IN VITRO RELEASE OF ENZYMES AND SURFACES MORPHOLOGY OF ENZYME COATED PELLETED GOAT FEED

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Special dedication to:

To my beloved parents:
(Misbah Bin Abu Yamin and Zainon Binti Abdullah)
Whose love, patience, kindness and prayers have brought me this far..

To my siblings:
(Hanysah Binti Misbah and Norhayati Binti Misbah)
For their endless encouragement, laughs, cries and tears..

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ABSTRACT

In the current study, effectiveness of mixed enzymes, β-mannanase (Hemicell L-W) and phytase (Finase L) coating on goat pelleted feed was investigated using a simulated rumen. Feed incorporated with enzymes coating is believed to be a beneficial method for the delivery of enzymes to targeted digestive areas. The coating effects were analyzed to understand the correlation of coating efficiency, profile of enzyme release and physical appearance. The coating process was conducted using a spray process. Freshly formulated basal ingredients consisted of palm kernel and wheat-based diet, were prepared using pelletizing method. After cooling, initial pellet and coated pellet were analyzed to determine the surface morphology and coating efficiency. The coated pellet from scanning electron microscope (SEM) showed a homogenous and smooth surface without any pores or cracks on its morphology. Micrographs from SEM showed that the coating was successfully segregated during spraying process. Cross-sectional morphology showed a thin plate-like characteristic with lots of twisting and turning routes that allow liquid to enter easily. The coated pellet surfaces showed a darker colour of brown (hue of red and green 4.10 ± 0.30, yellow and blue 14.70 ± 0.30, lightness 37.57 ± 0.51) compared with initial pellet (red and green 5.00 ± 0.35, yellow and blue 15.97 ± 0.25, lightness 39.43 ± 0.29) which demonstrates a significant difference ($P<0.05$) through colour measurement analysis. The difference was attributed to the original dark brown of mixed enzymes colour. In this study, the different absorbance values obtained between initial pellet and coated pellet indicated a high coating efficiency of 75 %. It indicated a high degree of protection *in vitro* in rumen. The thickness of coating detected had been infused at range of 195 to 269 ± 52 µm. The mixed enzyme showed a successful delayed on its release profile in simulated rumen fluid (pH of 6.8), whereby the release profile showed a constant discharged at an average of 8 % on coated pellet compared to the uncoated pellet. The highest peak of enzyme release was observed at 96.7 % after six hour. The high release profiles at 0 to 30 minutes on both uncoated and coated pellet at 16.6 % and 45.8 % were contributed from activation of enzymes on feed ingredients. The coated feed has improved the resistance time until it was transferred to the next phase of gut, abomasum. Results of surface morphology and coating efficiency provided valuable information for incorporation of enzymes coating into goat feed.

