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NUTRITIONAL AND RUMINAL DEGRADABILITY OF OIL PALM FRONDS IMPROVEMENT USING ENZYME EXTRACT FROM FILAMENTOUS FUNGI

MOHAMMAD AZRI BIN AZMI

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By

MOHAMMAD AZRI BIN AZMI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirement for The Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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October 2020

Chairman : Hasliza Abu Hassim, PhD
Faculty : Veterinary Medicine

In Malaysia, ruminant production still struggles to fulfill the local demands for human consumption. It is due to high cost which feeding cost constitute to 70% of the total production cost. The industry relies on locally available feed resources such as crop residues and agricultural by-products, apart from supplementation with concentrates made up from mainly imported ingredients. Malaysia is one of the most prominent producers of palm oil and produces approximately 30 million tonnes of oil palm fronds annually as agricultural by-product. However, its effective utilization is hindered by its high lignin content and low protein content. The presence of the lignin creates a barrier which prevent the access of rumen microbe toward the fermentable substrate. Several methods have been explored in removing the lignin content of the agricultural by-product. Biological pretreatment presents as viable option in enhancing the usage of agricultural by-product. Fungi is the best organism to degrade lignin due to its ability to produce lignolytic enzymes. In Objective 1, 11 fungi were isolated from rotten oil palm fronds. Their enzymes activity was determined. In Objective 2, based on enzymes activity in Objective 1, F1, F2 and F4 showed the most conducive enzyme activity profile compared to other fungi. The isolated F1, F2 and F4 identified as Trichoderma harzianum MK027305, Trichoderma harzianum MK027306, and Fusarium solani MK027309, respectively. In Objective 3, the oil palm fronds were pretreated with the enzymes extract extracted from the selected fungi. The enzyme extracts were obtained using 15, 30 and 45 days of solid-state fermentation. The oil palm fronds were then subjected to pretreatment using the enzyme extracts and tested for its in vitro ruminal degradability. From the study, it is found that the oil palm fronds pretreated with enzyme extract from the fungi Trichoderma harzianum MK027305 at 15 days of solid-state fermentation produces the highest total gas production at 13.5 mL of gas produced while highest methane production was observed on oil palm fronds pretreated with enzymes extract from Trichoderma harzianum MK027306 at 30 days of solid-state fermentation at 8.25 mL. The highest fatty acid production was exhibited by oil palm fronds pretreated by Fusarium solani MK027309 at 45 days of fermentation at 58.151 mmol/dl for acetic acid, 22.747 mmol/dl for propionic acid and 65.093 mmol/dl for butyric acid. The pretreatment of oil palm fronds with enzyme extract from Fusarium solani MK027309 at 45 days of solid-state fermentation

showed an increase of 21.67% total volatile fatty acid produced as compared to diet with untreated OPF with increase of 35.29% of apparent rumen degradable carbohydrate for g/g of OPF. In conclusion, the pretreatment using enzyme extract from Fusarium solani MK027309 after 45 days of fermentation via solid state fermentation showed successful result in increasing the ruminal degradability of oil palm fronds by 35.29%.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENAMBAHBAIKAN NILAI NUTRISI DAN KEBOLEHLERAIAN RUMEN PELEPAH KELAPA SAWIT MENGGUNAKAN EKSTRAK ENZIM DARI FUNGI BERFILAMEN

