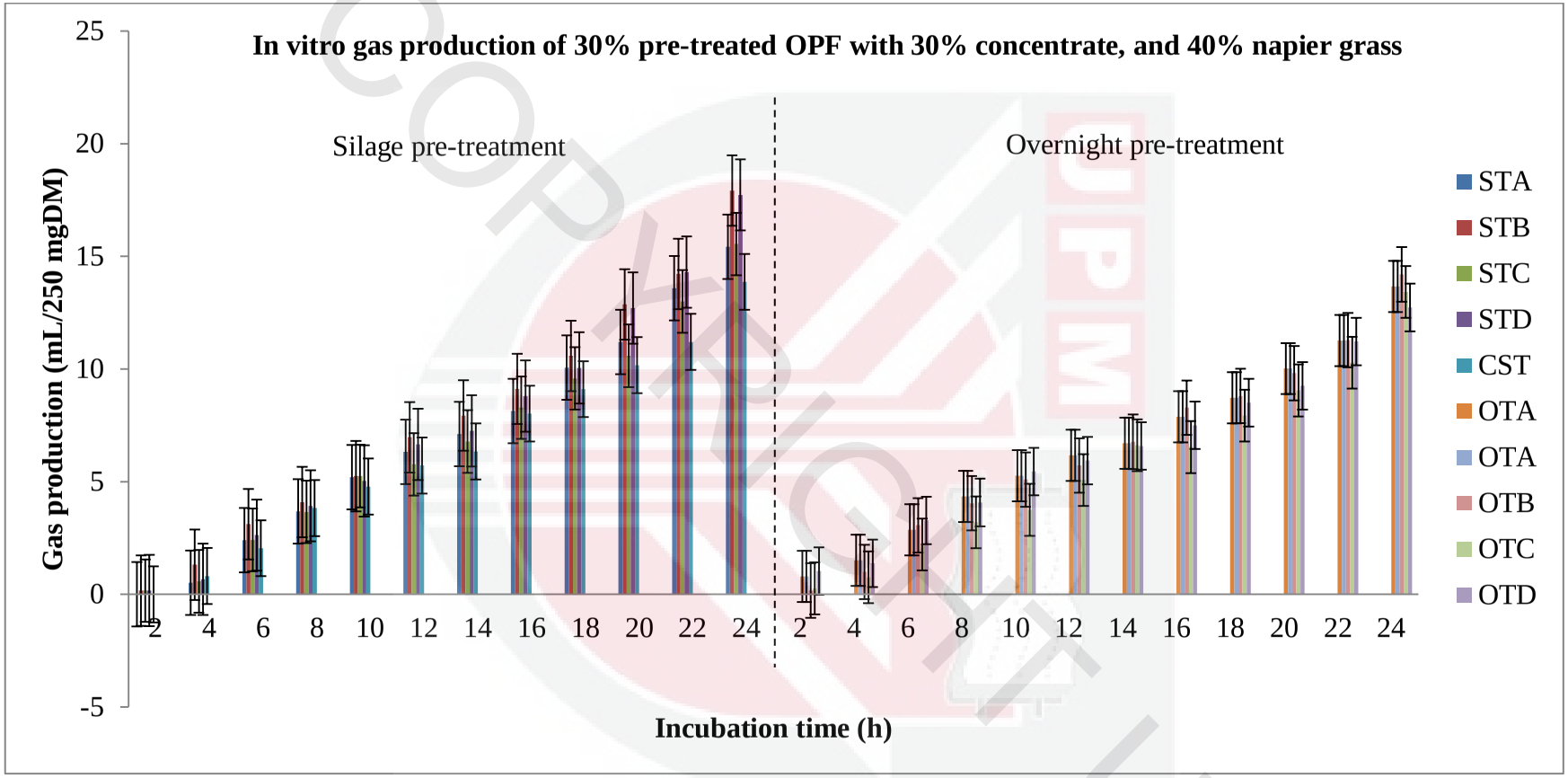


Figure 5.3: *In vitro* total gas production (mL/ 250 mg DM) against incubation time (h) of 10 samples for 24 h. *Notes*: Data shown are the average of triplicates with standard error bars. Notes: Means that do not share a superscripts letter are significantly different, where (p<0.05) is considered as significant value. STA: Silage treatment A (individual fungi *S. commune*), STB: Silage treatment B (individual fungi *P.mellea*), STC: Silage treatment C (co-culture fungi), STD: Silage treatment D (mixed enzyme extract), CST: Control silage treatment, OTA: Overnight treatment A (individual fungi *S. commune*), OTB: Overnight treatment B (individual fungi *P.mellea*), OTC: Overnight treatment C (co-culture fungi), OTD: Overnight treatment D (mixed enzyme extract), COT: Control overnight treatment.

Table 5.3: Gas production, apparent methane and pH of *in vitro* rumen fermentation after 24 h incubation

(SEM = Standard error of mean)

Sample	pH	(A) Total gas production (mL/ 250 mg DM)	(B) CH_4 (mL)	(C) Total gas production – CH_4 (A - B = C)
Silage pre-treatment				
STA	7.12[a]	15.43[abc]	3.67[d]	11.76[ab]
STB	7.10[a]	17.92[c]	2.67[c]	15.25[b]
STC	7.09[a]	15.55[abc]	2.83[c]	12.72[ab]
STD	7.14[a]	17.73[bc]	3.00[cd]	14.73[ab]
CST	7.08[a]	13.87[a]	2.83[c]	11.04[a]
Overnight Pre-treatment				
OTA	7.14[a]	13.67[a]	1.67[b]	12.00[ab]
OTB	7.14[a]	14.20[ab]	1.67[b]	12.53[ab]
OTC	7.09[a]	13.42[a]	1.33[ab]	12.09[ab]
OTD	7.11[a]	12.73[a]	0.83[a]	11.90[ab]
COT	7.13[a]	12.84[a]	1.00[ab]	11.84[ab]
SEM	0.01	0.56	0.29	0.41
***p*-value**	0.903	<0.001	<0.001	0.008

Notes: Means that do not share a superscripts letter are significantly different, where (p<0.05) is considered as significant value. CH_4: Methane gas production, STA: Silage treatment A (individual fungi *S. commune*), STB: Silage treatment B (individual fungi *P.mellea*), STC: Silage treatment C (co-culture fungi), STD: Silage treatment D (mixed enzyme extract), CST: Control silage treatment, OTA: Overnight treatment A (individual fungi *S. commune*), OTB: Overnight treatment B (individual fungi *P.mellea*), OTC: Overnight treatment C (co-culture fungi), OTD: Overnight treatment D (mixed enzyme extract), COT: Control overnight treatment.

5.3.3.2 Volatile fatty acid

The VFA production and ARDC of the pre-treated samples after 24 h of *in vitro* rumen fermentation are tabulated in Table 5.4. The acetate (45.50%) in average showed higher values compared to propionate (9.85%), and the

lowest is butyrate (1.83%) in all samples. All silage pre-treated and overnight pre-treated OPF samples showed 26.34%, 49.24%, 22.82% significantly (p<0.05) higher average acetate, propionate, and butyrate levels compared to the blank sample, respectively. In average, the total cumulative gas production of silage pre-treatment is 1.16% higher than overnight pre-treatment. However, in silage pre-treatment, STC (15.56%) and CST (16.85%) showed lower total cumulative gas production than overnight pre-treatment (OTA (17.51%), OTB (19.73%), and OTC (16.26%)). The ARDC values also increased 45.33% significantly (p<0.05) for all samples compared to blank, and the highest ARDC is in STD (5.20 mg). However, there is no significant difference (p>0.05) of ARDC between STD, with most of the samples except for OTA (4.33 mg) and OTC (4.34 mg). STD also showed the highest production of acetate, propionate, butyrate and total cumulative gas production. STD (49.26%) showed significantly (p<0.05) higher production of acetate compared to OTA (41.62%) and OTC (41.74%). However, there is no significant difference (p>0.05) compared to the other samples, including the control group.

Table 5.4: Volatile fatty acid production and apparent rumen degradable carbohydrate of the pre-treated samples after 24 hours of *in vitro* rumen fermentation.
(SEM = Standard error of mean)

Parameters	Silage pre-treatment					Overnight pre-treatment					Blank	SEM	*p*-value
	STA	STB	STC	STD	CST	OTA	OTB	OTC	OTD	COT			
Total VFA (mmol/L incubation)	20.44^{d}	20.20^{bcd}	15.56^{bc}	20.36^{cd}	16.85^{bcd}	17.51^{bcd}	19.73^{bcd}	16.26^{b}	19.19^{bcd}	18.54^{bcd}	11.03^{a}	0.85	<0.001
Acetate (%)	46.48^{b}	48.77^{b}	45.73^{b}	49.26^{b}	46.30^{b}	41.62^{b}	45.23^{b}	41.74^{b}	43.86^{b}	46.04^{b}	26.53^{a}	1.87	<0.001
Propionate (%)	10.31^{b}	10.67^{b}	9.74^{b}	11.18^{b}	10.11^{b}	8.17^{b}	10.71^{b}	8.21^{b}	9.69^{b}	9.57^{b}	3.35^{a}	0.66	<0.001
Butyrate (%)	2.02^{b}	2.11^{b}	1.87^{ab}	1.88^{ab}	1.88^{ab}	1.84^{ab}	1.58^{ab}	1.82^{ab}	1.91^{ab}	1.34^{ab}	1.15^{a}	0.09	0.006
Others (%)	41.19^{ab}	38.45^{ab}	42.66^{ab}	37.68^{a}	41.72^{ab}	48.37^{b}	42.48^{ab}	48.22^{b}	44.54^{ab}	43.05^{ab}	69.74^{c}	2.65	<0.001
ARDC (mg)	4.93^{bc}	5.16^{bc}	4.78^{bc}	5.20^{c}	4.87^{bc}	4.33^{b}	4.79^{bc}	4.34^{b}	4.65^{bc}	4.72^{bc}	2.61^{a}	0.22	<0.001

Notes: Means that do not share a superscripts letter are significantly different, where ($p< 0.05$) is considered as significant value. Silage treatment A (individual fungi *S. commune*), STB: Silage treatment B (individual fungi *P.mellea*), STC: Silage treatment C (co-culture fungi), STD: Silage treatment D (mixed enzyme extract), CST: Control silage treatment, OTA: Overnight treatment A (individual fungi *S. commune*), OTB: Overnight treatment B (individual fungi *P.mellea*), OTC: Overnight treatment C (co-culture fungi), OTD: Overnight treatment D (mixed enzyme extract), COT: Control overnight treatment.

5.4 Discussion

5.4.1 Pre-treatment of oil palm frond

The two pre-treatment either in silage or overnight method produced a good quality of pre-treated OPF. The physical characteristics of the pre-treated OPF showed an acceptable condition of silage which is in pH 4 with no mold growth. In addition, fresh aroma, firm structure, and pleasant colour of pre-treated OPF silage were also observed. According to Abdullah et al. (2000), good quality silage recorded when the pH around 4, as only lactic acid fermentation and homofermentative bacteria were preserved.

5.4.2 Nutritional composition

The CP content in the silage pre-treatment is 8% significantly higher (p<0.05) than overnight pre-treatment in overall. The CP content in a study by Ebrahimi et al. (2014) also increased 12% in OPF silage with additives (cellulase or lactic acid bacteria (LAB)) compared to the raw OPF. The CP of the OPF silage in the study by Ebrahimi et al. (2014) is also 16% higher compared to the CP in this current study. Many factors influence the difference in CP content of the OPF silage. According to Wahyuni et al. (2012), the OPF used with higher composition of petiole than leaflet will potentially contain more CP, and the plant age (mature OPF has lower CP content due to higher DM and lignin content) also influences the overall nutritional composition of the OPF. The nutritional composition is varies

depends on various factors such as geographical conditions, soil chemistry, fertilizers applied, agronomic practices, and even the age of the OPF depending on the nutrient level uptake in the plant tissues (Ooi et al., 2017). In the current study, the average lignin content of silage pre-treated OPF is 14% lower compared to overnight pre-treatment, concurrently increased 1.4% cellulose content of silage pre-treated OPF. This is because of the NDF of silage pre-treated OPF is 3% lower than overnight pre-treatment. The partial hydrolysis of fiber happened better in silage pre-treatment as compared to overnight pre-treatment caused that to be happened (Wahyuni et al., 2012).

5.4.3 *In vitro* rumen fermentation analysis

The *in vitro* gas production increased with the increasing incubation time within 24 h as the rumen microflora digest the feed supplied (Rahman et al., 2011). According to Astuti et al. (2020), the gas production in *in vitro* rumen fermentation will indicate the substrate digestibility as the gas produced from direct (e.g. CO_2 and CH_4) and indirect substrate fermentation (e.g. organic acids and bicarbonate buffers). It is also reported that the different fungal strains and action mechanisms resulted in different gas production by rumen microbes (Rusli et al., 2019).

The apparent methane gas production is 38% higher in silage pre-treatment when compared to overnight pre-treatment as the anaerobic reaction in silage produced higher acetic acid (CH_3COOH). This acetic acid contributes

to the splits of acetate into methane and CO_2 by methanogenic bacteria. The methanogenic bacteria also used hydrogen as an electron donor and CO_2 as an acceptor to produce methane (Yunqin et al., 2010). The production of methane gas is also influenced by the amount of carbohydrates digested. The dissolution and depolymerization of OPF during silage pre-treatment increase the carbohydrate release and also caused higher methane gas production compared to overnight pre-treatment. Carbohydrate is part of the cellulose makeup in plants, and the high amount of cellulose digestion by rumen microbes will increase methane gas production (Li et al., 2018).

5.4.4 Total gas production

The total cumulative gas production of OPF pre-treated with co-culture of the enzyme (STC, OTC) extract in both silage and overnight pre-treatment showed the lowest values. The co-culture of enzyme extract is low in preserving carbohydrates and did not improve the cellulose and hemicellulose release from OPF. A study reported that there are inhibitory effects on wood degradation by co-culture fungi between *Lenzites betulinus*, *Trametes orientalis*, and *Trametes vetulina,* which the deadlock interaction was generated. It resulted in lower degradation and saccharification of woody biomass compared to mono-culture (Wang et al., 2014).

