ผลของการเสริมเอนไซม์ย่อยเยื่อใยต่อกระบวนการหมักย่อย ของข้าวโพดหมักและฟางข้าว

นายนพรัตน์ ผกาเชิด

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีการผลิตสัตว์
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2557

EFFECTS OF EXOGENOUS FIBROLYTIC ENZYME SUPPLEMENTATION ON *IN VITRO* AND *IN VIVO* FERMENTATION OF CORN SILAGE AND RICE STRAW

Noppharat Phakachoed

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Animal Production Technology

Suranaree University of Technology

Academic Year 2014

EFFECTS OF EXOGENOUS FIBROLYTIC ENZYME SUPPLEMENTATION ON *IN VITRO* AND *IN VIVO* FERMENTATION OF CORN SILAGE AND RICE STRAW

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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นพรัตน์ ผกาเชิด : ผลของการเสริมเอนไซม์ย่อยเยื่อใยต่อกระบวนการหมักย่อยของข้าวโพดหมักและฟางข้าว (EFFECTS OF EXOGENOUS FIBROLYTIC ENZYME SUPPLEMENTATION ON *IN VITRO* AND *IN VIVO* FERMENTATION OF CORN SILAGE AND RICE STRAW) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.วิศิษฐิพร สุขสมบัติ, 179 หน้า.

ทำการทดลอง 3 งานทดลองในหลอดทดลองเพื่อศึกษาผลของการเสริมเอนไซม์ย่อยเยื่อใยต่อกระบวนการหมักย่อยของข้าวโพดหมักและฟางข้าว โดยใช้เทคนิคผลผลิตแก๊สในหลอดทดลอง 48 ชั่วโมงในการบ่ม การจัดทรีทเมนต์เป็นแบบแฟคทอเรียลในแผนงานทดลองแบบสุ่มสมบรูณ์ โดยมีการทำการทดลองซ้ำ 2 ครั้ง และแต่ละครั้งมีจำนวนซ้ำ 4 ซ้ำ เอนไซม์ที่ใช้ในการทดลองเป็นผลิตภัณฑ์ทางการค้า 8 ชนิด ที่ประกอบด้วยเอนไซม์เอนโดกลูคาเนส เอกโซกลูคาเนส และไซแลน-เนส (E1 E2 E3 E4 E5 E6 E7 และ E8) การวัดผลผลิตแก๊สที่เกิดขึ้นทำการวัดที่ชั่วโมงที่ 3 6 12 24 และ 48 หลังจากบ่มในตู้บ่ม การวัดการย่อยได้ของวัตถุแห้ง เยื่อใยที่ไม่ละลายในสารฟอกที่เป็นกลาง เยื่อใยที่ไม่ละลายในสารฟอกที่เป็นกรด และปริมาณของกรดไขมันระเหยได้ ทำการวัดที่ชั่วโมงที่ 24 และ 48 หลังจากบ่มในตู้บ่ม การทดลองที่ 1 พบว่า การเสริมเอนไซม์ย่อยเยื่อใยไม่มีผลต่อการย่อยได้ของวัตถุแห้งของข้าวโพดหมักและปริมาณแก๊สที่เกิดขึ้น แต่อย่างไรก็ตามการเสริมเอนไซม์ย่อยเยื่อใยสามารถเพิ่มการย่อยได้เยื่อใยที่ไม่ละลายในสารฟอกที่เป็นกลาง และเยื่อใยที่ไม่ละลายในสารฟอกที่เป็นกรดของข้าวโพดหมัก ซึ่งระดับที่สามารถเพิ่มการย่อยได้ขึ้นอยู่กับชนิดของเอนไซม์ที่เสริม ในการทดลองที่ 2 การเสริมเอนไซม์ย่อยเยื่อใยทุกชนิดสามารถเพิ่มการย่อยได้ของวัตถุแห้ง เยื่อใยที่ไม่ละลายในสารฟอกที่เป็นกลาง เยื่อใยที่ไม่ละลายในสารฟอกที่เป็นกรด และปริมาณแก๊สที่เกิดขึ้น อย่างไรก็ตามการย่อยได้ของวัตถุแห้ง เยื่อใยที่ไม่ละลายในสารฟอกที่เป็นกลาง เยื่อใยที่ไม่ละลายในสารฟอกที่เป็นกรด และปริมาณแก๊สที่เกิดขึ้น ไม่มีความสัมพันธ์กันระหว่างชนิดของเอนไซม์และชนิดของข้าวโพดหมัก การทดลองที่ 3 พบว่า การเสริมเอนไซม์ย่อยเยื่อใยไม่มีผลต่อการย่อยได้ของวัตถุแห้งของฟางข้าวและปริมาณแก๊สที่เกิดขึ้น แต่เอนไซม์ทุกชนิดที่เสริมสามารถเพิ่มการย่อยได้เยื่อใยที่ไม่ละลายในสารฟอกที่เป็นกลาง เยื่อใยที่ไม่ละลายในสารฟอกที่เป็นกรดของฟางข้าว แต่อย่างไรก็ตามการแสดงผลของเอนไซม์ที่เสริมแต่ละชนิดแตกต่างกันขึ้นอยู่กับระดับที่ทำการเสริมของเอนไซม์แต่ละชนิด จากผลในการทดลองในหลอดทดลอง ควรมีการศึกษาผลของการเสริมเอนไซม์ E1 และ E2 ในตัวสัตว์ที่ใช้ข้าวโพดหมักและฟางข้าวเป็นอาหารหยาบ