Oleh

MOHAMMAD AZRI BIN AZMI

Oktober 2020

Pengerusi : Hasliza Abu Hassim, PhD
Fakulti` : Perubatan Veterinar

Di Malaysia, pengeluaran ruminan masih belum dapat memenuhi permintaan tempatan. Antara faktor yang menghalang pertumbuhan sektor ternakan ruminan adalah kos pemakanan yang tinggi yang merangkumi 70% dari keseluruhan kos pengeluaran. Industri ruminan bergantung pada bahan tempatan dengan penambahan palet yang diperbuat dari bahan import. Bahan yang biasanya dijadikan makanan ruminan adalah bahan buangan dan sisa dari pemprosesan tanaman. Malaysia merupakan salah sebuah pengeluar minyak sawit terbesar dunia dan menghasilkan 30 juta tan pelepah kelapa sawit setahun. Walau bagaimanapun, penggunaan pelepah kelapa sawit secara efektif dihalang oleh kandungan ligninnya yang tinggi dan kandungan protein yang rendah. Lignin, beserta dengan selulosa dan hemiselulosa membentuk biomas lignoselulosa. Kehadiran biomas tersebut membentuk penghalang yang menghalang akses mikrob rumen. Ia juga mampu membuang enzim dari kawasan reaksi melalui proses penyerapan. Beberapa kaedah telah digunakan bagi menghapuskan kandungan lignin dalam sisa pertanian. Kaedah prarawatan biologi merupakan satu cara yang paling sesuai bagi tujuan membuang kandungan lignin. Fungi adalah organisma yang terbaik bagi membuang kandungan lignin disebabkan kebolehan menghasilkan enzim lignolitik. Kajian terdahulu menunjukkan pengurangan kos sebanyak 28% apabila menggunakan sisa pertanian yang melalui proses prarawatan. Dalam Objektif 1, 11 fungi telah berjaya diisolasi dari pelepah kelapa sawit reput. Seterusnya, aktiviti enzim fungi tersebut ditentukan. Dalam Objektif 2, berdasarkan kadar aktiviti enzim dalam Objektif 1, fungi F1, F2 dan F4 didapati mempunyai kadar aktiviti enzim yg kondusif bagi tujuan prarawatan untuk membuang kandungan lignin dari biomas lignoselulosa kerana fungi tersebut menunjukkan aktiviti enzim lignolitik yang tinggi dan aktiviti enzim selulolitik dan enzim hemiselulolitik yang rendah. Seterusnya, spesis fungi yang dipilih tersebut dikenal pasti. Fungi yg dipilih terdiri daripada F1 (*Trichoderma harzianum* MK027305), F2 (*Trichoderma harzianum* MK027306) dan F4 (*Fusarium solani* MK027309*)*. Dalam Objektif 3, pelepah kelapa sawit melalui proses prarawatan dengan ekstrak enzim dari fungi yang dipilih. Pelepah yang telah menjalani proses prarawatan kemudiannya diuji untuk kebolehleraian rumen secara *in vitro*. Dari eksperimen tersebut, pelepah kelapa

sawit yang mengalami proses prarawatan oleh ekstrak enzim dari *Trichoderma harzianum* MK027305 yang melalui proses penapaian pepejal selama 15 hari merekodkan gas tertinggi dengan jumlah gas terhasil sebanyak 13.5 mL. Pengeluaran gas metana tertinggi direkodkan oleh pelepah kelapa sawit yang menjalani proses prarawatan oleh ekstrak enzim dari *Trichoderma harzianum* MK027306 yang melalui proses penapaian pepejal selama 30 hari dengan pengeluaran gas sebanyak 8.25 mL. Asid lemak tertinggi direkodkan oleh pelepah kelapa sawit yang melalui proses prarawatan dengan ekstrak enzim dari *Fusarium solani* MK027309 menjalani proses penapaian pepejal selama 45 hari yang merekodkan kandungan asid asetik sebanyak 58.151 mmol/dl, kandungan asid propionik sebanyak 22.747 mmol/dl dan asid butirik sebanyak 65.093 mmol/dl. Proses prawatan menggunakan ekstrak enzim dari *Fusarium solani* MK027309 yang menjalani proses penapaian pepejal selama 45 hari manunjukkan peningkatan asid lemak meruap sebanyak 21.67% dan karbohidrat mudah hadam sebanyak 35.29%. Kesimpulannya, pengunaan ekstrak enzim dari *Fusarium solani* MK027309 yang selepas penapaian pepejal selama 45 hari untuk prarawatan pelepah kelapa sawit menunjukkan kejayaan dalam meningkatkan kebolehleraian rumen sebanyak 35.29%.