The STD showed the highest total cumulative gas production, acetate, propionate, butyrate percentages, and average ARDC value. The mixed enzyme extract of PM + SC successfully improved the delignification and

carbohydrate preservation. The pre-treatment of bagasse with the mixed enzyme extract from the fungus *Cladosporium cladosporioides* and commercial xylanase produced synergy when the sugar release was five times higher than the single enzyme pre-treatment (Ji et al., 2014). *Phanerina mellea* and *Schizophyllum commune* combination enzymes produced synergism and improve the delignification of ensiled OPF.

In the current study, the comparison between silage pre-treatment and overnight pre-treatment methods was done. Overall, the an 8% increase in CP content, a 14% decrease in lignin, 2.44% higher cellulose, 5% higher total gas production in *in vitro* study, and 1.16% higher in total cumulative gas production in VFA analysis in silage pre-treatment compared to overnight pre-treatment showed that the silage pre-treatment is a promising method to be used in the enzyme extract pre-treatment of OPF. The ensiling process can be divided into four phases which are aerobic, followed by fermentation, stable, and feed out. The aerobic phase begins after the OPF enters the silo, during which respiration and proteolysis of plant enzymes occur. The plant sugar is degraded into CO_2 and H_2O while plant proteases degrade protein into amino acid and ammonia, and some peptides and amides. The aerobic phase usually happens seven days before the anaerobic microorganisms start to grow and the fermentation phase starts (Borreani et al., 2018). Since the delignification process is an oxidative process in which ligninolytic enzymes use oxygen to exhibit reactions on lignin, the enzyme extract pre-treatment of OPF happened in the aerobic phase of ensiling. The oxidative process of the pre-treatment not only helps

delignification but also helps to reduce the O_2 composition in the silage's aerobic phase before entering the fermentation phase, which demands an anaerobic environment.

5.5 Conclusion

The enzyme extract pre-treatment of silage pre-treated OPF has higher gas production, acetate, propionate, and butyrate percentage and ARDC value than overnight pre-treatment. The silage pre-treatment is a promising method to be used in the pre-treatment of OPF with enzyme extract from WRF. The OPF pre-treatment by STB and STD showed promising pre-treatment. The CF content of STB and STD showed a significantly lower (p<0.05) value compared to overnight pre-treatment of OPF and no significant difference (p>0.05) in lignin content between silage pre-treatment. Even though, STB shows the highest value (15.25 mL/250mg DM) in *in vitro* total gas production, there is no significant difference (p>0.05) in *in vitro* total gas production between silage pre-treatment. The STD showed the highest acetate, propionate, butyrate, and ARDC with no significant difference (p>0.05) between STD, STA, and STB. In the current study, STB and STD were further used in an *in vivo* study.

CHAPTER 6

EFFECTS OF OIL PALM FROND PRE-TREATED WITH ENZYME EXTRACT FROM WHITE ROT FUNGI ON THE DIGESTIBILITY, SERUM BIOCHEMICAL PROFILES, GROWTH PERFORMANCE, AND MEAT QUALITY OF GOATS

6.1 Introduction

The utilization of oil palm frond (OPF) as animal feed has been discussed in many research studies (Rahman et al., 2015; Ebrahimi et al., 2012; Dahlan et al., 2000). Through nutritional composition studies and feed analysis methods, the OPF has been proven to have the potential utility as an animal feed ingredient (Saminathan et al., 2012). Nevertheless, various factors lead to the failure of OPF to meet the ruminant's optimal nutritional requirement. One main factor is the high lignocellulosic content leads to low nutritional value and energy, which results in ruminants with poor growth performance (Rahman et al., 2011). The application of OPF as a whole feedstuff also still discouraged, with only 30% amount of OPF was recommended to be included in total mixed ratio (TMR) (Kum & Zahari, 2011).

The rapidly increasing number of areas under cultivation of oil palm in Malaysia at second hand increased the fibrous by-products that are possibly used in the animal feed industry (Dahlan, 2000). This idea gives a mutual interest to both industries as the oil palm industry may free from pollutants problems, and concomitantly, the farmers can utilize the OPF as the feed source for the animals due to the limited grazing pastures. The low micronutrient composition of OPF to be used in the diet inclusion may be the

highlight of various studies. It may negatively affect the growth performance and production of the animals (Ebrahimi et al., 2013). However, the issue is also diversified to the consequences of consuming meat to human health. Feeding the raw or unprocessed OPF potentially reduced the weight gain of the ruminants due to the low protein content. It also contains >50% of moisture, which may lead to low palatability, undesirable taste of the feed, and finally reduce the DM nutrient intake by ruminants (Dahlan, 2000). Treatment of OPF to modify the structural arrangement of OPF element indirectly improves their nutritional composition are vital to allow more efficient digestion by rumen microbes. Exogenous enzyme treatment is a biological treatment that has become research attention as it provides specific treatment to degrade lignin and increase the cellulose that is used by rumen microbes.

The application of exogenous enzymes in animal feed has been used in various studies (Jacobsen et al., 2018; Thammiah et al., 2017; Taghizadeh & Nobari, 2012); however, the effect on the growth performance of the animal is still under investigation. Meanwhile, pelleting or ensiling was said to improve the digestion characteristics of OPF, however, the study needed to be better documented (Dahlan et al., 2000). The ability to increase the delignification of the feed by exogenous enzymes is tough to predict based on the biochemical characterization alone (Colombatto et al., 2003). Various factors influence the effectiveness of the enzyme treatment of OPF in improving the overall performance of the animals.

The enzyme extracted from *Ceriporiopsis subvermispora* probably gives

better delignification to OPF compared to *Phanerina mellea* as the different fungi produced different enzyme activity. The different effectiveness also could be due to the palatability, feed intake or animal performance during feeding. For example, feeding the treated wheat straw with commercial enzyme increased goat's digestibility and average daily gain (ADG) (Salem et al., 2011). It is a fact that various enzymes are widely commercialized and used for ruminants (Colombatto et al., 2007).

The beef and dairy products contain high saturated fatty acid (SFA) caused by the extensive bio-hydrogenation of polyunsaturated fatty acid (PUFA) that is unhealthy for human consumption. The inclusion of OPF in the ruminant's diet is said to potentially improve the meat quality and human health concurrently due to the enrichment of intramuscular and subcutaneous fat with n-3 PUFA of the meat (Ebrahimi et al., 2013). A diet rich in unsaturated fatty acids is good for human health by reducing the risk of stroke and coronary diseases. The goat's meat is said to be healthier for human when it contains less saturated fatty acid and relatively high unsaturated fatty acid (Ivanović et al., 2016).

For example, the meat quality of goats fed OPF reported by Musnandar et al. (2011) was improved as the rib-eye area was increased, while the protein, lipid, and water content showed no difference compared to goats fed with grass. The pre-treated OPF with fungi (*Lentinussajor- caju*) inclusion gives no significant effect on the meat and carcass characteristics of crossbreed male goats compared to untreated OPF (Chanjula et al., 2017). Therefore,

the current study aims to determine the effect of feeding the enzyme pre-treated OPF silage, which the enzyme extracted from white rot fungi (WRF) (*Phanerina mellea* and *Schizophyllum commun*) on the growth performance, digestibility, serum biochemical profiles, carcass and meat quality of goats by comparison to the feeding inclusion of untreated OPF in total mix ratio (TMR).

6.2 Materials and Methods

6.2.1 Study site and experimental diets

This study was conducted at Animal Research Centre, Institute of Tropical Agriculture and Food Security (ITAFoS) Universiti Putra Malaysia (UPM), Serdang, Malaysia. This study was done for 16 weeks with 14 days for animal adaptation to diet and daily management. All procedures involving the animals were approved by Institutional Animal Care and Use Committee (IACUC) UPM with reference number UPM/IACUC/AUP-R010/2022. Napier grass was freshly harvested from the ITAFoS farm area every day by cutting similar quality (harvested from the same pasture) throughout the experimental period to provide similar nutrient composition. Fresh OPF was collected from palm oil tree plantations area in Pusat Pertanian Putra, Universiti Putra Malaysia (PPP, UPM) which located side by side to the goat's farm. The harvested napier grass and OPF were chopped using chopper machine before fed to animals freshly, while the commercial concentrate were supplied too.

6.2.2 Animals and experimental design

Sixteen healthy male Boer cross-breed goats (*Capra aegagrus hircus*) were used at the age of eight to ten months with an average initial body weight (BW) of 17 ±0.5 to 20 ±0.5 kg which is the age of goats under of good pastoral conditions (first 12 months) were selected (Lu, 2001). All goats were kept in the individual pen in the same housing area to reduce biases from environmental factors and were managed under an intensive farming system. The goats were fed with Napier grass (40%) + concentrate (30%) + OPF (30%) from 4% body weight. The feed formulation was the same but different in OPF pre-treatment. Goats were divided into four groups, namely,

T1 (n=4), treatment, T2 (n=4), T3 (n=4), and control (CON) (n=4) (Figure 6.1). Each group was fed twice daily at 09:00 am and 17:30 pm. The clean water was supplied *ad-libitum*, and each pen's the mineral block was ready. The body weight (BW), average daily gain (ADG), and body condition score (BSC) were determined as the parameters for the growth performance study. An equal number of goats per group were maintained until slaughter.

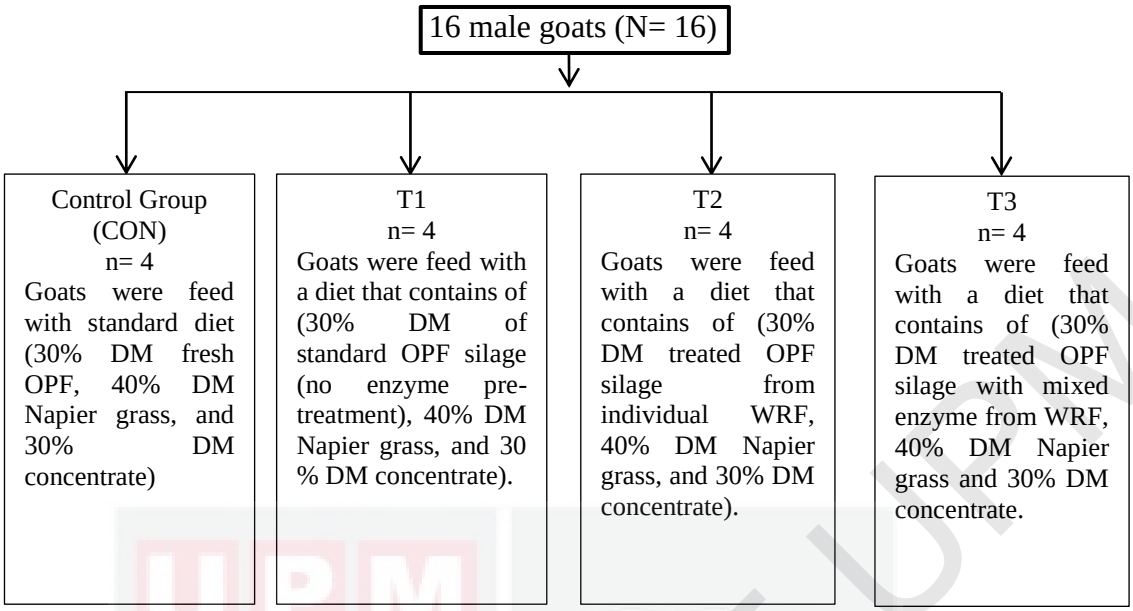


Figure 6.1: Experimental diets of different groups of goats

6.2.3 Sample preparation and analysis

All groups were fed with Napier grass (40 % DM) + concentrate (30 % DM) + OPF (30 % DM). The OPF of treatment 1 (T1) is the OPF silage without enzyme pre-treatment. In contrast, the OPF of treatment 2 (T2) is the OPF silage pre-treated with individual enzymes extracted from *Phanerina mellea* and the OPF of treatment 3 (T3) is the OPF silage pre-treated with mixed enzyme extract of *Phanerina mellea* + *Schizophyllum commune*. The treatment group (T1, T2, and T3) were compared with control group (CON) which offered with the fresh OPF. The OPF was collected from palm oil tree plantations area in Pusat Pertanian Putra, Universiti Putra Malaysia (PPP, UPM) (2.983592895423529, 101.71000476197045).

The fresh OPF was chopped using chopper machine before loading into the silo. The silage pre-treatment was done with the supplementation of 5%/ kg

molasses (Yunus et al., 2000), 3.1 mL/kg enzyme extract from WRF, and water up to 20.4 mL/kg followed (Colombatto et al., 2004) with slight modification. The chopped OPF was packed in a silo before tightly sealed to provide an anaerobic condition. The silages were incubated for 21 days at an ambient temperature. The nutritional composition, dry matter (DM), ash, crude protein (CP), crude fiber (CF), and ether extract (EE) of the total mix ratio (TMR) of the feed given was done before the feeding trial. Fecal samples from each animal were collected monthly for further nutritional composition analysis. The apparent digestibility of the nutritional composition between the ingested TMR and feces were calculated. All analyses were performed according to certified procedures of AOAC 2007. The nutritional composition analysis was performed in 3 replicates.

6.2.4 Measurement of feed intake, body weight, average daily gain and body condition score.

The amount of daily offered feeds and morning refusal per goat were weighted and calculated to measure the daily feed intake. The initial body weight (BW) of the goats was recorded at the beginning of the study by two consecutive weighs in the morning before feeding at 08:00 am. The weight gain of each animal was recorded at 14 days intervals before feeding and reported monthly until at the end of 16 weeks of the study. The average daily gain (ADG) was calculated as the difference between final live weight and initial live weight divided by the number of days of feeding trial (112 days). The body condition score (BCS) was measured according to Ghani et al. (2017), which used 1-5 scale system. Determination of the scoring was made

by several characteristics of the goats as tabulated in Table 6.1.