ทำการทดลอง 3 การทดลองในตัวสัตว์ เพื่อศึกษาผลของการเสริมเอนไซม์ย่อยเยื่อโดยใช้โคเจาะกระเพาะโคนมลูกผสมโฮลส์ไตน์ฟรีเชี่ยน โดยใช้แผนการทดลองแบบจัตุรัสลาติน โคเจาะ-

กระเพาะทุกตัวจะได้รับอาหารข้น 17 เปอร์เซ็นต์โปรตีน ปริมาณ 3 กิโลกรัมต่อวันร่วมกับฟางข้าวและน้ำสะอาดแบบไม่จำกัด (การทดลองที่ 4) โคเจาะกระเพาะทุกตัวจะได้รับอาหารข้น 21 เปอร์เซ็นต์โปรตีน ปริมาณ 3 กิโลกรัมต่อวันร่วมกับข้าวโพดหมักและน้ำสะอาดแบบไม่จำกัด (การทดลองที่ 5) และโคเจาะกระเพาะทุกตัวจะได้รับอาหารข้น 21 เปอร์เซ็นต์โปรตีน ปริมาณ 3 กิโลกรัมต่อวันร่วมกับฟางข้าวและน้ำสะอาดแบบไม่จำกัด (การทดลองที่ 6) การทดลองของแต่ละช่วงการทดลองแบ่งเป็น 21 วันของแต่ละช่วงการทดลอง 14 วันสำหรับการปรับตัว และอีก 7 วันสำหรับการเก็บตัวอย่างและวัดการย่อยได้ การทดลองที่ 4 พบว่า การเสริมเอนไซม์ไซแลน-เนสไม่มีผลต่อการกินได้วัตถุแห้ง ระดับ pH ปริมาณแอมโมเนียไนโตเจน การย่อยได้ของวัตถุแห้งและการย่อยได้ เยื่อใยที่ไม่ละลายในสารฟอกที่เป็นกรดของฟางข้าว แต่อย่างไรก็ตาม การเสริมเอนไซม์ไซแลนเนสที่ระดับ 20 กรัมต่อตัวต่อวัน สามารถเพิ่มการย่อยได้เยื่อใยที่ไม่ละลายในสารฟอกที่เป็นกลางของฟางข้าว การเสริมเอนไซม์ไซแลนเนสไม่มีผลต่อการย่อยได้ของเฮมิเซลลูโลส การทดลองที่ 5 และ 6 พบว่า การสริมเอนไซม์ย่อยเยื่อใยทั้ง 2 ชนิดไม่มีผลต่อการกินได้วัตถุแห้ง ปริมาณกรดไขมันระเหยได้ ระดับ pH ปริมาณแอมโมเนียไนโตเจน และปริมาณน้ำตาลกลูโคสในเลือด การเสริมเอนไซม์ย่อยเยื่อใยสามารถเพิ่มการย่อยได้ของวัตถุแห้ง เยื่อใยที่ไม่ละลายในสารฟอกที่เป็นกลาง และเยื่อใยที่ไม่ละลายในสารฟอกที่เป็นกรดของข้าวโพดหมัก หลังจากบ่มในกระเพาะหมักที่ชั่วโมงที่ 24 48 และ 72 (การทดลองที่ 5) และการเสริมเอนไซม์ย่อยเยื่อใยสามารถเพิ่มการย่อยได้ของเยื่อใยที่ไม่ละลายในสารฟอกที่เป็นกลาง และเยื่อใยที่ไม่ละลายในสารฟอกที่เป็นกรดของฟางข้าว หลังจากบ่มในกระเพาะหมักที่ชั่วโมงที่ 24 48 และ 72 (การทดลองที่ 6) จากผลการทดลองในตัวสัตว์ ระดับที่เหมาะสมในการเสริมเอนไซม์ E1 และ E2 สำหรับข้าวโพดหมัก คือ 0.5 และ 0.1 มิลลิลิตรต่อกิโลกรัมน้ำหนักแห้งข้าวโพดหมัก ตามลำดับ และระดับที่เหมาะสมในการเสริมเอนไซม์ E1 และ E2 สำหรับฟางข้าว คือ 2.0 มิลลิลิตรต่อกิโลกรัมน้ำหนักแห้งฟางข้าว