ABSTRAK

Dalam kajian ini, keberkesanan gabungan enzim *β-mannanase* (*Hemicell L-W*) dan *phytase* (*Finase L*) disalut pada pelet dedak kambing telah dikaji melalui kaedah simulasi rumen. Penggabungan dedak dengan salutan enzim dipercayai merupakan kaedah terbaik bagi memastikan keselamatan penyampaian enzim ke bahagian penghadaman tertentu. Kesan salutan dianalisis untuk meningkatkan pemahaman tentang hubungan keberkesanan salutan, profil pembebasan enzim dan rupa fizikalnya. Proses salutan dijalankan melalui kaedah semburan. Adunan bahan formulasi yang masih segar terdiri daripada isirong kelapa sawit dan juga bahan asas gandum disediakan melalui proses pempeletan. Selepas penyejukan, pelet asal dan pelet bersalut dianalisis bagi menentukan morfologi permukaan dan keberkesanan salutannya. Morfologi permukaan didapati lebih rata dan licin pada pelet bersalut berbanding pelet asal tanpa kesan lubang dan retak menerusi mikroskop elektron pengimbas (SEM). Mikrograf SEM menunjukkan salutan berjaya disebarkan melalui proses semburan. Morfologi keratan rentas menunjukkan ciri seperti plat nipis dengan banyak laluan piuhan dan putaran yang membenarkan bendalir masuk dengan mudah. Warna coklat permukaan pelet bersalut didapati lebih gelap (rona warna merah dan hijau 4.10 ± 0.30, kuning dan biru 14.70 ± 0.30, pencahayaan 37.57 ± 0.51) berbanding warna pelet asal (merah dan hijau 5.00 ± 0.35, kuning dan biru 15.97 ± 0.25, pencahayaan 39.43 ± 0.29) yang menunjukkan terdapat perbezaan yang signifikan ($P<0.05$) melalui analisis pengukuran warna. Perbezaan warna terhasil dari campuran warna asal enzim iaitu coklat gelap. Kajian ini menunjukkan terdapat peningkatan kecekapan salutan yang tinggi pada 75 % hasil perbandingan nilai penyerapan pelet asal dan pelet bersalut. Ini menunjukkan keberkesanan perlindungan *in vitro* adalah tinggi di dalam rumen. Ketebalan salutan secara infusi didapati di antara 195 hingga 269 ± 52 µm. Kombinasi enzim telah menunjukkan profil pembebasan berjaya dilambatkan dalam simulasi bendalir rumen (pH 6.8), di mana profil pembebasan berkadar malar pada purata 8 % dari pelet bersalut berbanding dengan pelet asal. Takat pembebasan enzim paling tinggi didapati pada 96.7 % selepas enam jam. Pada minit 0 ke 30 memperlihatkan profil pembebasan yang tinggi pada kedua-dua pelet asal dan bersalut pada kadar 16.6 % dan 45.8 % disebabkan oleh pengaktifan enzim oleh ramuan dedak. Pelet dedak bersalut telah memperbaiki masa ketahanan sehingga ia beralih ke fasa usus yang seterusnya, abomasum. Hasil kajian morfologi permukaan dan keberkesanan salutan memberikan informasi berguna untuk salutan kombinasi enzim terutama sekali pada dedak kambing.

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LIST OF ABBREVIATIONS

CIE	-	Commission Internationale de l'E´ clairage
Co.	-	Company
CR-10	-	Colour Reader-10
E.C.	-	European Community
e.g.	-	For example,
et al.	-	and others
etc.	-	et cetera/ and so forth
EU	-	European Union
Eq.	-	Equation
FAO	-	Food and Agriculture Organization
GI	-	Gastrointestinal
i.e.	-	that is
L*a*b*	-	L* = Lightness, a*= red and green, b*= yellow and blue characteristic
MFM Feedmill Sdn Bhd	-	Malayan Flour Mill Feedmill Sendirian Berhad
MSDS	-	Material Safety Data Sheet
no.	-	by number
p-value	-	Level of significance
®	-	statutory notice of right trademarks
SEM	-	Scanning Electron Microscopy
TEM	-	Transmisson Electron Microscopy
™	-	Trademarks right
TM 3000	-	Tabletop Microscopy 3000
UV/VIS Spectrophotometer	-	Ultraviolet-Visible Spectrophotometer

LIST OF SYMBOLS

abs	-	Absorbance
α	-	Alpha
β	-	Beta
cm	-	Centimeter
D	-	Delta
g	-	Gram
kg	-	Kilogram
K_2HPO_4	-	di-potassium hydrogen phosphate
kV	-	Kilo-Volts
M	-	Molar
mA	-	Milliampere
mg/mL	-	milligrams per millilitre
ml	-	Millilitre
mL	-	Millilitre
ml g^{-1}	-	millilitre per gram
mm	-	Millimeter
µm	-	Micrometer
mol/liter	-	molar per liter
NaCl	-	sodium chloride
NaOH	-	*sodium hydroxide*
nm	-	nanometer
rpm	-	revolutions per minutes
P	-	Phosphorus
pH	-	power of Hydrogen
PSI	-	pound per square inch
°	-	Degree
%	-	Percentage

^{o}C	-	degree Celsius
$<$	-	Less than
λ	-	Wavelength
$\pm$	-	both plus and minus operations
$=$	-	equality