Table 6.1: Body condition score scale characteristics

Scale	Descriptions
1 (Very thin and emaciated)	Sharp and prominent spinous and transverse processes with no fat cover.
2 (Thin)	Less sharp spinous processes with sharp transverse processes. Less fat cover
3 (Medium or moderately lean)	Smooth and rounded spinous and transverse processes. Some fat cover. Ideal body condition score.
4 (Moderately fat)	Well covered and rounded spinous and transverse processes. Thick muscle
5 (Very fat/ obese)	Undetected spinous processes. Muscle full with thick fat cover

6.2.5 Blood collection and serum biochemical profiles analysis

Blood samples were obtained from the jugular vein about one hour prior to the morning feed once a month, throughout the 16 weeks of feeding trials. Each sample comprised 5 mL and was collected in plain and EDTA Vacutainer tubes. After collection, the samples were placed on ice and centrifuged (SH120-II) within 24 hours at 1000 x g for 20 minutes to separate the plasma and serum. The serum were then stored at -80^{o}C subsequent analysis. Before analysis, the serum samples were completely thawed. The samples were then analysed for biochemical profiles including glucose, cholesterol, total protein, urea, triglyceride, calcium, sodium, potassium, and chlorine, by using Siemens Dimension Xpand Plus blood chemistry analyser

(USA) and compared with the standard normal range of serum biochemical profiles of Boer goats by Bani et al. 2008 (Affan et al., 2019). All measurements were made in one run with triplicate for each sample.

6.2.6 Slaughtering procedure

The goats were transferred from the Animal Research Center, Institute of Tropical Agriculture and Food Security (ITAFoS) to the Faculty of Agriculture slaughterhouse by lorry (Hicom with cage). The goats were restrained before being slaughtered according to Muslim (Halal) standard slaughter procedure MS1500:2009 by certified slaughter using sharp knives and severing the throat and major blood vessels in the neck (Department of Standards Malaysia, 2009). Non-carcass parts of goats such as head, skin, feet, omental, stomach, intestine, mesenteric and genitals were determined as others, while kidney, heart, liver, spleen, and lung were weighed and determined as offal.

6.2.7 Carcass measurements

Within 45 min after slaughtering, the 0 h pH of the dressed carcass was recorded using a portable pH meter (HANNA Hi8314 with an INGOLD type electrode Metrohm) before the hot carcass was weighed and stored in the chiller (4^{o}C). The rumen digesta was collected and stored at -20^{o}C before volatile fatty acid (VFA) analysis. After 24 h, the cold carcass was weighed, and the pH after 24 h was recorded. The rack and loin cuts were separated

between the 12th and 13th ribs. The cross-sectional surface of the *longissimus dorsi* (LD) muscle (rib-eye area) was subsequently measured. The carcass was deboned, and the major muscle, fat and bone were weighed separately to determine the carcass's physical composition.

6.2.8 Meat quality evaluation

The current study used the longissimus dorsi (LD) of the goats for every meat quality evaluation analysis. The LD was collected after the carcass was chilled at 4°C. Meat quality analysis involved were nutritional composition analysis, cooking loss percentage, Warner-Bratzle shear force, water holding capacity percentage, and colour L*, a*, and b* determination. Nutritional composition analysis was conducted according to procedures of AOAC 1990 (Chemists & Cunniff 1990). The nutritional composition analysis was performed in 3 replicates. The cooking loss percentage was assessed by immersing 30 g of LD muscles that were kept in thin-walled polyethylene plastic bag in 80°C water bath until the internal temperature of the meat was at 80°C. Warner-Bratzle shear force was assessed by using TA.HD Plus, stable micro system texture analyser machine by Mason Technology. Cooked LD muscle from cooking loss assessment was carried out and cut (13 mm X 13 mm cross section) before being analysed. Water holding capacity percentage assessment used 20 g of LD muscle and was kept in a polyethylene plastic bag before storing in the chiller at 4°C for seven days. The weight before and after 7 days was recorded. Color L*, a*, and b* were

determined using the ColorFlex ® system (Hunterlab, Reston, VA, USA). The L* value relates to Lightness, the a* value to the Red-Green hue where a positive value relates to the red intensity and the b* value to the Yellow-Blue, where a positive value relates to yellow.

6.2.9 Statistical analysis

Data on feed intake, BW changes, serum biochemical profiles, carcass measurement and meat quality evaluation were analyzed using IBM SPSS statistics 27 and presented as mean ± SEM (standard error of mean). Analysis of variance (ANOVA) was applied with $p<0.05$, followed by Tukey's test.

6.3 Results

6.3.1 Nutritional composition of total mixed ratio and feces of goat

The nutritional composition of the total mixed ratio (TMR) offered to goats in different treatment groups were tabulated in Table 6.2. The nutritional composition of TMR was compared to the nutritional composition of the feces shown in Table 6.3 as to determine the effect of feeding OPF pre-treated silage inclusion on the digestibility of goats. The CP of the TMR of T3 (82.86 g/kg DM) is significantly higher ($p<0.05$) compared to T1 (81.03 g/kg DM) diets which T3 showed the highest mean CP. While, T2 (82.41 g/kg DM) shows no significant difference ($p>0.05$) compared to T3 and CON (81.35

g/kg DM) diets. In general, the mean CP in TMR is 39.2% higher compared to the mean CP in feces for all diets, and in contrast, the mean CF in TMR is 31.3% lower compared to the mean CF in the feces. The mean CF in T3 (319.18 g/kg DM) of TMR is significantly lower (p<0.05) than the other diets. The mean EE in all silage treatment diets of TMR showed 2.18% significantly lower (p<0.05) value compared to the CON diet.

Table 6.2: Nutritional composition of total mixed ratio for goats in different treatment group

Parameters	Diets				SEM	*p*-Value
	CON	T1	T2	T3		
DM (g/kg DM)	601.65^{a}	600.21^{a}	597.88^{a}	598.17^{a}	0.77	0.34
Ash (g/kg DM)	99.10^{a}	100.22^{a}	101.37^{a}	100.66^{a}	0.41	0.94
Crude Protein (g/kg DM)	81.35^{ab}	81.03^{a}	82.41^{bc}	82.86^{c}	0.42	<0.001
Crude Fiber (g/kg DM)	338.67^{b}	338.99^{b}	339.09^{b}	319.18^{a}	4.27	<0.001
Ether Extract (g/kg DM)	44.92^{c}	42.20^{a}	42.97^{ab}	43.82^{b}	0.51	<0.001

Notes: Means that do not share a superscripts letter are significantly different, where (p<0.05) is considered as significant value. CON (30% DM OPF, 40% DM Napier grass and 30% DM concentrate); T1 (30% DM of untreated OPF silage, 40% DM Napier grass and 30% DM concentrate); T2 (30% DM *P. mellea* enzyme extract treated OPF silage, 40% DM Napier grass and 30% DM concentrate); T3 (30% DM mixed enzyme extract treated OPF silage, 40% DM Napier grass and 30% DM concentrate).

Table 6.3: Nutritional composition of feces in different treatment group for over 4 months

Parameters	Diets				SEM	*p*-Value
	CON	T1	T2	T3		
DM (g/kg DM)	487.31^{a}	483.26^{a}	489.28^{a}	488.14^{a}	1.13	0.21
Ash (g/kg DM)	123.98^{a}	126.04^{a}	127.04^{a}	125.66^{a}	0.55	1.00
Crude Protein (g/kg DM)	35.94^{a}	35.59^{a}	35.76^{a}	35.71^{a}	0.06	0.78
Crude Fiber (g/kg DM)	667.97^{a}	667.81^{a}	621.74^{a}	595.40^{a}	15.54	0.19
Ether Extract (g/kg DM)	31.87^{a}	30.30^{a}	25.93^{a}	27.44^{a}	1.17	0.20

Notes: Means that do not share a superscripts letter are significantly different, where (p<0.05) is considered as significant value. CON (30% DM OPF, 40% DM Napier grass and 30% DM concentrate); T1 (30% DM of untreated OPF silage, 40%

DM Napier grass and 30% DM concentrate); T2 (30% DM *P. mellea* enzyme extract treated OPF silage, 40% DM Napier grass and 30% DM concentrate); T3 (30% DM mixed enzyme extract treated OPF silage, 40% DM Napier grass and 30% DM concentrate).

6.3.2 Feed intake

The feed intake and the apparent digestibility of OPF pre-treated silage inclusion in TMR were tabulated in Table 6.4. There is no significant difference (p>0.05) between the feed intake of all diets. However, the DM intake of T3 is 4.21% significantly higher (p<0.05) compared to T2 diets. No significant difference (p>0.05) between the DM intake of T3 with CON and T1 diet and the CON and T1 diet showed no significant difference (p>0.05) with T2 diet as well. The T3 (47.15 g/kg DM) is significantly higher (p<0.05) CP intake compared to the CON (45.40 g/kg DM) and T1 (45.41 g/kg DM) diet. There is no significant difference (p>0.05) between T3 and T2 (46.65 g/kg DM) diet. The CP apparent digestibility of T3 (56.91%) diet is significantly higher (p<0.05) compared to T1 (56.07%), and CON (55.81%), however, no significant different (p>0.05) compared to T2 (56.61%) diet. There is no significant difference (p>0.05) between each group for EE intake, directly resulted in no significant different (p>0.05) of EE apparent digestibility between CON, T1, T2, and T3 diets.

Table 6.4: Total feed intake and apparent digestibility of goats in different treatment diets.

Parameters	Diets				SEM	*p*-Value
	CON	T1	T2	T3		
Feed intake (g)	1132.96[a]	1112.24[a]	1099.10[a]	1175.57[a]	14.48	0.697
DM Intake (g/kg DM)	831.13[ab]	790.31[ab]	780.13[a]	849.13[b]	14.21	0.016
CP Intake (g/kg)	45.40[a]	45.41[a]	46.65[ab]	47.15[b]	0.44	0.009
CP Apparent digestibility (%)	55.81[a]	56.07[ab]	56.61[bc]	56.91[c]	0.25	0.134
EE Intake (g/kg)	15.05[a]	11.90[a]	17.04[a]	16.38[a]	0.99	0.523
EE Apparent digestibility (%)	28.95[a]	28.23[a]	39.62[a]	37.36[a]	2.51	0.271

Notes: Means that do not share a superscripts letter are significantly different, where (p<0.05) is considered as significant value. CON (30% DM OPF, 40% DM Napier grass and 30% DM concentrate); T1 (30% DM of untreated OPF silage, 40% DM Napier grass and 30% DM concentrate); T2 (30% DM *P. mellea* enzyme extract treated OPF silage, 40% DM Napier grass and 30% DM concentrate); T3 (30% DM mixed enzyme extract treated OPF silage, 40% DM Napier grass and 30% DM concentrate).

6.3.3 Growth performance

The growth performance was assessed by determining BW, ADG, and BCS as tabulated in Table 6.5, 6.6, and 6.7, respectively. The first parameter to assess the growth performance is BW. The mean initial BW showed a similar value between the treatments and CON diets, which is between 17 to 20 kg. After 16 weeks of the feeding trial, T3 led higher mean final BW (23.50 kg) followed by CON, T2, and T1 diet. The changes in T3 BW after 16 weeks showed a significant difference (p<0.05) in BW gain with the increment of 4.15 kg compared to T1 (2.45 kg) and CON (2.53 kg) diets. However, there is no significant difference (p>0.05) compared to the T2 diet. While no

significant difference (p>0.05) was detected between the T1, CON, and T2 diets.

The second parameter to assess the growth performance is through ADG assessment (Table 6.6). The ADG was calculated every month for every animal until 16 weeks of the feeding trial. In the first month of the feeding trial, the mean ADG of T3 (37.50 g/d) was the highest compared to CON (22.48 g/d), T1 (22.50 g/d) and T2 (35.00 g/d). However, no significance difference (p>0.05) is shown between diets. The final month showed that T2 (34.52 g/d) was the highest followed by T3 (32.49 g/d), T1 (25.00 g/d), and CON (25.00 g/d) diet. However, no significant difference (p>0.05) can be seen between each diet. The total mean ADG of T3 (35.84 g/d) is significantly higher (p<0.05) compared to CON (21.04 g/d) and T1 (20.42 g/d). However, there is no significant difference (p>0.05) between T3 and T2 diets. The ADG of T2 (33.13 g/d) diet is higher compared to CON and T1 diet, however, there is no significant difference (p>0.05) can be seen between each diet.

The third parameter to assess the animal's growth performance is BCS analysis (Table 6.7). Scale from 1 (very thin) to 5 (very fat) used in the BCS system. At the initial of the study, all goats in control and treatment diets showed BCS <3. The score increased to 25% of BCS ≥3 in the first month for T2 and T3 but not for CON and T1 diet. In the second month, T3 showed 75% of BCS ≥3, while T2 and CON increased to 50% of BCS ≥3 and T1 to 25% of BCS ≥3. The third month showed no increment of BCS ≥3 for all

diets. The final month showed that the CON diet is maintained with 50% of BCS ≥3 together with T1. However, T2 diet showed increased to 75% of BCS ≥3, while T3 diet to 100% of BCS ≥3.

Table 6.5: Body weight changes of goats throughout feeding trial

Parameters	Diets				SEM	*p*-Value
	CON	T1	T2	T3		
Initial BW (kg)	19.60[a]	18.63[a]	17.63[a]	19.18[a]	0.37	0.558
Final BW (kg)	22.13[a]	21.08[a]	21.60[a]	23.33[a]	0.45	0.256
BW gain (kg)	2.53[a]	2.45[a]	3.98[ab]	4.15[b]	0.42	0.008

Notes: Means that do not share a superscripts letter are significantly different, where (p<0.05) is considered as significant value. CON (30% DM OPF, 40% DM Napier grass and 30% DM concentrate); T1 (30% DM of untreated OPF silage, 40% DM Napier grass and 30% DM concentrate); T2 (30% DM *P. mellea* enzyme extract treated OPF silage, 40% DM Napier grass and 30% DM concentrate); T3 (30% DM mixed enzyme extract treated OPF silage, 40% DM Napier grass and 30% DM concentrate).

Table 6.6: Average daily gain of goats for every month throughout feeding trial

Month	Diets				SEM	*p*-Value
	CON	T1	T2	T3		
First month (g/d)	22.48[a]	22.50[a]	35.00[a]	37.50[a]	3.49	0.165
Second month (g/d)	22.50[a]	23.33[a]	35.83[a]	35.83[a]	3.82	0.202
Third month (g/d)	21.67[a]	20.00[a]	37.50[a]	35.83[a]	4.51	0.063
Final month (g/d)	25.00[a]	25.00[a]	34.52[a]	32.49[a]	2.90	0.082
Total ADG (g/d)	21.04[a]	20.41[a]	33.13[ab]	35.84[b]	3.75	0.006

Notes: Means that do not share a superscripts letter are significantly different, where (p<0.05) is considered as significant value. CON (30% DM OPF, 40% DM Napier grass and 30% DM concentrate); T1 (30% DM of untreated OPF silage, 40% DM Napier grass and 30% DM concentrate); T2 (30% DM *P. mellea* enzyme extract treated OPF silage, 40% DM Napier grass and 30% DM concentrate); T3 (30% DM mixed enzyme extract treated OPF silage, 40% DM Napier grass and 30% DM concentrate).