สาขาวิชาเทคโนโลยีการผลิตสัตว์ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2557 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

NOPPHARAT PHAKACHOED : EFFECTS OF EXOGENOUS FIBROLYTIC ENZYME SUPPLEMENTATION ON *IN VITRO* AND *IN VIVO* FERMENTATION OF CORN SILAGE AND RICE STRAW. THESIS ADVISOR : ASSOC. PROF. WISITIPORN SUKSOMBAT, Ph.D., 179 PP.

EXOGENOUS FIBROLYTIC ENZYME/CORN SILAGE/RICE STRAW

Three *in vitro* experiments were conducted to evaluate the effect of enzyme additives supplementation on fermentation of corn silage and rice straw using a 48 h *in vitro* gas production technique. The experiments were conducted as factorial in completely randomized designs each with two runs and four replicates. Eight enzyme additives were commercial products of endoglucanase, exoglucanase, and xylanase (E1, E2, E3, E4, E5, E6, E7, and E8). Gas production (GP) was measured at 3, 6, 12, 18, 24, and 48 h after incubation. Degradability of dry matter (DMD), neutral detergent fiber (NDFD), and acid detergent fiber (ADFD), and volatile fatty acid concentrations (VFA) (total and individual molar proportions) were determined after 24 and 48 h. Experiment I showed that DMD and total GP were unaffected by enzyme additives, but all enzyme additives increased NDFD and ADFD of corn silage, with the optimum dose rate depended on the enzyme additive. In experiment II, all enzyme additives increased DMD, NDFD, ADFD and total GP of the four corn silage substrates. However, for all parameters DMD, NDFD, ADFD and total GP, there were no enzyme × corn silage substrate interactions. In experiment III, DMD and total GP were unaffected by enzyme additives, but all enzyme additives increased the NDFD and ADFD of rice straw. However, each fibrolytic enzyme additive showed a different response depending on the enzyme dose rates. Based on the responses observed in the

in vitro experiments, enzyme E1 and E2 should be further evaluated with *in vivo* studies using diets based on corn silage and rice straw.