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CHAPTER 1

INTRODUCTION

Generally, by developing coatings on various agglomerates form such as pellets or other shaped will primarily strengthen their palatability through inclusion of special ingredients or additives that can directly perceived. Coatings can be classified as proteins, lipids, and polysaccharides, alone or in combination. They function as defense over oxygen and moisture during manufacturing, handling, and storage to prevent damage on the feed. Coatings also increase its safety value by its natural biocide performance or comprise together with antimicrobial component (Valverde *et al*., 2005). Applying liquid material and drying it to acceptable moisture level is a way of coating preferences. However, this procedure was not preferable on animal feed pellets that consists numerous nutrients in its diets with standard optimum inclusion (Humphry *et al*., 1997). Post-pellet spray application supply with liquid phytase is recognized as providing option to improve the losses of solid enzymes prior to conditioning. Anyhow, these systems need extra workforce and investments (Encarnacoa, 2016).

In animal science, enzymes act as mediator of livestock and poultry digestive systems. It has attracted the interest of researchers and nutritionists to establish exogenous fibrolytic enzymes in animal diet for productivity enhancement. Well performances had been shown on monogastric animal such as poultry and pig, upon applying enzymes in its feeds. Not only that, a ruminant response to exogenous fibrolytic enzymes was varied depending on different source of enzyme, application method, and types of diets and livestocks (Bedford and Schulze, 1998; Wang and McAllister, 2002).

Nonetheless, adaptation of enzymes technology in ruminants is still being sceptic and provoking. The established enzyme products that consider as nutritional on animal feed are also used in non-feed productions such as pulp and paper, fuel and textile. It is because fibrolytic enzymes that increase the animal productivity is perceived as a complex technology. Most of recent researches tend to focus on these additive applications (Beauchemin *et al*., 2004). However, Nsereko *et al*. (2002) had showed a successful improvement in rising up the bacteria quantity in rumen by supplementing an exogenous fibrolytic enzyme in dairy cow feed. The experiments used hemicelluloses and secondary products of digestion, which are cellulose. Through advancement of enzyme technology in the past twenty years, efficacy and matching activities between enzymes and their target substrates had been established. One of the prominent technologies that help address this challenge is the use of selected and suitable enzymes for ruminant consumption.

1.1 Research Overview

The European Union prohibited the use of antibiotic as feed additive starting from 2006. The policy was then gradually adapted by non-European Countries resulting in restriction of antibiotic application in animal feed. Benchaar *et al*. (2008) explained that nowadays the communities are more aware on antibiotic that transmit the potentially resistant microbial from monogastric animal to human. It includes ruminants whereby microbiologist and nutritionist attempt to find a new supplementary to improvise the efficiency and productivity of animals especially livestock.

Martin and Nisbet (1992) highlighted that attention have been shown in previous years on manipulation of ruminal microflora to increase animal feed performance and elucidate related issues in current feeding system. Balancing pH of digestive system by improving the lactate-performance capability of ruminal microbial without using antibiotics or ionophores has the potential to resolve the economic needs of animal production with regards to rumen acidosis. The growing concern by consumers on antibiotics in the animal nutrition and its quality create motivation to investigate and develop new non-antibiotic, or "natural" feed additives. As a result, current exposure on safety, quality and animal environmental performance encounter the strategy to use "natural" additives to increase productivity, to reduce risk on animal transmit potential pathogen to human, to minimize resistancy of antibiotic on genes evolution and to diminish the spreading of pollutions (Chaucheyras-Durand *et al*., 2008).

Undeniably, studies on suitable enzymes in feeds could be very challenging. An enzyme must indicate a better performance on animal growth over systems, which is already established and highly recognized by previous practitioner on animal genetics, nutrition, in order to enhance the management and feeding activities (Anderson and Hsiao, 2009). A review by Barletta (2011) indicated that feed enzymes have played a significant role in increasing the efficiency of meat and egg production. In general, animals use enzymes to degrade the consumed feed produced either by the animal itself, or by natural presence in digestive system. Currently, the types of enzymes used in animal dietary are those that break down proteins, fibre, starch and phytate. Therefore, this research aims to primarily benefit others research interests that is targeting an increase on nutrient applying to ruminants feed. The understanding over ruminal extraction will be considered initial and informatics in relation with the ecosystem and nutrition resource to optimize the digestive efficiency.