Table 6.7: The condition of body condition score of goats of different diets throughout feeding trial

Feeding duration	Diets							
	CON (n=4)		T1 (n=4)		T2 (n=4)		T3 (n=4)	
	BCS<3 (%)	BCS≥3 (%)	BCS<3 (%)	BCS≥3 (%)	BCS<3 (%)	BCS≥3 (%)	BCS<3 (%)	BCS≥3 (%)
Initial	n = 4 (100%)	n = 0 (0%)	n = 4 (100%)	n = 0 (0%)	n = 4 (100%)	n = 0 (0%)	n = 4 (100%)	n = 0 (0%)
First month	n = 4 (100%)	n = 0 (0%)	n = 4 (100%)	n = 0 (0%)	n = 3 (75%)	n = 1 (25%)	n = 3 (75%)	n = 1 (25%)
Second month	n = 2 (50%)	n = 2 (50%)	n = 3 (75%)	n = 1 (25%)	n = 2 (50%)	n = 2 (50%)	n = 1 (25%)	n = 3 (75%)
Third month	n = 2 (50%)	n = 2 (50%)	n = 3 (75%)	n = 1 (25%)	n = 2 (50%)	n = 2 (50%)	n = 1 (25%)	n = 3 (75%)
Final month	n = 2 (50%)	n = 2 (50%)	n = 2 (50%)	n = 2 (50%)	n = 1 (25%)	n = 3 (75%)	n = 0 (0%)	n = 4 (100%)

Notes: Scale 1: very thin and emaciated, Scale 2: thin, Scale 3: medium or moderately lean, Scale 4: moderately fat, Scale 5: very fat/obese. BCS<3: body condition score 1 and 2. BSC ≥3: body condition score 3, 4, and 5. CON (30% DM OPF, 40% DM Napier grass and 30% DM concentrate); T1 (30% DM of untreated OPF silage, 40% DM Napier grass and 30% DM concentrate); T2 (30% DM *P. mellea* enzyme extract treated OPF silage, 40% DM Napier grass and 30% DM concentrate); T3 (30% DM mixed enzyme extract treated OPF silage, 40% DM Napier grass and 30% DM concentrate).

6.3.4 Serum biochemical profiles

The mean concentration of glucose, cholesterol, total protein, urea, triglyceride, calcium, sodium, potassium, and chlorine of the animals in different diets following 16 weeks of feeding trials were tabulated in Table 6.8. The blood glucose, cholesterol, urea, triglyceride, calcium, sodium, potassium, and chlorine overall mean were in the standard range of Boer goats and similar between each diet which did not show any clinical sign and the goats were in healthy conditions. There is no significance difference (p>0.05) between each group, even though some of the data showed a significance difference (p<0.05) in the early phase of the feeding trial. The

mean blood total protein in the CON diet is significantly higher (p>0.05) compared to the T2 diet. However, no significant difference (p>0.05) compared to T1 and T3. At the same time, there is no significant difference (p>0.05) between treatments group T1, T2, and T3.

Table 6.8: Effect of different diet on serum biochemical profiles for over 16 weeks of feeding trials

Attribute	Diets					SEM	p-Value
	CON	T1	T2	T3	Standard Range		
Glucose (mmol/L)							
0 week	3.95^{b}	2.18^{a}	3.03^{ab}	2.08^{a}	2.70 - 4.20	0.38	<0.001
4 weeks	3.43^{a}	2.83^{a}	2.73^{a}	2.45^{a}	2.70 - 4.20	0.18	0.214
8 weeks	3.40^{a}	2.53^{a}	2.80^{a}	2.23^{a}	2.70 - 4.20	0.22	0.072
12 weeks	2.65^{b}	1.73^{a}	2.18^{ab}	2.08^{ab}	2.70 - 4.20	0.16	0.046
16 weeks	1.23^{a}	1.45^{a}	1.63^{a}	1.70^{a}	2.70 - 4.20	0.09	0.106
Overall mean	2.93^{a}	2.14^{a}	2.47^{a}	2.11^{a}	2.70 - 4.20	0.17	0.230
Cholesterol (mmol/L)							
0 week	4.57^{b}	3.47^{ab}	2.63^{ab}	2.24^{a}	0.9 - 11.7	0.45	0.020
4 weeks	3.40^{a}	2.92^{a}	2.96^{a}	3.23^{a}	0.9 - 11.7	0.10	0.697
8 weeks	3.56^{a}	3.47^{a}	3.35^{a}	2.71^{a}	0.9 - 11.7	0.17	0.395
12 weeks	3.03^{a}	3.09^{a}	3.16^{a}	2.83^{a}	0.9 - 11.7	0.06	0.785
16 weeks	2.76^{a}	2.82^{a}	2.59^{a}	1.91^{a}	0.9 - 11.7	0.18	0.191
Overall mean	3.46^{a}	3.15^{a}	2.94^{a}	2.58^{a}	0.9 - 11.7	0.19	0.068
Total protein (g/L)							
0 week	81.65^{a}	69.90^{ab}	68.20^{a}	74.40^{ab}	35 - 130	2.60	0.027
4 weeks	78.70^{b}	71.60^{ab}	68.65^{a}	76.50^{ab}	35 - 130	1.98	0.034
8 weeks	70.28^{a}	72.55^{a}	63.05^{a}	70.53^{a}	35 - 130	1.80	0.451
12 weeks	63.30^{a}	67.55^{a}	58.43^{a}	67.45^{a}	35 - 130	1.87	0.081
16 weeks	75.58^{a}	63.85^{a}	61.15^{a}	62.60^{a}	35 - 130	2.87	0.094
Overall mean	73.90^{b}	69.09^{ab}	63.90^{a}	70.39^{ab}	35 - 130	1.79	0.030
Urea (mmol/L)							
0 week	6.50^{a}	6.28^{a}	5.85^{a}	6.13^{a}	0.2 - 4.4	0.12	0.854
4 weeks	6.48^{a}	6.33^{a}	6.50^{a}	6.53^{a}	0.2 - 4.4	0.04	0.997
8 weeks	6.15^{a}	6.28^{a}	6.80^{a}	6.90^{a}	0.2 - 4.4	0.16	0.136
12 weeks	4.50^{a}	3.68^{a}	4.18^{a}	4.28^{a}	0.2 - 4.4	0.15	0.451
16 weeks	6.30^{a}	6.60^{a}	6.70^{a}	6.88^{a}	0.2 - 4.4	0.11	0.610
Overall mean	5.99^{a}	5.83^{a}	6.01^{a}	6.14^{a}	0.2 - 4.4	0.05	0.975
Triglyceride (mmol/L)							
0 week	0.28^{a}	0.32^{a}	0.27^{a}	0.24^{a}	0.3 - 11.1	0.01	0.657

4 weeks	0.27[a]	0.36[a]	0.27[a]	0.28[a]	0.3 - 11.1	0.02	0.321
8 weeks	0.25[a]	0.27[a]	0.25[a]	0.27[a]	0.3 - 11.1	0.01	0.635
12 weeks	0.16[a]	0.19[a]	0.16[a]	0.17[a]	0.3 - 11.1	0.01	0.715
16 weeks	0.20[a]	0.20[a]	0.21[a]	0.19[a]	0.3 - 11.1	0.00	0.782
Overall mean	0.23[a]	0.27[a]	0.23[a]	0.23[a]	0.3 - 11.1	0.01	0.665

Attribute	**Diets**					**SEM**	**p-Value**
	CON	T1	T2	T3	Standard Range		
Calcium (mmol/L)							
0 week	2.51[a]	2.33[a]	2.27[a]	2.20[a]	0.2 - 1.1	0.06	0.077
4 weeks	2.49[a]	2.50[a]	2.46[a]	2.47[a]	0.2 - 1.1	0.01	0.940
8 weeks	2.70[a]	2.70[a]	2.82[a]	2.70[a]	0.2 - 1.1	0.03	0.853
12 weeks	1.58[a]	1.41[a]	1.46[a]	1.56[a]	0.2 - 1.1	0.04	0.593
16 weeks	1.65[a]	1.59[a]	1.63[a]	1.67[a]	0.2 - 1.1	0.02	0.299
Overall mean	2.19[a]	2.11[a]	2.13[a]	2.12[a]	0.2 - 1.1	0.02	0.996
Sodium (mmol/L)							
0 week	140.95[a]	143.68[a]	134.10[a]	134.33[a]	142.00-155.00	2.08	0.150
4 weeks	155.90[a]	157.63[a]	143.05[a]	145.48[a]	142.00-155.00	3.17	0.379
8 weeks	147.58[a]	150.50[a]	150.70[a]	146.88[a]	142.00-155.00	0.85	0.480
12 weeks	111.13[a]	97.33[a]	100.18[a]	103.58[a]	142.00-155.00	2.58	0.140
16 weeks	146.78[a]	152.95[a]	148.68[a]	150.23[a]	142.00-155.00	1.13	0.104
Overall mean	140.47[a]	140.42[a]	135.34[a]	136.10[a]	142.00-155.00	1.19	0.965
Potassium (mmol/L)							
0 week	5.10[a]	5.08[a]	4.55[a]	4.93[a]	3.50-6.70	0.11	0.218
4 weeks	5.88[a]	5.75[a]	5.20[a]	5.00[a]	3.50-6.70	0.18	0.121
8 weeks	5.35[a]	5.38[a]	5.65[a]	5.30[a]	3.50-6.70	0.07	0.490
12 weeks	4.13[a]	4.05[a]	4.25[a]	4.00[a]	3.50-6.70	0.05	0.791
16 weeks	4.90[a]	4.95[a]	4.83[a]	4.88[a]	3.50-6.70	0.02	0.950
Overall mean	5.07[a]	5.04[a]	4.90[a]	4.82[a]	3.50-6.70	0.05	0.889
Chlorine (mmol/L)							
0 week	108.28[a]	112.88[a]	104.33[a]	105.43[a]	99.00-100.00	1.65	0.185
4 weeks	120.95[a]	123.23[a]	112.10[a]	114.35[a]	99.00-100.00	2.29	0.465
8 weeks	114.30[a]	118.28[a]	119.55[a]	116.00[a]	99.00-100.00	1.01	0.450
12 weeks	84.40[a]	76.23[a]	76.98[a]	80.80[a]	99.00-100.00	1.63	0.173
16 weeks	116.55[a]	118.18[a]	118.58[a]	118.10[a]	99.00-100.00	0.39	0.800
Overall mean	108.90[a]	109.76[a]	106.31[a]	106.94[a]	99.00-100.00	0.70	<0.001

Notes: Means that do not share a superscripts letter are significantly different, where (p<0.05) is considered as significant value. CON (30% DM OPF, 40% DM Napier grass and 30% DM concentrate); T1 (30% DM of untreated OPF silage, 40% DM Napier grass and 30% DM concentrate); T2 (30% DM *P. mellea* enzyme extract treated OPF silage, 40% DM Napier grass and 30% DM concentrate); T3 (30% DM mixed enzyme extract treated OPF silage, 40% DM Napier grass and 30% DM concentrate).

6.3.5 Volatile fatty acid analysis

The mean pH, volatile fatty acid (VFA), and ARDC value of rumen fluid collected from goats of different diets was tabulated in Table 6.9. The mean pH of rumen fluid is around 5.73 to 5.81 with T3 showed the highest with no significant difference (p>0.05) between each diet. The mean total VFA in T3 (39.44 mmol/L) is significantly higher (p<0.05) compared to T2 (30.12 mmol/L), T1 (22.07 mmol/L), and CON (28.18 mmol/L) diet. The average acetate (46.45%) showed the highest value, followed by propionate (10.52%) and butyrate (3.56%). The acetate content of T3 (51.88%) showed the highest value compared to the other diets. However, there is no significant difference (p>0.05) between each diet. The dietary treatments also did not affect the propionate and butyrate content as there is no significant difference (p>0.05) between each diet. The apparent rumen-degradable carbohydrates (ARDC) value of T3 showed the highest value compared to the other diets, with a value of 5.72 mg. However, each diet has no significant difference (p>0.05).

Table 6.9: Volatile fatty acid (VFA) of rumen fluid collected from goats of different diets.

Parameters	Diets				SEM	*p*-Value
	CON	T1	T2	T3		
pH	5.81[a]	5.77[a]	5.74[a]	5.73[a]	0.02	0.965
Total VFA (mmol/L incubation)	28.18[ab]	22.07[a]	30.12[ab]	39.44[b]	3.12	0.015
Acetate (%)	47.45[a]	41.18[a]	45.30[a]	51.88[a]	1.93	0.104
Propionate (%)	10.40[a]	9.29[a]	10.75[a]	11.22[a]	0.36	0.114
Butyrate (%)	2.72[a]	3.64[a]	4.16[a]	3.72[a]	0.26	0.091
Others (%)	39.44[a]	45.90[a]	39.19[a]	33.19[a]	2.25	0.093
ARDC (mg)	5.13[a]	4.67[a]	5.22[a]	5.72[a]	0.19	0.101

Notes: Means that do not share a superscripts letter are significantly different, where ($p < 0.05$) is considered as significant value. CON (30% DM OPF, 40% DM Napier grass and 30% DM concentrate); T1 (30% DM of untreated OPF silage, 40% DM Napier grass and 30% DM concentrate); T2 (30% DM *P. mellea* enzyme extract treated OPF silage, 40% DM Napier grass and 30 % DM concentrate); T3 (30% DM mixed enzyme extract treated OPF silage, 40% DM Napier grass and 30% DM concentrate).

6.3.6 Carcass characteristics

The mean carcass characteristics of each diet were tabulated in Table 6.10. Dressing out % was calculated by hot carcass to the final BW of the goats. The dressing out % was 42.40 – 44.08%, which has no significant difference (p>0.05) between each diet. The muscle weight % of T2 (26.18%) and T3 (26.38%) are significantly higher (p<0.05) than T1 (23.63), with T3 showed the highest value. However, no significant difference (p>0.05) between T2 and T3 to CON (24.38%) diet. The fat weight % of all treatment diets is greater compared to the CON, diet with T2 and T3 are significantly higher (p<0.05) by 0.72 and 0.89% to the CON diet. No significant difference (p>0.05) between fat weight % in T1 and CON diet. The offal, others, and bone weight % showed no significant difference (p>0.05) between each diet.