Three *in vivo* experiments were evaluated for the effect of fibrolytic enzymes in fistulated crossbred non-lactating Holstein Friesian cows in Latin squares design. All cows were fed approximately 3 kg/d of concentrate containing 17% CP together with *ad libitum* rice straw (experiment IV), 21% CP together with *ad libitum* corn silage (experiment V) and 21% CP together with *ad libitum* rice straw (experiment VI) and clean water. Each period in the Latin square design lasted for 21 d, 14 d for adaptation to diets and 7 d for ruminal sample collection and *in vivo* disappearance trial. Experiment IV showed that the enzymes did not change dry matter intake (DMI), ruminal pH, NH_3-N concentrations, DMD or ADFD. However, NDFD increased when xylanase was added at 20 g/cow/day. Hemicellulose degradability was unaffected by the supplementation of xylanase. Experiment V and VI showed that the enzyme additives had no effect on DMI, total volatile fatty acid (VFA), ruminal pH, NH_3-N and blood glucose concentration. The enzyme additives increased DMD, NDFD, and ADFD of corn silage after 24, 48, and 72 h of ruminal incubation in experiment V. The enzyme additives increased NDFD and ADFD of rice straw after 24, 48, and 72 h of ruminal incubation in experiment VI. The optimum dose rates of enzymes E1 and E2 were 0.5 and 1.0 ml/kg of corn silage DM respectively, and optimum dose rates of enzymes E1 and E2 were 2.0 ml/kg of rice straw DM.

School of Animal Production Technology Student's Signature_____

Academic Year 2014 Advisor's Signature_____

Co-advisor's Signature_____

ACKNOWLEDGEMENTS

First of all, I would like to express my sincere thanks to my thesis advisor, Assoc. Prof. Dr. Wisitiporn Suksombat for his teaching, advice, invaluable help, continuous guidance and encouragement throughout the course of this research. I would also like to thank Asst. Prof. Dr. Pipat Lounglawan, my co-advisor, for helping, guidance and supports of the thesis. In addition, I would like to express my sincere thanks to Prof. Dr. Karen Beauchemin (senior scientist of Lethbridge Research Centre, Agriculture and Agri-Food Canada) for her teaching, advice, invaluable help, continuous guidance and encouragement throughout the course of this research. I would not have achieved this far and this thesis would not have been completed without all the support that I have always received from them. I would like to thank staff of dairy group, University farm and the Center of Scientific and Technological Equipment and my friends in Animal Production Technology, Suranaree University of Technology for helpful suggestion. Most importantly, I would like to give the biggest thank to my family for their love, support, suggestion and giving me the opportunity to pursue my graduate studies.

Noppharat Phakachoed

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LIST OF ABBREVIATIONS

AC	=	Acetate
ADF	=	Acid detergent fiber
ADFD	=	Degradability of acid detergent fiber
BU	=	Butyrate
CP	=	Crude protein
DM	=	Dry matter
DMD	=	Degradability of dry matter
DMI	=	Dry matter intake
DP	=	Degrees of polymerization
GP	=	Gas production
MPS	=	Microbial protein synthesis
NDF	=	Neutral detergent fiber
NDFD	=	Degradability of neutral detergent fiber
PR	=	Propionate
TGP	=	Total gas production
TMR	=	Total mixed ration
VFA	=	Volatile fatty acids

CHAPTER I

INTRODUCTION

1.1 Rationale of the study

Fibrolytic enzyme feed additives have potential to improve fiber digestion and productivity of ruminants. Forages are high in fiber content, which can limit intake and digestibility of feed by ruminants (Jung and Allen, 1995). Rumen microorganisms produce enzymes that hydrolyze fiber; however, the complex cell wall structure and limited residence time of forage in the rumen limits the extent of fiber digestion by ruminants (Wang and McAllister, 2002). Many studies have evaluated the use of fibrolytic enzyme additives to overcome this limitation (as reviewed by Beauchemin, Colombatto, Morgavi, and Yang, 2003), with most research focused on cellulases and xylanases that degrade cellulose and hemicellulose, respectively, the major constituents of plant cell walls. Primarily xylanases and cellulases are concentrated extracts resulting from bacterial or fungal fermentations that have specific enzymatic activities.