Fermentation of carbohydrates and proteins in rumen are interrelated and the limit of fermentation in rumen can be determined by the rate of passage that in return influences nutrient availability. Moreover, it will elicits complicates of accurate prediction on nutrients supply by microbial transformations of feeds in the rumen before intestinal digestion for optimal performance (Nocek and Russell, 1988; Sklan and Tinsky, 1996; Calsamiglia *et al*., 2007). Gouin (2004) reviewed that controlled release of the ingredients can improve the effectiveness of additives, broaden the ingredient application range and to ensure the optimal dosage. In this study, efficiency of release is one of the major objectives, which need to be elucidated in order to establish the optimum level of enzymes release.

At the beginning of experiment, following hypotheses were being considered as preliminaries of this study:

1. Improved efficiencies of feed could be observed between initial and pelleted feed coated with combination β-mannanase and phytase for goat feeding.

2. Optimum release profile could be obtained on goat feeding with pelleted feed coated with mixed enzymes.

1.2 Problem Statement

Various observations had been made on pressure and temperature activities on feed processing, such as extrusion, pelleting and others. Ingredients that are volatile on heat and/ or easy to dissolve in water such as enzymes do not survive long. In addition, handling and post-production process exposure which include warehousing and logistic (transportation) will also contribute to the degradation of products' nutrients.

On the other hand, digestive system of animals is also one of the factors that contribute to reduction and lose of the valuable nutrients. Therefore, diminishing of numerous nutrients before reach to the targeting digestive area is believed as a waste to feed manufacturer (Street *et al.*, 2009). Hence, the ability of newly developed supplements to adapt to the manufacturing condition such as conditioning and pelleting, onto the rough conditions (steam quality, force, heat and control of time) of feed production is necessary (Thomas *et al.*, 1998; Igbasan *et al.*, 2000). Supplementing enzymes in pelleted feed products must consider on whether it would be affected by process that involve high temperatures (above 80 °C) in short periods of time (Kirk *et al.*, 2002). Thus, a reliable and efficient enzymes coating is favourable to secure the transfer to target area and continuously its beneficial effect to improve on an animal performance.

Nowadays, most consumers' are very concern about animal feed source and demand for healthy food. Encouragement over healthy and safe ruminant diets has attracted researchers to improve on animal feed (Carreño *et al.*, 2015). It includes manipulation of ruminal fermentation patterns to improve animal performance (Hu *et al.*, 2005). In ruminant, the largest compartment of the four-compartment stomach is the rumen that is mainly the place for fermentation of fibrous feed that would diminish nutrients to supply on its target area (LaCount *et al.*, 1996).

The complexity of combinatorial microbial action in digestive system is closely related with its nutrient. The major bacterial that hydrolyzes fiber, sugars, starches and proteins are mostly anaerobe such as bacteria, protozoa and fungi. A deficiency of nutrients, which suits the microbial feeding, could affect on growth, lessen down their habitats, negatively influences the digestive performance and feed consumption especially on fibrous diets (Puga *et al.*, 2001). Several studies conducted had acknowledged the effectiveness of incorporated enzymes in various diets, which are mainly corns, cereals, soybean meals or barley (Beauchemin *et al*., 1995; Feng *et al*., 1996), as well as the component of the diet to which the enzyme additive had been added (Krause *et al*., 1998; McAllister *et al*., 1999; Knap *et al.*, 2003). Combinatorial enzyme also had been agreeable to be beneficial in high concentrate diets (Beauchemin *et al*., 1997) and can improve the hydrolysis as concluded by Sun *et al*. (2011). The specific mixed enzymes used in this research were β-mannanase and phytase.