The rib eye area of T2 (13.90 cm^2) and T3 (14.85 cm^2) are significantly higher (p<0.05) compared to T1 (10.26 cm^2) diets. However, there is no significant difference (p>0.05) between the rib eye area of T2 and T3 diets to CON (13.68 cm^2). T3 showed the highest value of rib eye area compared to the other diet.

Table 6.10: Carcass characteristic of goats

Parameter	Diets				SEM	*p*-Value
	CON	T1	T2	T3		
Final BW (kg)	22.13[a]	21.08[a]	21.60[a]	23.50[a]	0.45	0.221
Hot carcass (kg)	9.38[a]	9.20[a]	9.35[a]	10.35[a]	0.22	0.071
Cold carcass (kg)	8.98[a]	8.48[a]	8.73[a]	9.60[a]	0.31	0.127
Dressing out (% BW)	42.40[a]	43.67[a]	43.44[a]	44.08[a]	0.21	0.190
Offal weight (% BW)	30.75[a]	31.05[a]	31.32[a]	30.49[a]	0.16	0.958
Others weight (% BW)	22.36[a]	21.27[a]	21.91[a]	21.32[a]	0.22	0.341
Muscle weight (% BW)	24.38[ab]	23.63[a]	26.18[b]	26.38[b]	0.59	0.017
Fat weight (% BW)	2.17[a]	2.52[ab]	2.89[b]	3.06[b]	0.17	0.003
Bone weight (% BW)	9.78[a]	9.39[a]	9.56[a]	9.83[a]	0.09	0.896
Rib eye area (cm^2)	13.68[ab]	10.26[a]	13.90[b]	14.85[b]	0.87	0.013

Notes: Means that do not share a superscripts letter are significantly different, where (p<0.05) is considered as significant value. CON (30% DM OPF, 40% DM Napier grass and 30% DM concentrate); T1 (30% DM of untreated OPF silage, 40% DM Napier grass and 30% DM concentrate); T2 (30% DM *P. mellea* enzyme extract treated OPF silage, 40% DM Napier grass and 30% DM concentrate); T3 (30% DM mixed enzyme extract treated OPF silage, 40% DM Napier grass and 30% DM concentrate).

6.3.7 Meat quality

Characteristics of longissimus dorsi (LD) meat of the goats were tabulated in Table 6.11. The carcass pH was collected from different meat parts of the goats were in the range of 6.53 – 6.72 for 0 h and 5.89 – 6.13 for pH after 24

h are not affected (p>0.05) by dietary treatment. No significant effect (p>0.05) of feeding goats with the inclusion of pre-treated ensiled OPF with enzyme extract to the meat cooking loss %, water holding capacity %, and shear force. The lightness (L) of the treatment diet is 7.36% higher compared to the CON in colour evaluation. However, there are also no significant differences (p>0.05) showed between the colour in the treatment diets and the CON.

The nutritional composition analysis of the meat was tabulated in Table 6.12. The mean DM, ash and EE showed no significant difference (p>0.05), which is in the range of 98.68 – 98.90, 1.05 – 1.19, and 7.88 – 12.94 g/kg DM, respectively. The mean CP content in all treatment diets showed a significantly higher (p<0.05) value compared to the CON diet, with T3 showed the highest value of 13.51 g/kg DM.

Table 6.11: Meat quality characteristics of goat

Parameter	Diets				SEM	*p*-Value
	CON	T1	T2	T3		
Carcass pH (0 h)	6.57[a]	6.72[a]	6.55[a]	6.53[a]	0.04	0.405
Carcass pH (24 h)	6.13[a]	6.10[a]	5.99[a]	5.89[a]	0.05	0.056
Cooking loss %	11.81[a]	16.86[a]	14.40[a]	17.15[a]	1.08	0.390
Water holding capacity %	99.04[a]	100.00[a]	98.33[a]	98.04[a]	0.38	0.621
Shear force (kg/cm^2)	768.43[a]	965.56[a]	974.26[a]	1116.54[a]	61.94	0.673
L*(Lightness)	38.17[a]	44.84[a]	44.09[a]	43.48[a]	1.31	0.095
a* (Redness)	10.69[a]	8.90[a]	8.49[a]	9.80[a]	0.42	0.149
b* (Yellowness)	11.46[a]	12.15[a]	10.89[a]	12.51[a]	0.31	0.453

Notes: Means that do not share a superscripts letter are significantly different, where (p<0.05) is considered as significant value. CON (30% DM OPF, 40% DM Napier grass and 30% DM concentrate); T1 (30% DM of untreated OPF silage, 40%

DM Napier grass and 30% DM concentrate); T2 (30% DM *P. mellea* enzyme extract treated OPF silage, 40% DM Napier grass and 30% DM concentrate); T3 (30% DM mixed enzyme extract treated OPF silage, 40% DM Napier grass and 30% DM concentrate).

Table 6.12: Nutritional composition analysis of goat meat

Parameter	Diets				SEM	*p*-Value
	CON	T1	T2	T3		
DM (g/kg DM)	989.0[b]	986.8[a]	987.1[a]	988.2[ab]	0.44	0.018
Ash (g/kg DM)	10.8[a]	10.5[a]	11.7[a]	11.9[a]	0.29	0.045
Crude Protein (g/kg DM)	111.3[a]	130.9[b]	132.3[b]	135.1[b]	4.71	<0.001
Ether Extract (g/kg DM)	78.8[a]	129.4[a]	83.7[a]	95.5[a]	9.87	0.515

Notes: Means that do not share a superscripts letter are significantly different, where (p<0.05) is considered as significant value. CON (30% DM OPF, 40% DM Napier grass and 30% DM concentrate); T1 (30% DM of untreated OPF silage, 40% DM Napier grass and 30% DM concentrate); T2 (30% DM *P. mellea* enzyme extract treated OPF silage, 40% DM Napier grass and 30% DM concentrate); T3 (30% DM mixed enzyme extract treated OPF silage, 40% DM Napier grass and 30% DM concentrate).

6.4 Discussion

6.4.1 Nutritional composition of total mixed ratio and feces of goat

The nutritional composition of TMR showed that the CP value is significantly higher (p<0.05) in T3 compared to T1 diets, and no significant difference (p>0.05) between T3, T2 and CON diets. The complete degradation of carbohydrates to carbon dioxide (CO_2) and water (H_2O) preserved the N content and increased the proportion of CP in the TMR (Tuyen et al., 2013). This significant difference in CP content also depends on the OPF's status, such as the age at which the mature OPF will possibly contain more petiole parts of the OPF and decreased CP content (Wahyuni et al., 2012). However, the enzyme pre-treatment in T2 and T3 degraded lignin, increasing the holocellulose release and the CP content indirectly due to the overall

energy improvements caused by the decreased of CF content in the TMR. The EE of the T1, T2 and, T3 were significantly lower compared to the CON diet may cause by the effect of delignification and the inconsistencies of sampling or TMR mixing (Wahyuni et al., 2012).

6.4.2 Growth performance

The ensiled OPF with the mixed enzyme extract pre-treatment (T3) showed to have the potential to be used and increase the growth performance of goats as the BW and total ADG showed partial significant difference ($p<0.05$) compared to CON diets. Wahyuni et al. 2012 reported that the goats offered TMR supplemented with the enzyme at 2 g/kg DM showed higher ADG than those offered TMR supplemented with the enzyme at 0, 4, and 6 g/kg DM. However, there is no significant difference ($p>0.05$) between the enzyme-treatment TMR compared to non-treated TMR. In this study, the enzyme from *Aspergillus spp.* BCC 274 used is not applied directly to the silage meanwhile was added to the concentrate portion before being mixed with the OPF silage (Wahyuni et al., 2012). According to Adesogan et al. (2014), the enzyme supplementation improved the ruminal fiber digestion, increasing the digestible energy intake and directly increasing the animal performance. However, the animal performance also depended on animal's physiological status during the experiments eventhough the increase of digestibility occurred (Adesogan et al., 2014).

6.4.3 Volatile fatty acid analysis

The mean total VFA in T3 diet is significantly higher (p<0.05) compared to T1 but not T2 and CON diets. The production of VFA and other gases mirrors microbial growth and their potential to degrade substrate performance (Zhang et al., 2017). The higher the total VFA produced, the better the organic matter (OM) fermentability by rumen microbes (Washaya et al., 2021). The total VFA of goats fed with OPF pre-treated with *Lentinus sajor-ceju* fungi increased significantly compared to non-treated OPF (Hamchara et al., 2018). The acetate: propionate (A: P) ratio between diets is similar, which indicates that the treatment did not increase the fiber intake by goats (Singh et al., 2006). In the current study, the VFA pattern was unaffected by the ensiled OPF treatment diets, and the raw OPF without enzyme pre-treatment is nutritive enough to be included in the goat diet formulation.

6.4.4 Carcass characteristics

The carcass characteristics of goats showed no obvious different between each diet. The muscle % of T2 and T3 diets is significantly different (p<0.05) compared to the T1 but not to the CON diet. The sequence is T3>T2>CON>T1 showing that the OPF silage without the enzyme treatment could reduce the nutrient composition of OPF. In contrast, the enzyme extract pre-treatment from WRF improves the cellulose and hemicellulose release from the OPF and allows the digestibility by rumen microbe, directly avoiding the nutrient reduction composition of the OPF silage. The mixed

enzyme extract further enhances the delignification of OPF by synergism between the crude enzymes.

The T3 diet shows higher muscle % compared to T2, however, no significant difference (p>0.05) between T3 and T2 diets. This result showed that probably the synergism between the crude enzymes does not happen. However, there are also possibilities that the synergism happened but the duration of feeding trial (16 weeks) is not enough or maybe more number of animals (n) needed in this study to be more significant. A study by Masran et al. (2016) reported that the lignin loss of corn cob pre-treated with crude enzyme from a single *P. florida* PF05 is 2.8% which is lower compared to the cocktail enzyme pre-treatment by the combination of *P. florida* PF05, *Pleurotus sajor-caju* PS07, and *Pleurotus eryngii* PE08 with 5.2% lignin loss. The mixture helps to maximize the delignification of a single type of ligninolytic enzyme (Masran et al., 2016). In the current study, the raw OPF offered to the goats are nutritive enough, as no significant difference (p>0.05) recorded between T2, T3 and CON diets. This also showed that the raw OPF can directly include in the animal diets without ensiling or enzyme pre-treatment.

The fat % of the T1, T2 and T3 diets is significantly higher (p<0.05) compared to the fat % in the CON diet. Even though the fat content of the treatment diet is higher compared to the CON diet, it is still in the normal range, which is 2.17 to 3.16% or 450 to 800 g. The fat content reported by Ebrahimi et al. (2013) showed that the goats fed with the inclusion of OPF

range from 630 to 780 g. Chanjula et al. (2017) reported that providing the inclusion of OPF pre-treated with fungal *Lentinussajor-caju* did not have apparent effects on carcass composition and muscle composition in finishing goats.

6.4.5 Meat quality

No adverse effect was identified due to dietary treatment on the meat quality of the goats as the meat pH, cooking loss, water holding capacity, shear force and color analysis showed no significant difference (p>0.05) between each diet. The meat pH in this study is above the range of *rigor mortis* which is between 5.6 and 5.8. This is a good sign as the low pH will influence the meat's tenderness, colour, and water-holding capacity (Chanjula et al., 2017). The colour of the muscle in this study also is not affected (p>0.05) by the dietary treatment and is in good condition as the L*, a*, and b* values are similar to the study reported by previous study (Chanjula et al., 2017; Adeyemi et al., 2015).

The nutritional composition analysis showed that the CP content of T1, T2 and T3 is significantly higher (p<0.05) compared to the CON diet. The higher CP content of the muscle is due to the lesser intramuscular fat composition and higher muscle composition. Usually, the different ages, breeds, sex and maturity of goats will cause a various meat quality and CP composition (Goetsch et al., 2011). In the current study, the goats used were in a similar range of age and initial weight. Age and maturity are not the reason for the

treatment diets to have higher CP than the CON diet. The diets given to a different group of goats is very similar. Overall, this resulted in a similar result between each diet in almost all experiments. However, the T3 diet showed the potential as it shows the highest final body weight, dressing out and muscle weight composition with good meat quality characteristics. T3 diet showed the highest CP content with 13.51 g/kg DM, which is reflected in the muscle weight % composition of a goat carcass. This indicated that the mixed enzyme pre-treatment of OPF can potentially improve the nutritive value of OPF and the carcass performance of the goats.

6.5 Conclusion

The T3 diet resulted in significantly higher BW gain and ADG compared to the CON and the T1 diets. The muscle weight (%), CP contain, and the total VFA of T3 diet showed a significant difference ($p<0.05$) compared to CON and T1 diets. There is no significant difference ($p>0.05$) between T3 and T2 indicated that no synergistic effect happened in the pre-treatment of mixed enzyme extract of *Schizophyllum commune* and *Phanerina mellea*. The analysis of meat and carcass in this study showed that including the OPF pre-treated silage in the TMR potentially improved the carcass performance of the goats and maintained the good meat quality. The T3 and T2 diets both showed potential in the pre-treatment of OPF silage and improve the overall performance of the goats.

CHAPTER 7

GENERAL DISCUSSION

Malaysia is currently the second largest producer and exporter of palm oil after Indonesia. This high volume of production directly generates huge amount of fibrous by-products which makes palm oil industry the predominant contributors in fibrous by-products production. Oil palm frond (OPF) is the most abundant among palm oil industry's fibrous by-products. It is available every day during pruning when harvesting the fruits. The replanting of oil palm trees will generate so much of this OPF waste that is potentially become a pollutants (Hermiati et al., 2013). According to Ooi et al. (2017), the estimation of annual collection rate of OPF for pruning and replanting activities at the average generation rate are 10.4 and 14.4 tons/ha, respectively, which shows an increasing trend every year. Due to their concern towards the environment and the availability of the OPF, researchers came out with the idea to fully utilize the OPF waste and turned into a value added product rather than left unprocessed on the open field.