Supplemental fibrolytic enzyme additives have been shown to improve *in vitro* fiber digestion and to enhance the nutritive value of both low (Yang and Xie, 2010) and high (Eun, Beauchemin, and Schulze, 2007) quality forages. Eun and Beauchemin (2007) and Eun et al. (2007) used alfalfa and corn silage as substrates and reported that supplemental enzymes increased digestion of dry matter (DM) and fiber when assessed *in vitro*, which was also observed *in vivo* (Rode, Yang, and

Beauchemin, 1999; Yang, Beauchemin, and Rode, 2000). In a study that used grass hay : concentrate (600 : 400 g/kg DM) as the substrate, fibrolytic enzymes increased total bacterial numbers (Giraldo, Ranilla, Tejido, and Carro, 2007), and cellulolytic bacteria were increased in the rumen simulation (i.e., Rusitec) fermenters using barley grain and alfalfa hay as substrates (Wang, McAllister, Rode, Beauchemin, Morgavi, Nsereko, Iwaasa, and Yang, 2001). *In vivo* studies have also shown positive responses when supplemental fibrolytic enzymes were fed to ruminants (Arriola, Kim, Staples, and Adesogan, 2011; Holtshausen, Chung, Gerardo-Cuervo, Oba, and Beauchemin, 2011), however, the effects were inconsistent with some studies reporting no effects on *in vivo* digestibility or animal performance (Lewis, Sanchez, Hunt, Guy, Pritchard, Swanson, and Treacher, 1999; Knowlton, McKinney, and Cobb, 2002).

It has been suggested that enzyme additives vary in effectiveness depending upon factors such as enzyme activity, type and dose of enzyme, type of diet, enzyme application method, and animal physiological status (Beauchemin et al., 2003). Thus, a major limitation to widespread commercial use of enzyme technology for ruminants is the uncertainty of effectiveness of enzyme products, as well as the variability in response for a given product depending upon the diet and feeding conditions. It is not yet possible to predict the potential of effects of feed enzymes from their biochemical characterization alone (Beauchemin, Colombatto, and Morgavi, 2004). Thus, conducting an *in vitro* bioassay that reflects the conditions of the rumen can be a useful means of identifying ideal enzyme candidates for use in feeding trials. Our study focused on corn silage and rice straw because it is fed to cattle in many parts of the world. While some previous feeding studies have evaluated supplemental enzymes using corn silage and rice straw based diets, it is not clear what enzyme activities and doses are most effective. It is important to establish optimum dose rate

of specific enzyme additives because dose rate directly affects the cost:benefit ratio of feeding enzymes to dairy cows to improve forage digestibility.

1.2 Research objectives

1. To study *in vitro* batch culture supplementation of fibrolytic enzyme on DM, NDF and ADF degradability, rumen fermentation profile, and total gas production of corn silage.

2. To study *in vitro* batch culture supplementation of fibrolytic enzyme on DM, NDF and ADF degradability, rumen fermentation profile, and total gas production of rice straw.

3. To study *in vivo* supplementation of fibrolytic enzyme on feed intake, DM, NDF and ADF degradability, and rumen fermentation profile of corn silage.

4. To study *in vivo* supplementation of fibrolytic enzyme on feed intake, DM, NDF and ADF degradability, and rumen fermentation profile of rice straw.

1.3 Research hypotheses

1. Supplementation of fibrolytic enzyme *in vitro* batch culture can increase DM, NDF and ADF degradability, total gas production and alter rumen fermentation profile of corn silage.

2. Supplementation of fibrolytic enzyme *in vitro* batch culture can increase DM, NDF and ADF degradability, total gas production and alter rumen fermentation profile of rice straw.

3. Supplementation of fibrolytic enzyme *in vivo* can increase feed intake, DM, NDF and ADF degradability, and alter rumen fermentation profile of corn silage.

4. Supplementation of fibrolytic enzyme *in vivo* can increase feed intake, DM, NDF and ADF degradability, and alter rumen fermentation profile of rice straw.