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APPROVAL

I certify that a Thesis Examination Committee has met on 19th October 2020 to conduct the final examination of Mohammad Azri bin Azmi on his thesis entitles **"NUTRITIONAL AND RUMINAL DEGRADABILITY OF OIL PALM FRONDS IMPROVEMENT USING ENZYME EXTRACT FROM FILAMENTOUS FUNGI"** in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The committee recommends that the student be awarded the degree in Master of Science.

Members of the Thesis Examination Committee were as follows:

Mohd Hezmee bin Mohd Noor, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Goh Yong Meng, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Abdul Munir bin Abdul Murad, PhD
Associate Professor
Department of Biological Sciences and Biotechnology
Universiti Kebangsaan Malaysia
(External Examiner)

_____

NOR AINI AB. SHUKOR, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Hasliza binti Abu Hassim, PhD
Associate Professor
Department of Veterinary Preclinical Sciences
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Mohd Termizi bin Yusof, PhD
Associate Professor
Department Microbiology
Faculty of Biotechnology
Universiti Putra Malaysia
(Member)

Zunita binti Zakaria, PhD
Professor
Department of Veterinary Pathology & Microbiology
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

_____

ZALILAH MOHD SHARIFF, PhD
Professor and Dean
School of Graduate Studies
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Signature: _____
Name of Member of Supervisory Committee: Prof. Dr. Zunita Zakaria

Signature: _____
Name of Member of Supervisory Committee: Assoc. Prof. Dr. Mohd Termizi Yusof

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LIST OF ABBREVIATIONS

°C	Degree Celsius
%	Percentage
±	Plus-Minus
ANOVA	Analysis of Variance
cm	Centimeters
DNA	Deoxyribonucleic acid
OPF	Oil Palm Fronds
GC	Gas chromatography
h	Hour
GC	Gas chromatography
M	Mole
mins	minutes
DNS	Dinitrosalicylic acid
ITS	Internal Transcribed Spacer
RNA	Ribonucleic Acid
w/v	Weight per volume
w/w	Weight per weight
GDP	Gross Domestic Product

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CHAPTER 1

INTRODUCTION

1.1 Background

Malaysia's meat production shows an increase of between 3-5% annually which is lower than increase of annual consumption (Nor et al., 2018). This increase in annual consumption is driven by rapid growth in economy. Indeed, this growing livestock industry leads to high demands for more animal feed. Despite the increase, the ruminant industry in Malaysia is still far from reaching self-sufficiency level with reported self-sufficiency level of 25.67% in 2013 (Ariff et al., 2015). This is due to lagging production increase as compared to increase in demand. One of the contributing reasons for this limitation is the high feeding cost to profitability ratio by the farmers, making the importation of meat to be more favourable as compared to local production (Ibragimov et al., 2016). In 2007, the government imported 140,402 metric tonnes of beef and beef-based products.

Feeding cost constitute a large portion of cost in livestock production industry which amounts to 60-70% of the total cost. Local ruminant industry depends on locally available feedstuffs with some supplementation provided by imported ingredients. Major local substance used are crop residue and agro-industrial by products. These include rice bran, copra cake, palm kernel cake (PKC), oil palm fronds (OPF), sago, tapioca and broken rice (Loh, 2002).

Malaysia is recognised as the second largest producer of palm oil. In the year 2008, Malaysia recorded production of 17.7 million tonnes of palm oil. This large production stems from large area used for plantation which was recorded at 4.5 million hectares (Abdullah and Sulaiman, 2013). These large-scale plantations produce large amount of oil palm by-product. One of the main by-products from oil palm plantation is the OPF which are obtained from pruning, felling and harvesting processes. Approximately, 30 million tonnes of OPF are produced yearly (Wan Zahari and Farid, 2011)

Although the OPF have been widely used in livestock production as animal feed, its effective usage is still limited due to its high lignin content and low protein content. The lignin content, along with cellulose and hemicellulose forms lignocellulosic biomass (Zhang and Zhao, 2010). The lignocellulosic biomass plays an important role in imparting strength to the plant cell wall and protects them from enzymatic degradation. The structural, physiochemical and compositional factor of the lignocellulosic biomass limit the degradability of cellulose and hemicellulose present in the OPF by rumen microbes (Binod et al., 2012). Indeed, this cellulose and hemicellulose made up the main fermentation substrate for microbial which will then be converted into volatile fatty acid. The volatile fatty acids constitute 70% of the ruminant's energy supply. The main volatile fatty acids produced from rumen fermentation are acetic acid, butyric acid and propionic acid (France and Dijkstra, 2005). Due to this limitation, the utilisation of OPF

solely as source of roughage for livestock is limited (Wanrosli et al., 2007). Thus, this prompts for research to be made to enhance the ruminal degradability of OPF.