In Malaysia, the utilization of OPF as a feedstock for ruminants has been taken place so many years ago. OPF is a potential solution to solve the problem of limited grass or other fodder other than hay and rice straw (Ooi et al., 2017). The nutritious of raw OPF for ruminant was proven by Zahari et al. (2003), which investigated that more than 40% degradability value by rumen degradation of OPF was observed. Even though, the OPF is more digestible compared to rice straw or sugarcane leaf hay due to the higher

carboxymethyl cellulase and xylanase activities and high fiber content such as neutral detergent fiber (NDF) (78.7%) and acid detergent fiber (ADF) (55.6%), the OPF still considered as low nutritional diet for ruminant due to its insufficient protein content (Ooi et al., 2017). The high composition of cellulose and hemicellulose of OPF was encapsulated by the lignin which prevents the penetration of rumen microbes. The high lignin and silica composition in OPF reduce the nutritional value of the OPF more when fed to ruminants (Dahlan, 2000). The highly resistant of lignin (an aromatic polymer) to microbial degradation prevent the carbohydrate release from the OPF. The lignin structure is needed to be degraded which predominantly responsible by filamentous fungi (Manavalan et al., 2015).

Filamentous fungi used as the biological pre-treatment of fibrous by-products when the applications managed to minimize the problems in physical and chemical pre-treatment such as high cost of production and not environmentally friendly. The filamentous fungi degrades lignin in the cell wall structure by producing extracellular ligninolytic enzymes (Ibarra et al., 2006). Other than that, the degradation of lignin by fungi also happened with the existence of ligninolytic's accessory enzymes. The colonization action of filamentous fungi in degrading lignin structure of the substrate also helps to increase the efficiency of lignocellulose enzyme. The pre-treatment of fungi allows the colonization of the cell lumina, which are the anatomical pores of the substrate. The non-enzymatic lignin degradation can be carried out by mycelia based on Fenton reaction. The depolymerization of cell wall structure by WRF free radicals cleaved the carbon-carbon linkages (Rouches

et al., 2016). The propagation of fungal mycelia caused cell wall erosion and destroyed the cell wall structure. Yu et al. (2010) scanned the morphological surface of corn straw after fungi pre-treatment by using a scanning electron microscope (SEM). There are some physical changes in the surface where irregular holes can be seen, which increases the surface area and porosity of the corn straw (Yu et al., 2010).

There are several well-known WRF that capable metabolizing lignin efficiently in a variety of fibrous by-products such as *Phanerochaete chrysosporium*, *Ceriporiopsis subvermispora*, *Phlebia subserialis*, and *Pleurotus ostreatus* (Isroi et al., 2011). In the current study, the fungi were cultured from the OPF collected from PPP, UPM before identified and assessed for the enzyme activity. We believe that there are still many other species of fungi that possibly provide better pre-treatment of OPF besides the effectiveness of the fungi pre-treatment is differ due to the particular fungi's nature (Chapter 3). Three species of WRF were identified which are *Marasmius palmivorus, Schizophyllum commune*, and *Phanerina mellea*. Even though the application of *Schizophyllum commune* in the pre-treatment of fibrous by-products is more familiar. However, the identification of *Marasmius palmivorus,* and *Phanerina mellea* extended the list of fungi that potentially improve the effectiveness of OPF pre-treatment. Previously, the fungi strains that applied in the pre-treatment of OPF were discussed in 2.4.1.

The enzyme activity result of identified fungi showed that *Phanerina mellea*

recorded a promising value with the highest average ligninolytic enzyme activity of 0.55 U/ml on day 10 of SSF (Chapter 4). The ligninolytic enzyme activity of *Phanerina mellea* is lower when compared to the other fungi such as *Trichoderma harzianum* or *Fusarium solani* studied by Azmi et al. (2022). It showed that *Trichoderma harzianum* or *Fusarium solani* might provide better pre-treatment of OPF compared to *Phanerina mellea*. Even though the method of assessment between these studies is similar, however, there are many indirect factors such as the OPF nutritional value used, environmental factor or many others, that may unfair to compare between this studies. The co-culture between WRF fungi shows no synergism between fungi combinations. More random selection of fungi needed to be involved in the co-culture of the identified WRF so that higher chance of the co-culture fungi to produce synergism. The RBBR media in this study is not involved MnP enzyme production for decolourization. This RBBR decolourization test is a rapid test and further assessment of the combination should be done to get more complete set of assessment. In the current study, the determination of enzyme activity of the co-culture fungi was done to mirror the RBBR decolourization test. The result showed that no synergism happened in the co-culture of fungi, but the antagonism happened does not affect or reduce the enzyme activity production (Chapter 4).

A good animal diet should result in high *in vitro* gas production, VFA and ARDC value in *in vitro* rumen fermentation analysis. In the current study, all silage pre-treatment showed higher gas production compared to overnight pre-treatment and STB showed the highest gas production, which resulted

from the higher CP content in nutritional composition study (Chapter 5). According to Charmley (2001), the increase amount and CP solubility in silage causing the increase of ruminal ammonia level. That reflects to higher CP content in silage pre-treatment compared to overnight pre-treatment. The chemical composition analysis also showed lower ADL and NDF in silage pre-treatment compared to overnight pre-treatment and STB showed the lowest value followed by STD. According to Chanjula et al. (2017), the cell wall breakdown can be indicated by the lower ADF and NDF and improved the nutritive value. This showed that the enzyme extract of *Phanerina mellea* increased the carbohydrate digestion of OPF which directly increased the methane gas production. There is a quadratic relationship between ADL and ADF content in the OPF, carbohydrate digestion and methane gas production. However, there is no significant difference (p<0.05) between the result of STB and STD diet.

The total VFA production result the highest total VFA gas production in STD followed by STB. However, there is no significant difference (p<0.05) showed between this treatment diets. Similar result also portray in ARDC value. In general, the STB and STD diets showed similar potential in the pre-treatment of OPF and the mixed enzyme extract of *Schizophyllum commune* and *Phanerina mellea* did not showed any synergistic effect when compared to STB.

Increase of ADG in ruminant can be directly explained the improvement of the available nutrient intake by the animal to grow (Salem et al., 2011). In the

current study, there is significant difference (p>0.05) between the total ADG of T3 diet compared to CON and T1 diets, even though, in contrast, there is no significant difference (p<0.05) with T2 (Chapter 6). This result indicated that there is increased in available nutrients to goats for deposition and growth on T3 diet. However, this result also support the result in *in vitro* study that there is no synergism happened between the mixed enzymes extract of *Schizophyllum commune* and *Phanerina mellea* on OPF silage pre-treatment. The significantly lower DM intake value (p<0.05) of T2 diets does not affect the ADG compared to T3 and even reinforce that the mixed enzyme extract of *Schizophyllum commune* and *Phanerina mellea* did not showed any synergistic effect.

The carcass and meat quality result in this study resulted in a similar value between each diet. The T3 diet showed the potential as it shows the highest final body weight, dressing out and muscle weight composition with good meat quality characteristics. T3 diet showed the highest CP content, which is reflected in the muscle weight % composition of a goat carcass (Chapter 6). Although most parameters in carcass characteristics, meat quality, and VFA showed no significant difference (p>0.05) between diets. There are some parameters such as muscle weight %, and CP content of meat showed significant difference (p<0.05) between T3 and CON diets that showed improvement of the enzyme extract pre-treatment on OPF silage. The parameters such as feed intake, BW gain and total ADG of T3 and T2 also showed significant difference (p<0.05) compared to T1 and CON diet reinforce the improvement of the enzyme extract pre-treatment. However, no

significant difference (p<0.05) of carcass characteristics and meat quality assessment between T3 and T2 once again proved that there is no synergism happened between the enzyme extract of *Schizophyllum commune* and *Phanerina mellea*.

CHAPTER 8

SUMMARY, CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH

8.1 Summary

In this current study, due to the high possibility that there are many other candidate fungi that may offer better delignification of OPF due to their natural characteristics, the filamentous fungi that was collected and cultured from the OPF of oil palm tree in PPP, UPM was first identified. After series of morphology, molecular and microscopic observations, the fungi were identified as *Marasmius palmivorus, Mucor fusiformis*, *Penicillium citrinum*, *Trichoderma asperellum*, *Schizophyllum commune*, *Phanerina mellea*, *Aspergillus neoellipticus*, and *Syncephalastrum racemosum*. Three of the fungi were identified as WRF which are *Marasmius palmivorus*, *Schizophyllum commune*, and *Phanerina mellea* (Chapter 3). The completion of the first objective was when the enzyme activity of the identified fungi was successfully determined and *Phanerina mellea* was identified to have the best ligninolytic and cellulolytic + hemicellulolytic combination at 10 days of pre-treatment incubation (Chapter 4).

After that, the study continued with co-culture of selected identified fungi to assess the synergistic effect between their culture combinations. The identified WRF was selected and cultured on the RBBR media to observe the decolourization due to the interaction between WRF. The assessment was continued with the enzyme activity determination of the co-cultured fungi to

assure the antagonist interaction result in the RBBR media observation. The enzyme activity determination was compared with the individual fungi and identified that the individual fungi *Phanerina mellea* still have better ligninolytic and cellulolytic + hemicellulolytic combination followed by the co-culture combination of *Schizophyllum commune* + *Phanerina mellea* (Chapter 4).

The study was continued with the pre-treatment of OPF with the comparison between silage and overnight pre-treatment. The *in vitro* gas production, VFA and ARDC of the pre-treated OPF was assessed and compared. The methane gas production and the chemical composition of the pre-treated OPF were also assessed. The silage pre-treatment showed more promising value of *in vitro* gas production, VFA and ARDC (Chapter 5). The third objective was completed when STB and STD showed the potential to be further used in animal trial.

The study was continued to achieve the 4th objective (Chapter 6) which is to assess the *in vivo* ruminal degradability, serum biochemical profiles and growth performance of goats fed with OPF silage pre-treated with enzyme extracts from WRF. The OPF was pre-treated in silage form and was included for 30% in the diet formulation offered to the goats. The diet was compared with the CON diet which 30% raw OPF was offered. The nutritional composition of the pre-treated OPF was assessed and the feed intake, ADG, BCS and the serum biochemical of the goats were determined.

The final objective of the study was achieved when the carcass and meat

quality of the goats was assessed (Chapter 6). The effect of feeding the OPF pre-treatment on goats was assessed with the carcass characteristics as the overall body compositions was measured such as hot and cold carcass, muscle, fat, bone and rib eye area. The meat quality was assessed based on their carcass pH, cooking loss, water holding capacity, shear force and colour. The nutritional composition and VFA of the rumen fluid collected also examined to see the effect of feeding OPF pre-treatment. Finally, the effect of the OPF pre-treatment by enzyme extracted from selected WRF were identified the potential of enzyme extract from *Phanerina mellea* and mixed enzyme extract of *Phanerina mellea* + *Schizophyllum commune* to be used in the OPF pre-treatment can be further explored as T3 and T2 diets both showed potential in the pre-treatment of OPF silage.

Since there is no significant difference (p>0.05) in overall performance between T3 and T2 diets, both enzyme pre-treatment methods shows its potential for further commercialise especially in OPF silage pre-treatment. Eventually, through this pre-treatment method, the farmers can provide better OPF silage with higher nutritional value compared to the OPF silage without pre-treatment to their animals. The method of the enzyme pre-treatment in silage may also be practiced in other forages to increase their nutritional value. With the optimum delignification by enzyme from WRF that can produced the best combination of high ligninolytic with low cellulolytic and hemecellulolytic enzyme activity, the nutritional value of the silage (other forages) can be improved and directly improved the overall performance of animals.

8.2 General conclusion

Based on the result of this study, it can be concluded that:

- *Marasmius palmivorus, Mucor fusiformis*, *Penicillium citrinum*, *Trichoderma asperellum*, *Schizophyllum commune*, *Phanerina mellea*, *Aspergillus neoellipticus*, and *Syncephalastrum racemosum* enzyme activity were successfully determined with *Phanerina mellea* best showed the enzyme activity production of the desired enzyme. The *Phanerina mellea* has an average ligninolytic enzyme activity value of 0.55 U/mL, and the average cellulolytic + hemicellulolytic is 0.20 U/mL on day 10 of SSF – Objective 1.

- The co-culture of the selected WRF was successfully assessed and there is no synergistic effect was identified between each combination. The enzyme activity of *Phanerina mellea* and co-culture of *Phanerina mellea* + *Schizophyllum commune* showed the most promising to be further used in *in vitro* rumen fermentation analysis– Objective 2.

- The *in vitro* ruminal degradability of OPF pre-treated with enzyme extract from WRF was successfully assessed with the enzyme extract pre-treatment of OPF silage shows higher total gas production, VFA and ARDC value compared to overnight pre-treatment. The STB and STD potentially used in the pre-treatment of OPF in *in vivo* study – Objective 3.

- The *in vivo* ruminal degradability and performance of goats fed with OPF silage pre-treated with enzyme extract from WRF were successfully

assessed with T3 and T2 diets both show the potential in OPF silage pre-treatment and improve the BW, ADG and BCS of the goats – Objective 4.

- The carcass characteristics and meat quality of goats fed with OPF silage pre-treated with enzyme extract from WRF were successfully assessed and showed that the inclusion of pre-treated OPF silage in the TMR improved the carcass characteristics and maintain the meat quality – Objective 5.

8.3 Recommendation

Based on the result of the current study, it is therefore suggested that future studies should focus on:

- To do more optimization on the pre-treatment of OPF based on the specific parameters. The lack of research studies on optimizing OPF pre-treatment generates the infirmity of the information. Besides that, due to the factors that affect the fungi pre-treatment, the nature of the fungi species, and the substrate, the optimization of the pre-treatment of OPF by using different fungi strain has to be enriched to find the best fungi that can pre-treat the OPF. I believe that, many other fungi out there possibly can pre-treat OPF better than *Ceriporiopsis subvermispora*, *Lentinula edodes*, *Ganoderma lucidum*, *Trichoderma harzianum, Fusarium solani* or even *Phanerina mellea*.

- The pre-treatment of OPF by combined method. Kainthola et al. (2021) stated that the capability of the fungi pre-treatment can be maximized with conventional breeding and molecular techniques to improve the delignification and preserve the maximum carbohydrate (Kainthola et al., 2021). Physical pre-treatment, such as socking with buffer (Dhiman et al., 2015) or ultrasonication (Sabarez et al., 2014) before enzymatic pre-treatment will help the penetration of the enzyme by loosening up the substrate structure. The combination will make the processing method become more feasible in controlling the temperature, humidity, or other possible things that may influence the quality of the product, besides help to reduce the cost of production due to less energy and investment required (Mtui, 2009). This method will also help to reduce the loss of carbohydrates which is also the challenge for up-scaling the bio pre-treated product.