1.4 Scope and limitation of the study

1. A 48 h *in vitro* batch culture method was used to examine the effects of commercial enzyme additives on the DM, NDF and ADF degradability, and rumen fermentation profile of corn silage and rice straw.

2. Fistulated crossbred Holstein Friesian cows from Suranaree University's dairy farm were used to examine the effects of commercial enzyme additives on feed intake, DM, NDF and ADF degradability, and rumen fermentation profile of corn silage and rice straw.

1.5 Expected results

1. Increase DM, NDF and ADF degradability, total gas production and alter rumen fermentation profile of corn silage through supplementation of fibrolytic enzyme *in vitro* batch culture.

2. Increase DM, NDF and ADF degradability, total gas production and alter rumen fermentation profile of rice straw through supplementation of fibrolytic enzyme *in vitro* batch culture.

3. Increase feed intake, DM, NDF and ADF degradability and alter rumen fermentation profile of corn silage through supplementation of fibrolytic enzyme *in vivo*.

4. Increase feed intake, DM, NDF and ADF degradability and alter rumen fermentation profile of rice straw through supplementation of fibrolytic enzyme *in vivo*.

1.6 References

Arriola, K.G., Kim, S.C., Staples, C.R., and Adesogan, A.T. (2011). Effect of fibrolytic enzyme application to low and high concentrate diets on the performance of lactating dairy cattle. **J. Dairy Sci.** 94: 832-841.

Beauchemin, K.A., Colombatto, D., Morgavi, D.P., and Yang, W.Z. (2003). Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. **J. Anim. Sci.** 81(E. Suppl. 2): 37-47.

Beauchemin, K.A., Colombatto, D., and Morgavi, D.P. (2004). A rationale for the development of feed enzyme products for ruminants. **Can. J. Anim. Sci.** 84: 23-36.

Eun, J.S., and Beauchemin, K.A. (2007). Assessment of the efficacy of varying experimental exogenous fibrolytic enzymes using *in vitro* fermentation characteristics. **Anim. Feed Sci. Technol.** 132: 298-315.

Eun, J.S., Beauchemin, K.A., and Schulze, H. (2007). Use of exogenous fibrolytic enzymes to enhance *in vitro* fermentation of alfalfa hay and corn silage. **J. Dairy Sci.** 90: 1440-1451.

Giraldo, L.A., Ranilla, M.J., Tejido, M.L., and Carro, M.D. (2007). Influence of exogenous fibrolytic enzymes and fumarate on methane production, microbial growth and fermentation in Rusitec fermenters. **Br. J. Nutr.** 98: 753-761.

Holtshausen, L., Chung, Y.H., Gerardo-Cuervo, H., Oba, M., and Beauchemin, K.A. (2011). Improved milk production efficiency in early lactation dairy cattle with dietary addition of a developmental fibrolytic enzyme additive. **J. Dairy Sci.** 94: 899-907.

Jung, H.G., and Allen, M.S. (1995). Characteristics of plant cell walls affecting intake and digestibility of forages by ruminants. **J. Anim. Sci.** 73: 2774-2790.

Knowlton, K.F., McKinney, J.M., and Cobb, C. (2002). Effect of a direct-fed fibrolytic enzyme formulation on nutrient intake, partitioning, and excretion in early and late lactation Holstein cows. **J. Dairy Sci.** 85: 3328-3335.

Lewis, G.E., Sanchez, W.K., Hunt, C.W., Guy, M.A., Pritchard, G.T., Swanson, B.I., and Treacher, R.J. (1999). Effect of direct-fed fibrolytic enzymes on the lactational performance of dairy cows. **J. Dairy Sci.** 82: 611-617.

Rode, L.M., Yang, W. Z., and Beauchemin, K.A. (1999). Fibrolytic enzyme supplements for dairy cows in early lactation. **J. Dairy Sci.** 82: 2121-2126.