Previous research has explored the ways to remove the lignin content of agricultural by-product such as rice and wheat straw and sugarcane bagasse via pretreatment. These pretreatment methods include physical, chemical, physicochemical and biological pretreatment. Physical pretreatment includes grinding and milling, microwave and extrusion. Chemical pretreatment includes alkali treatment acid treatment, organic solvent, ozonolysis and ionic liquid. The physicochemical pretreatment includes steam explosion, wet oxidation and carbon dioxide explosion. The biological pretreatment is the pretreatment method in which enzymatic degradation was utilised instead. These pretreatment methods aim to disrupt hydrogen bond in crystalline cellulose, break down cross-linked matrix of lignin and hemicellulose, and raising porosity and surface area of cellulose for subsequent enzymatic hydrolysis in agricultural by-product. Pretreatment is generally viewed as least technologically mature method apart from being expensive (Laser et al., 2002). The expensiveness of the pretreatment method especially chemical, physical and physicochemical pretreatment is contributed by their use of steam and chemical products.

Apart from that, the need for corrosion resistant reactor further adds cost to these pretreatment methods (Rabelo et al., 2009). These pretreatment methods produce compounds which may interfere with subsequent fermentation process. Thus, biological pretreatment has been chosen as a safe and cheap method in removing the lignin content of agricultural by-products. Apart from that, biological pretreatment also requires less energy, modest environmental conditions and have no chemical requirement. Pretreatment of wheat straw by using colonization of white rot fungi shows degradation of lignin up to 18.5% (Dinis et al., 2009). However, colonization of fungi results in large amount of biomass loss (Rahman et al., 2011). This may be avoided by only using enzyme extract from the fungi. Previous research shows pretreatment using white rot fungi saw an increase of 12% of OPF ruminal degradability (Hassim et al., 2012). Apart from that, previous study has shown that the feeding cost was reduced by 28% when 40% of concentrated feed mixture was replaced with biologically pretreated sugarcane bagasse (Abdel-Aziz, 2002).

1.2 Problem statement

Lignin bound with cellulose and hemicellulose have been a culprit in preventing effective use of OPF as it may interfere with the ruminal fermentation due to limited access of microbes to fermentable carbohydrate such as cellulose and hemicellulose. Biological pretreatment has shown great potential over their chemical, physical and physicochemical counterparts in breaking down these lignin-hemicellulose bonds. Furthermore, these pretreatments also bring along several issues such as higher cost and usage of chemical that may hamper subsequent fermentation. Biological pretreatment using inoculation on fungi have shown great potential despite their high dry matter loss. Enzymatic pretreatment is a great alternative to prevent this. In fact, previous study has shown an increase up to 12% in ruminal degradability of OPF when pretreated using

white rot fungi. Apart from that, previous study proved that 40% replacement of concentrated feed mixture with biologically pretreated sugarcane bagasse which is another type of fibrous agricultural by-product resulted in 28% of feed cost reduction. However, different fungi yield different activity of enzyme cocktail. It is imperative to test these fungi's enzyme activity and their delignification ability and further assess the effect on ruminal degradability of OPF.

1.3 Objectives

1.3.1 General Objectives

To assess the effectiveness of OPF pretreated with enzyme extract from filamentous fungi in improving ruminal degradability of OPF.