Feed enhancing enzymes are enzymes that increase the efficiency of feed utilization by improving feed digestability (Knap *et al*., 2003). Dean *et al*. (2013) explained that applying enzyme to feeding is attractive because the feed consist variety of hydrolyze composition (e.g., cellulase, xylanase, and amylase) which can be directly fermented by ruminal bacteria in rumen. Therefore, a thorough process is needed to ensure even distribution of added enzyme in the feed. Besides that, it is also discussed that enzymes efficacy is positively correlated with supplemental enzymes on nutrients rations (Bowman *et al*., 2002) which contradict with Yang *et al*. (1999) findings. This variance of results and findings indicate the need and importance of further examination on enzyme application methodology in feed with regards to the mechanism and effect of enzyme delivery in rumen.

In developed countries, nutrition of people is considered as high priority, not only the nutritional value but also type of component in their food such as colour, flavour or texture that improves the quality of health, of which preference is on natural-based ingredients. This is the key to establish the beneficial foods (Imram, 1999; Grashorn, 2005). On the other hand, animal feed also can be beneficial with the supplementation of value added material that can be seen on its appearance (Shaharuddin *et al.*, 2014). Quality perspective on visual perception or in other word visual appearance is evaluated through its attributes or specific feature on objects (Amookht *et al*., 2014) and also to establish a colour feature (MacDougall, 2010). In this experiment design, several feed appearance attributes were studied such as colour and morphology in order to analyze the homogenity of spray process on the feed surface.

As highlighted by Krishnamachari *et al*. (2011), observation on particle by particle through scanning and transmission of electron microscopes is beneficial but also has its own disadvantages. Eventhough observation using Scanning Electron Microscopy (SEM) and Transmisson Electron Microscopy (TEM) may need longer in time than some other equipment such as infrared spectroscopy, X-ray diffraction and others, the texture particles of any particles can easily be detected on its feature among numerous others with extra precaution on sample screening. SEM and TEM are used to study the mode of imaging combination which produces a morphological feature such as shape, size, roughness and other particles. In this research, surface morphology of coated pellet of feed was scanned through SEM to visualize the coated structure.

Following are several research questions elated with this study:

1. How well the coating of mixed enzymes which are sprayed on feed pellet contribute to efficiency of delivery on rumen system especially for goat intake?

2. Why feeding coated feed pellet with enhanced combination of enzymes is suitable to improve feed digestibility and increase its efficiency on goat/ lamb growth performance?

3. What measurement of attributes can be determined on feed pellet which are sprayed with combinatorial enzymes consisting of β-mannanase and phytase as consumer attraction and goat feeding?

1.3 Objectives of This Study

The objectives of this study are as the following: -

1. To determine the coating efficiency of mixed commercial enzymes, the physical attributes and surface morphology of pelleted goat feed;

2. To investigate the enzyme/ coating release efficiency in liquid media simulating rumen fluid; and

3. To evaluate the surface morphology and measurement of colour on pelleted goat feed sprayed with mixed enzymes using scanning electron microscopy techniques.

1.4 Scopes of This Study

Below are the scopes which had been studied to achieve above objectives:

1. Investigation on feed efficiency of pelleted feed coated with mixed β-mannanase and phytase.

2. Analysis of enzyme coating efficiency through spraying process on pelleted goat feed.

3. Determination of the optimum level of release profile using combinatorial enzymes in rumen.

1.5 Thesis Outline

Mainly, this research investigated the effect of coating efficiency in commercial pelleted goat feed in liquid media simulating rumen fluid, using mixed β-mannanase and phytase to improve the delivery efficiency of enzymes. This study was conducted to analyze the effect of coating pelleted goat feed which was sprayed with combination enzymes using COMCO liquid system. It was exhibited a possibility of coating pelleted feed in order to deliver nutrient to target area that is goat rumen, whereby efficiency of its coated enzyme on digestive process in rumen would be determined and measured by UV/ VIS Spectrophotometer. In parallel, the optimum level of release efficiency would be obtained upon release of mixed β-mannanase and phytase in rumen.

In this study, the physical appearance of enzyme coated pelleted feed were compared to examine the differentiation of colour and surface feature of both pelleted feed as well as the homogenity of coating using Colour Reader and Scanning Electron Microscope.

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