Wang, Y., McAllister, T.A., Rode, L.M., Beauchemin, K.A., Morgavi, D.P., Nsereko, V.L., Iwaasa, A.D., and Yang, W. (2001). Effects of an exogenous enzyme preparation on microbial protein synthesis, enzyme activity and attachment to feed in the rumen simulation technique (Rusitec). **Br. J. Nutr.** 85: 325-332.

Wang, Y., and McAllister, T.A. (2002). Rumrn microbes, enzymes and feed digestion: A review. **Asian-Aust. J. Anim. Sci.** 15: 1659-1676.

Yang, H.J., and Xie, C.Y. (2010). Assessment of fibrolytic activities of 18 commercial enzyme products and their abilities to degrade the cell wall fraction of corn stalks in in vitro enzymatic and ruminal batch cultures. **Anim. Feed Sci. Technol.** 159: 110-121.

Yang, W.Z., Beauchemin, K.A., and Rode, L.M. (2000). A comparison of methods of adding fibrolytic enzymes to lactating cow diets. **J. Dairy Sci.** 83: 2512-2520.

CHAPTER II

LITERATURE REVIEW

2.1 Feed for ruminant animals

Livestock feeds provide the basic nutrients required for animal production, including energy, proteins, minerals, and vitamins. Feed for ruminant animals may be broadly classified as concentrate and roughage depending on their composition.

2.1.1 Concentrates

Concentrates are feeds that contain a high density of nutrients, usually low in crude fiber content (less than 18% of dry matter) and high in total digestible nutrients. Concentrates may be fed in raw or milled forms as individual feeds or may be blended or formulated into balanced rations for particular production purposes. Concentrates may be high in energy, referred to as energy concentrates, or high in protein, with over 20% crude protein, referred to as protein concentrates.

2.1.2 Roughages

Roughages or forages are the edible parts of plants with a low density of nutrients, with crude fiber content over 18% of DM and low in total digestible nutrients. Forages can provide feed for grazing animals or that can be harvested for feeding that includes the classes of feed such as fresh, herbage, hay and silage, browse, and straws. Forage consists largely of carbohydrate in the form of fiber, and its digestion is accomplished through the enzymic action of the rumen microbes. Forages are a potential feed for ruminant animals, as ruminants are best adapted to the

utilization of plant cell walls for conversion of fibrous feed sources into milk and meat products.

2.2 Plant cell walls

The plant cell wall is a complex macromolecular structure that surrounds and protects the cell. Cell walls are important features of plant cells that perform a number of essential functions, including providing shape to the many different cell types needed to form the tissues and organs of a plant. The composition of cell wall varies largely between plant species, tissues within the plant and also between different stages of growth (Fisher, Burns, and Moore, 1995; McDougall, Morrison, Stewart, and Hillman, 1996). Plant cell walls are usually divided into two categories : primary walls that surround growing cells or cells capable of growth and secondary walls that are thickened structures containing lignin and surrounding specialized cells such as vessel elements or fiber cells (Figure 2.1).

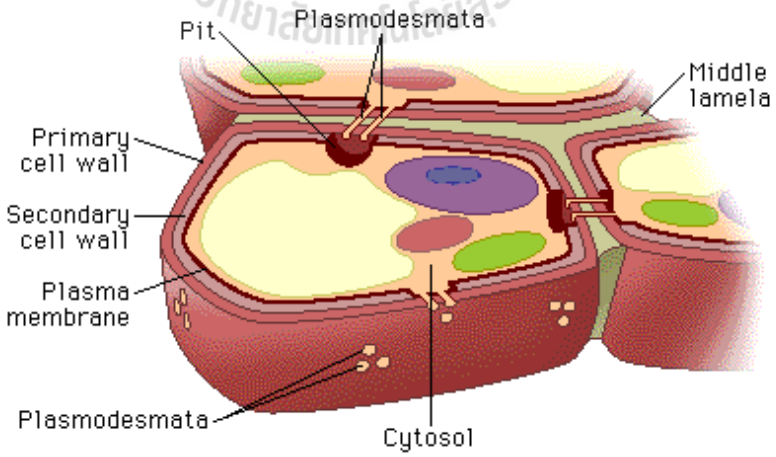

Figure 2.1 Schematic representation of a plant cell and wall development.