- To co-culture and find *Phanerina mellea* possible combination that can produce synergistic effect and enhance the enzyme activity for the pre-treatment of OPF.

- To elongate the study period of feeding trial (more than 16 months) and increase the animal numbers ($n > 4$). The longer feeding trial with more animals will strengthen the data and increase the possibility to produce a more significant comparison between diets (especially between T2 and T3 diets).

- The study on the comparison between all identified fungi that showed best in the pre-treatment of OPF should be evaluated to identify the best fungi in OPF pre-treatment. For example, fungi such as *Lentinula edodes* best showed by Rahman et al. (2011), *Ganoderma lucidum* by Rusli et al. (2019), *Trichoderma harzianum* by Azmi et al. (2019) should be compare with *Phanerina mellea*.

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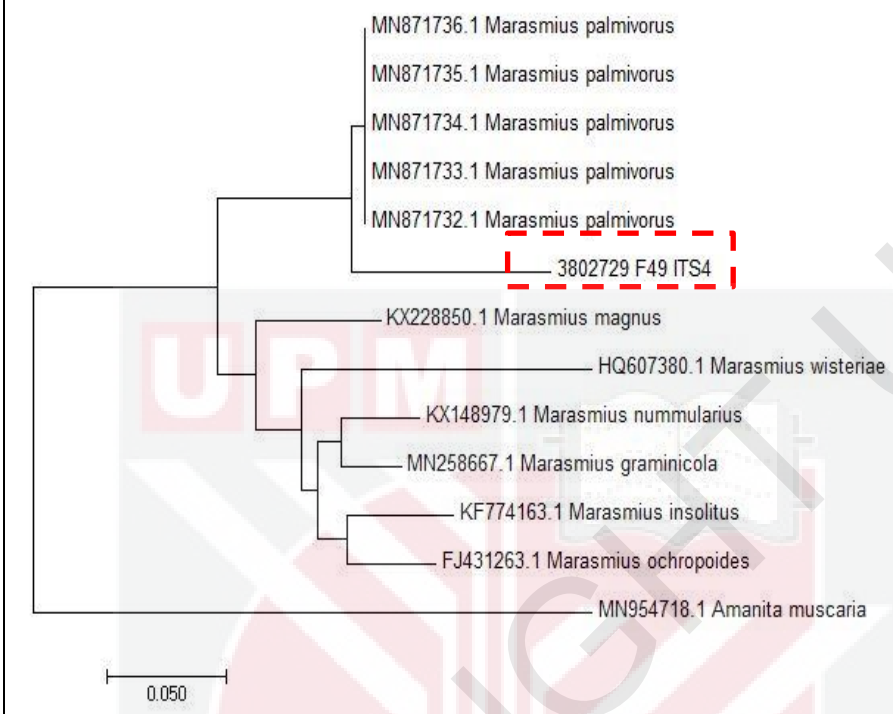
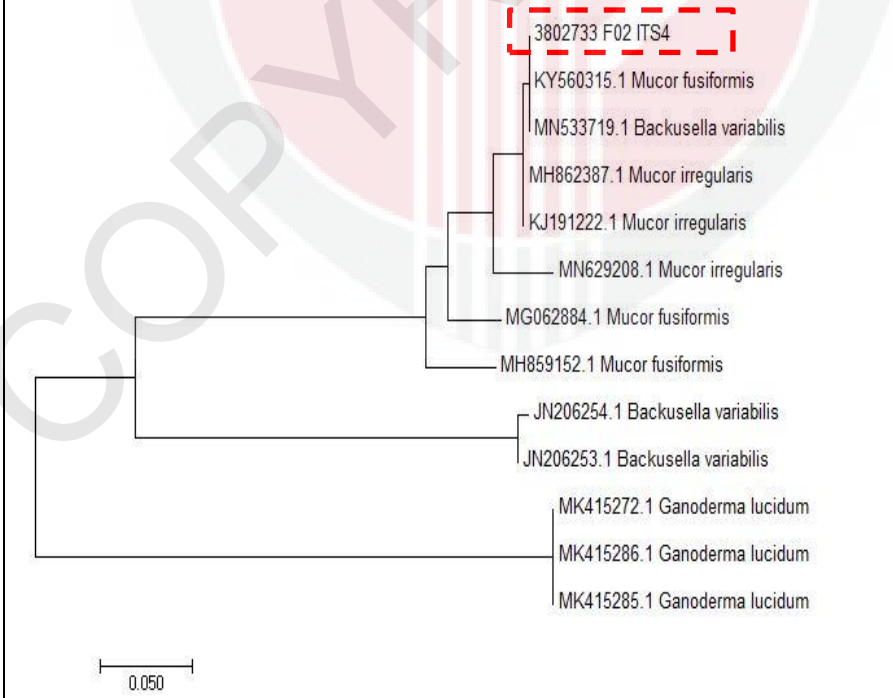
APPENDICES

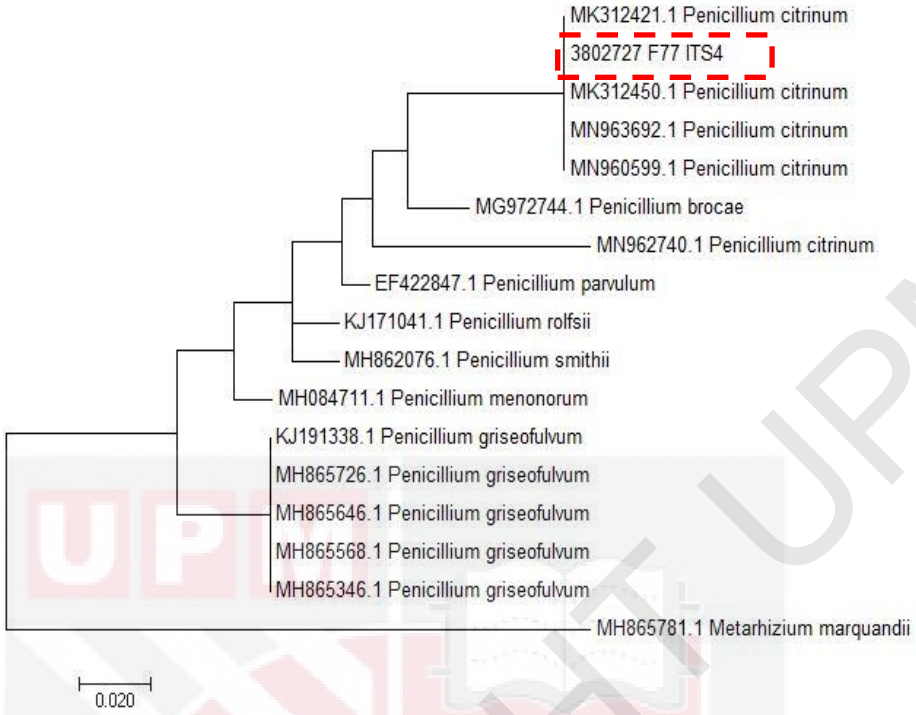
F49



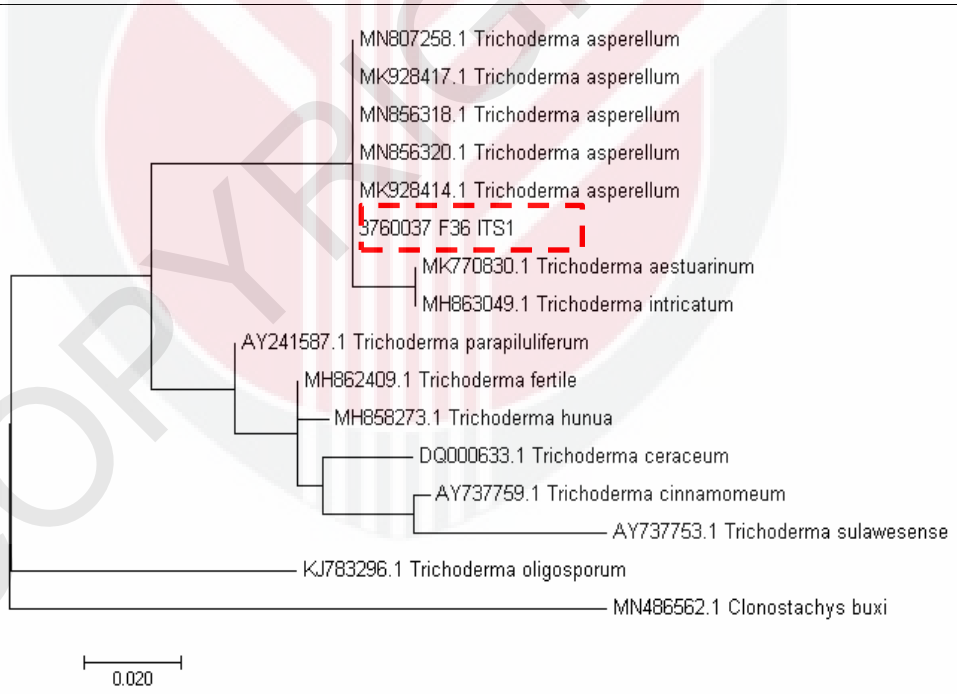
F02

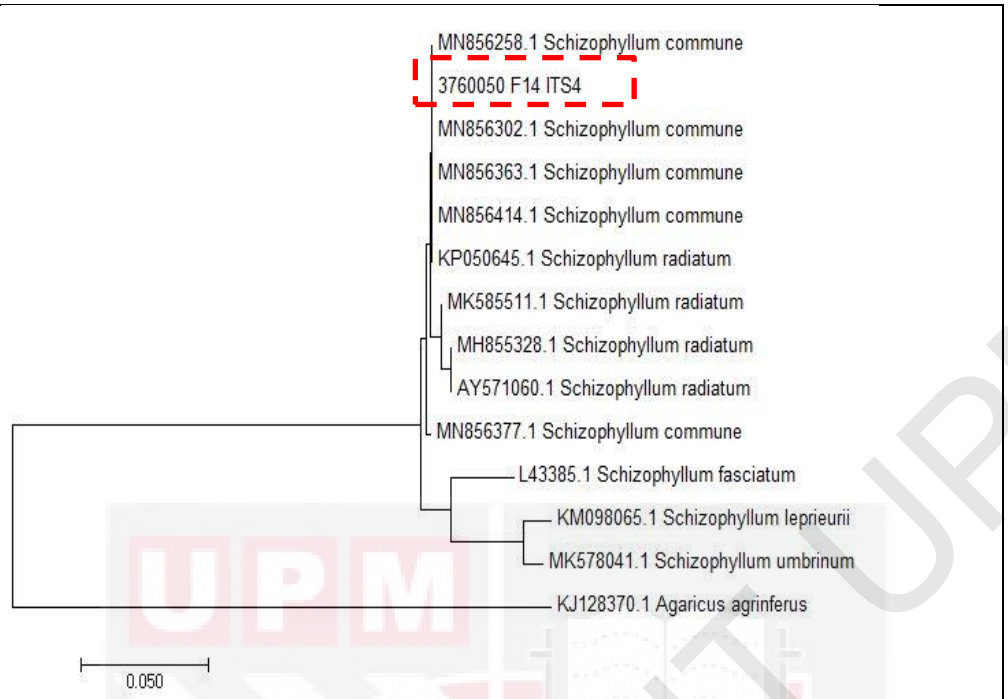
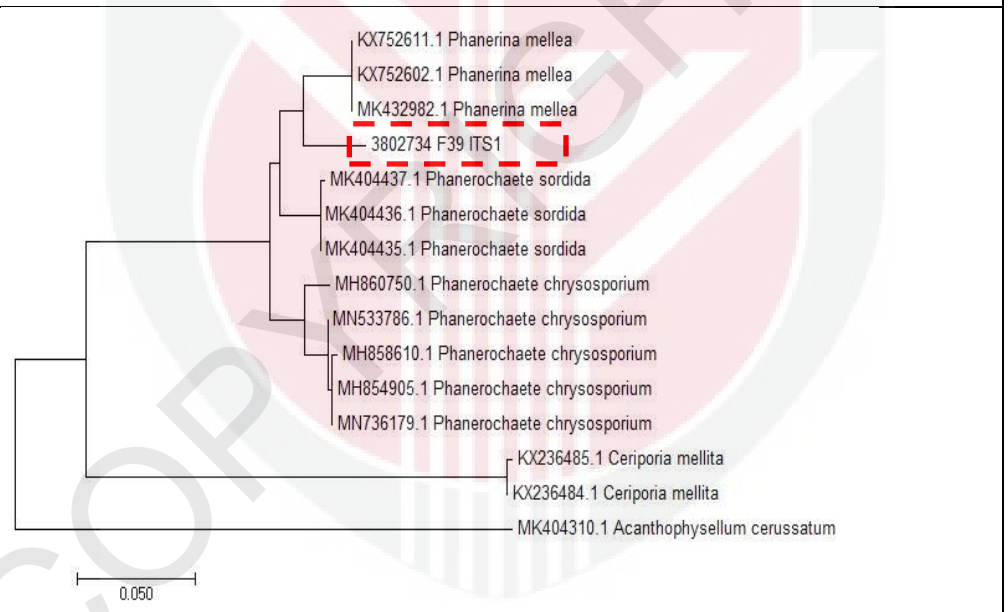