1.3.2 Specific Objectives

a) To isolate fungi from OPF and determine their enzyme activity

b) To select the optimal fungi and determine their species

c) To assess the *in vitro* ruminal degradability of OPF pretreated with enzyme extract from selected fungi

1.4 Hypothesis

Using enzyme extract from filamentous fungi with high lignolytic enzyme activity to perform pretreatment on OPF, will lower the lignin content and disrupt the lignocellulosic bond. Effectively, allowing rumen microbe to access fermentable cellulose and hemicellulose and further increasing the ruminal degradability of OPF by producing more volatile fatty acids such as acetate, propionate and butyrate by fulfilling the ruminants' dietary requirements.

1.5 Significance of Study

Malaysia has a large area allocated for oil palm plantation significant with its role as a major player in global palm oil production. This large-scale plantation produces large amount of oil palm by-product such as palm kernel cake, empty fruit bunch, palm oil mill and OPF. Approximately 45,000,000 tonnes of OPF are produced annually from felling and trimming process.

The OPF are commonly used as source of roughage and animal, particularly for livestock. The OPF have no report of toxicity problems and are palatable for ruminants. Their utilisation as roughage have shown no detrimental effect toward the animals. Apart from that, the inclusion of OPF in beef cattle and dairy cows diet with inclusion levels of 30% with good formulation could support live weight gain between 0.6 and 0.8 kg/d and milk yield of 22 l/d respectively (Zahari and Alimon, 2005). Despite that, OPF is known to have high lignin content, forming lignocellulosic biomass. The lignocellulosic biomass is present to impart strength to the plant. However, their presence causes the OPF to have poor nutritive value due to limited access of rumen microbe to fermentable cellulose and hemicellulose. Their presence also limits other enzymes' effectiveness due to their ability to absorb the enzyme, effectively removing them from the reaction site.

Pretreating the OPF using the enzyme extract from fungi might help in improving its ruminal degradability. White rot fungi which belong to the phylum basidiomycetes have been extensively researched due to their ability to produce lignolytic enzymes such as manganese peroxidase, laccase and lignin peroxidase (Asgher et al., 2013; Wan and Li, 2010). However, other fungi from different phylum might show better lignolytic enzyme activity (Singh and Singh, 2014). The filamentous fungi have the ability to produce the enzymes in large amount (Xiaoyun et al., 2012). These enzymes break the lignin-hemicellulose bond, remove the lignin and provide access to the fermentable cellulose and hemicellulose by the rumen microbes. Hence, improving the effectiveness of OPF' usage as viable livestock feed as the rumen microbe have more access to the fermentation substrate in the form of cellulose and hemicellulose, further increasing the amount of volatile fatty acids production. This can provide positive effect in term of growth of the animal as volatile fatty acid made up 70% of energy source of the ruminants. Apart from that, enzymatic pretreatment with enzyme extracted from filamentous fungi is also cost effective as it does not require expensive setup and is safe as it free from any strong chemical such as acids and alkali. The enzyme extraction can be done using solid state fermentation method. The filamentous fungi are notorious for their enzyme producing capability and the inexpensive solid state fermentation setup further minimize the cost for large scale enzyme production. The pretreatment using enzyme extract from fungi is also applicable to all fibrous agricultural by-product as reported by various study (Asgher et al., 2013; Wan and Li., 2010; Maeda et al., 2011).

The increased effectiveness also means lower cost for the industry as it will utilise agricultural by-product which is normally discarded. Furthermore, improved nutritional value may translate into better growth performance and higher quality meat and milk at a fraction of conventional feeding cost. Higher quality meats also improve its value. Previous study has shown that biological pretereatment on similar agricultural by-

product have indeed reduce the cost of feeding. Indeed, 40% replacement of concentrated feed with pretreated agricultural by-product have improve the cost of feeding by 28%. Apart from that, the increased effectiveness also means the planting space competition between plantations for livestock and human use will be lowered.

Lower feeding cost may also be reflected on the increased production of livestock. Indeed, Malaysia is still lagging behind in fulfilling its local demand for meat and resorted to importing the meat. This may cause lower retail price of meat for the masses. Indeed, it is imperative to identify the fungi that shows the optimal enzyme activity from their cocktail to be used as pretreatment agent to overcome the lignocellulosic concern in feeding this agricultural by-product.

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