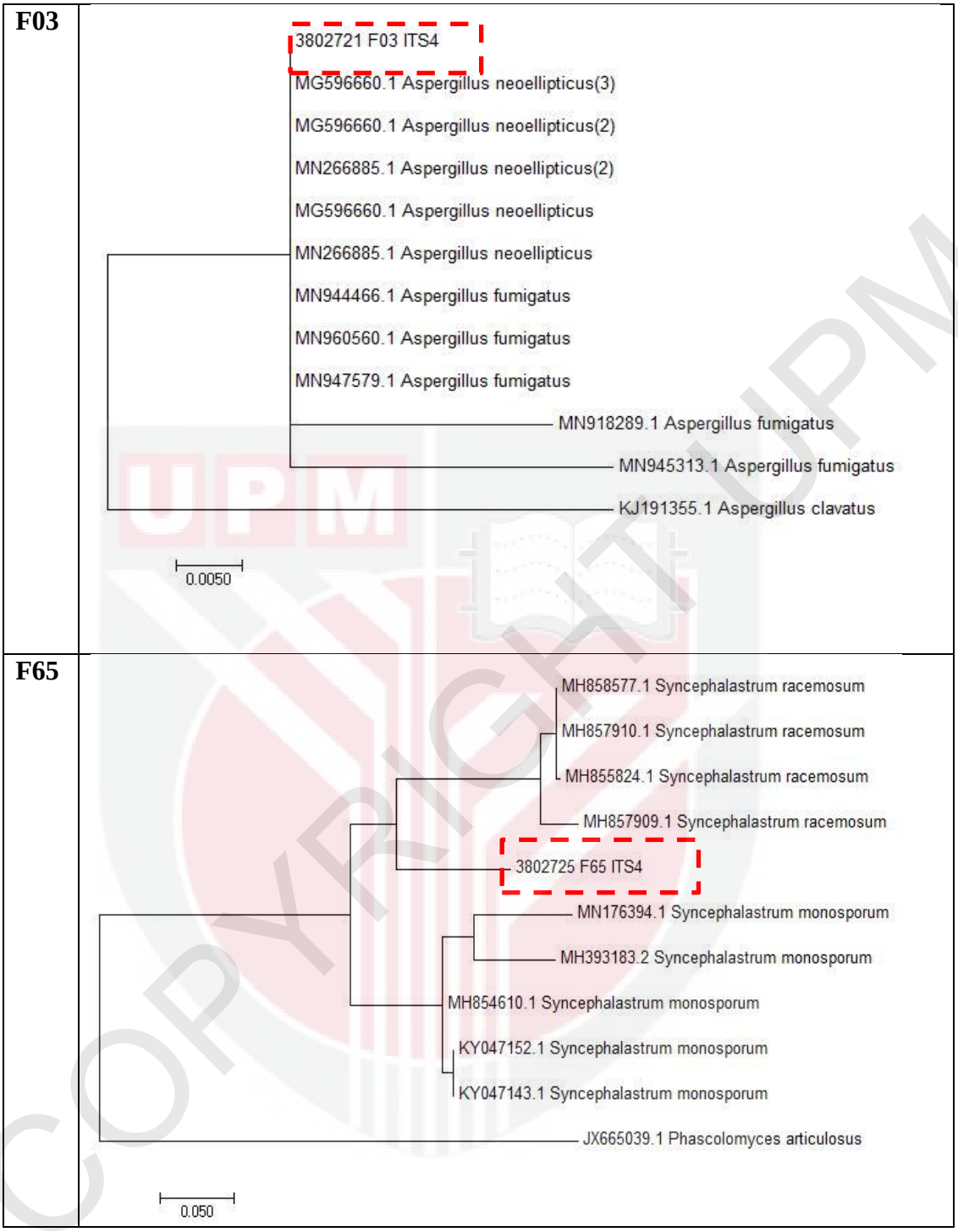
F77



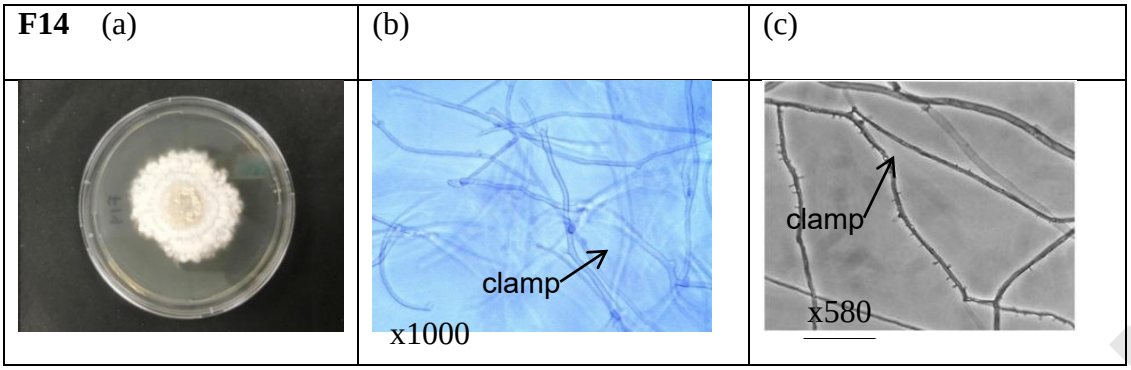
F32



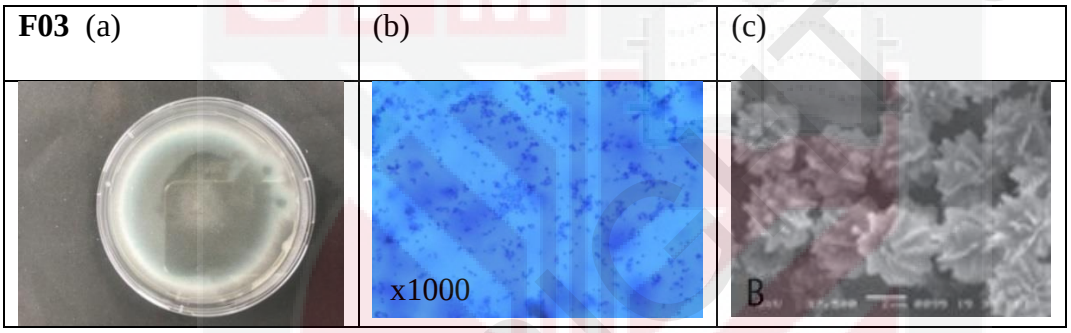
F14	
F39	



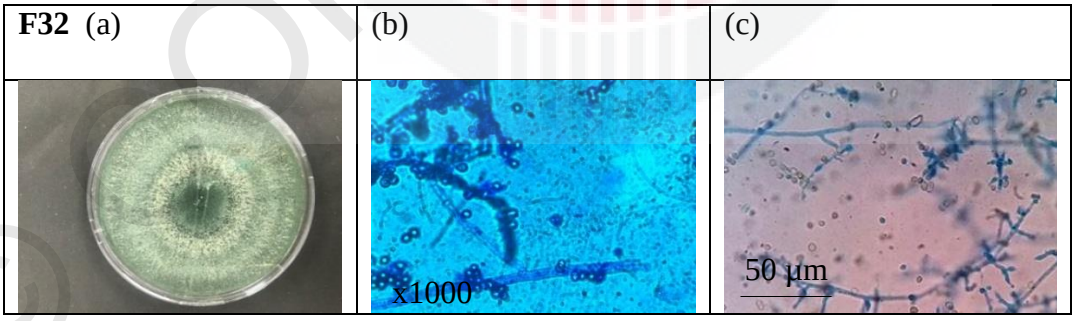
Appendix A1: Phylogenetic tree of isolated fungi from OPF sample.



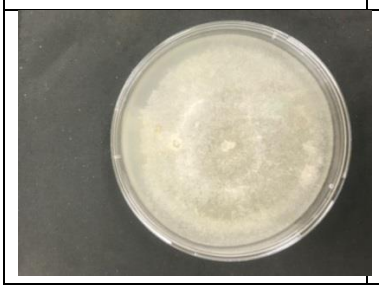
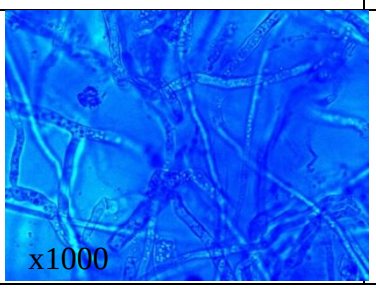
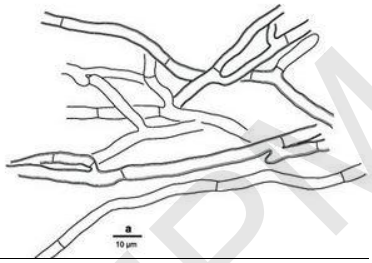
Appendix A2: The microscopic structure of *S. commune* (a) morphology on PDA agar media, (b) The microscopic structure of *S. commune*, (c) The morphology reference of *S. commune* (Sigler et al., 1999).



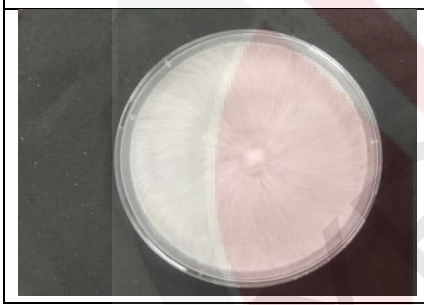
Appendix A3: The microscopic structure of *A. ellipticus* (a) morphology on PDA agar media, (b) The microscopic structure of *A. ellipticus*, (c) SEM image of *A. ellipticus* (Zafar et al., 2017).



Appendix A4: The microscopic structure of *T. asperellum* (a) morphology on PDA agar media, (b) The microscopic structure of *T. asperellum*, (c) The morphology reference of *T. asperellum* (Podder & Ghosh, 2019)

F39 (a)	(b)	(c)
		

Appendix A5: The microscopic structure of *P. mellea* (a) morphology on PDA agar media, (b) The microscopic structure of *P. mellea* (c) The morphology reference of *P. mellea* (Miettinen et al., 2016).

F49 (a)	(b)	(c)
		

Appendix A6: The microscopic structure of *M. palmivorus* (a) morphology on PDA agar media, (b) The microscopic structure of *M. palmivorus*, (c) The morphology reference of *M. palmivorus* (Tamur et al., 2019).

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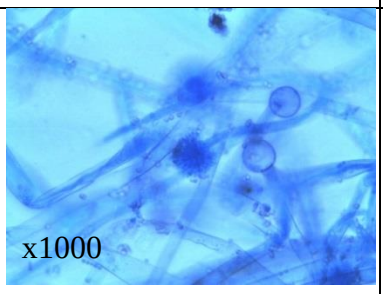
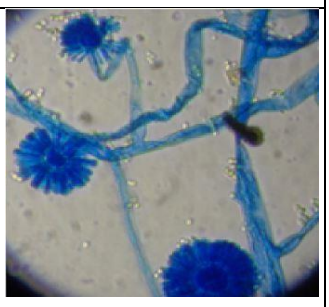
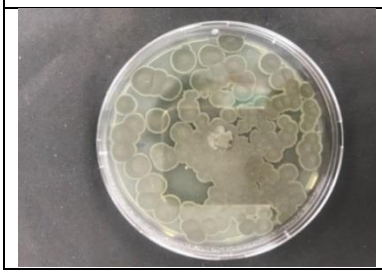
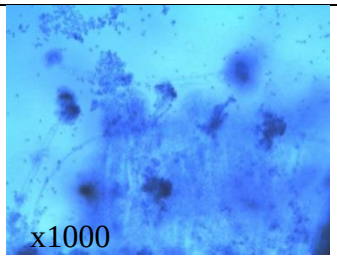
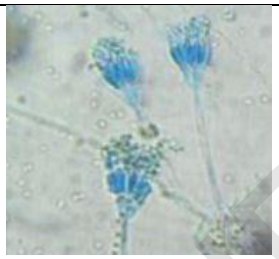
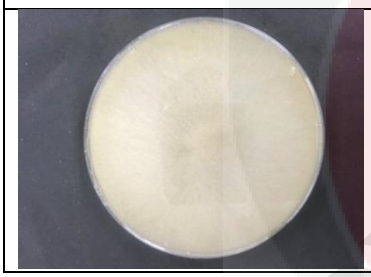
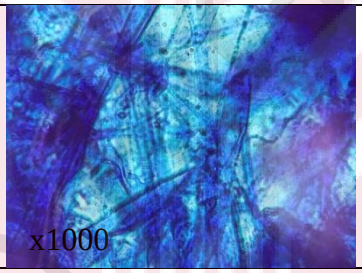
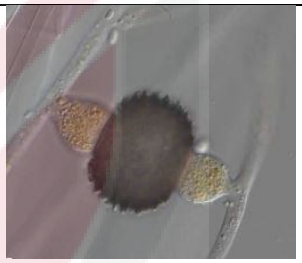
		

Appendix A7: The microscopic structure of *S. racemosum* (a) morphology on PDA agar media, (b) The microscopic structure of *S. racemosum*, (c) The

morphology reference of *S. racemosum* (Raju et al., 2020).

F77 (a)	(b)	(c)
		

Appendix A8: The microscopic structure of *P. citrinum* (a) morphology on PDA agar media, (b) The microscopic structure of *P. citrinum*, (c) The morphology reference of *P. citrinum* (Saif et al., 2020).

F02 (a)	(b)	(c)
		

Appendix A9: The microscopic structure of *M. fusiformis* (a) morphology on PDA agar media, (b) The microscopic structure of *M. Fusiformis*, (c) The morphology reference of *M. fusiformis* (Walther et al., 2013).

Appendix A10: Fungi sampling at TPU, UPM oil palm tree plantation

BIODATA OF STUDENT

Ahmad Fariz Nicholas was born on 8th of July 1989 in Kampung Balung Cocos, Tawau, Sabah. He is the last child of six Nicholas Berman and Norazmah Abdullah children. He completed his bachelor degree in Applied Biology in 2014 from University Technology Mara (UiTM). Upon completion, he continued his master degree in Nanoscience due to inner interest under Prof. Dr. Mohd Zobir Hussein supervision. He eventually got the motivation to pursue PhD in Nutrition as encouraged by his parents and friends under Associate Prof. Dr. Hasliza Abu Hassim supervision.

LIST OF PUBLICATIONS

Nicholas, A. F., Hassim, H. A., Zainudin, M. H. M., Zakaria. Z., Yusof. M. T., Nayan. M. N. M., Azmi. A. F. M. (2024). Isolation and Screening of Indigenous Filamentous Fungi Producing Ligninolytic, Cellulolytic and Hemicellulolytic Enzymes from Decomposed Oil Palm Frond. *Journal of Science and Technology*, Vol. 32 (6).

Nicholas, A. F., Hassim, H. A., Zainudin, M. H. M., Zakaria. Z., Yusof. M. T., Nayan. M. N. M., Azmi. A. F. M. (2025). Co-culture of white rot fungi and the effect of oil palm frond pre-treated with enzyme extract on in vitro rumen fermentation. *Journal of Food Biochemistry*.