From : http://www.phschool.com/science/biology_place/biocoach/plants/walls.html.

Plant cell walls contain a wide range of additional compounds that modify their mechanical properties and permeability. The major polymers are cellulose, hemicellulose, pectin and lignin, which limited ability to digest by animals. However, have bacteria and other microbial populations in their digestive tracts can ferment these compounds partially into usable nutrients for ruminant animals (Figure 2.2). Plant cell walls typically consist of about 35-50% cellulose, 20-35% hemicellulose and 10-25% lignin by dry mass (Sticklen, 2008).

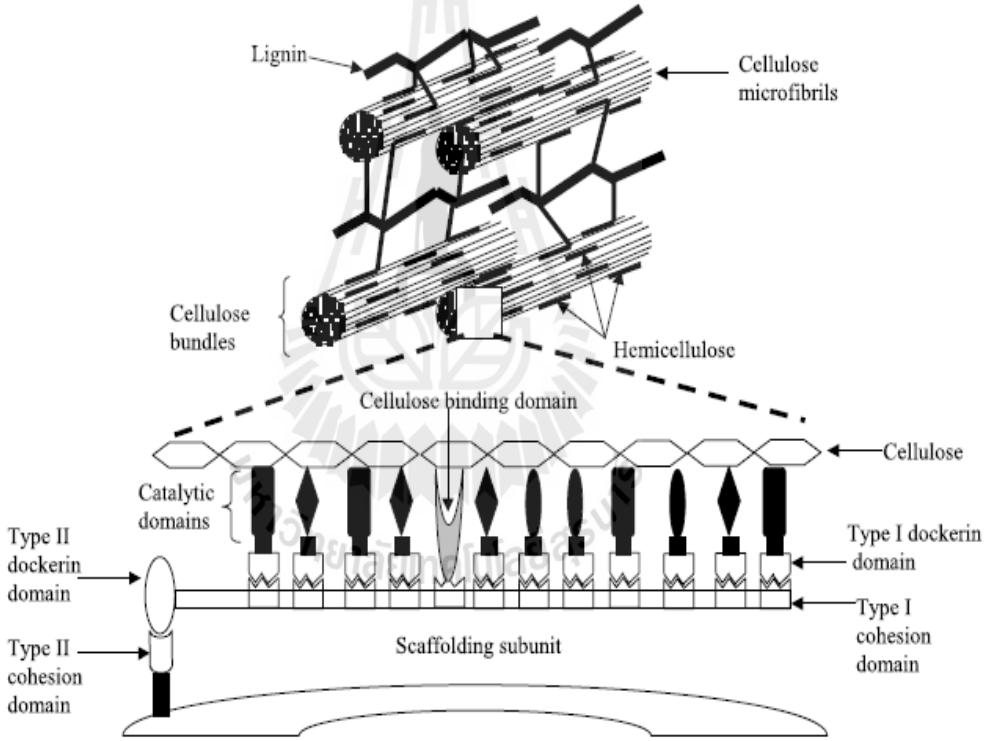

Figure 2.2 Idealized representation of fiber and its component cellulose, hemicellulose, and lignin

From : Krause et al., 2003.

2.2.1 Cellulose

The cellulose chains are organized together into progressively more complex assemblies at increasing size scales. The chemical structure of cellulose, which is a linear polymer of β-(1,4)-linked D-glucose monomer units, is in fact quite simple. Typically, cellulose chains in primary plant cell walls have degrees of polymerization (DPs) in the range from 5000 to 7500 glucose monomer units, with the DP of cellulose from wood being around 10,000 and around 15,000 for cellulose from cotton. The basic repeating unit of cellulose is cellobiose, the β-(1,4)-linked disaccharide of D-glucose. Although cellulose functions as the rigid, loadbearing component of the cell wall, the rigidity of the cellulose microfibril is strengthened within a matrix of hemicelluloses and pectins (Figure 2.3).

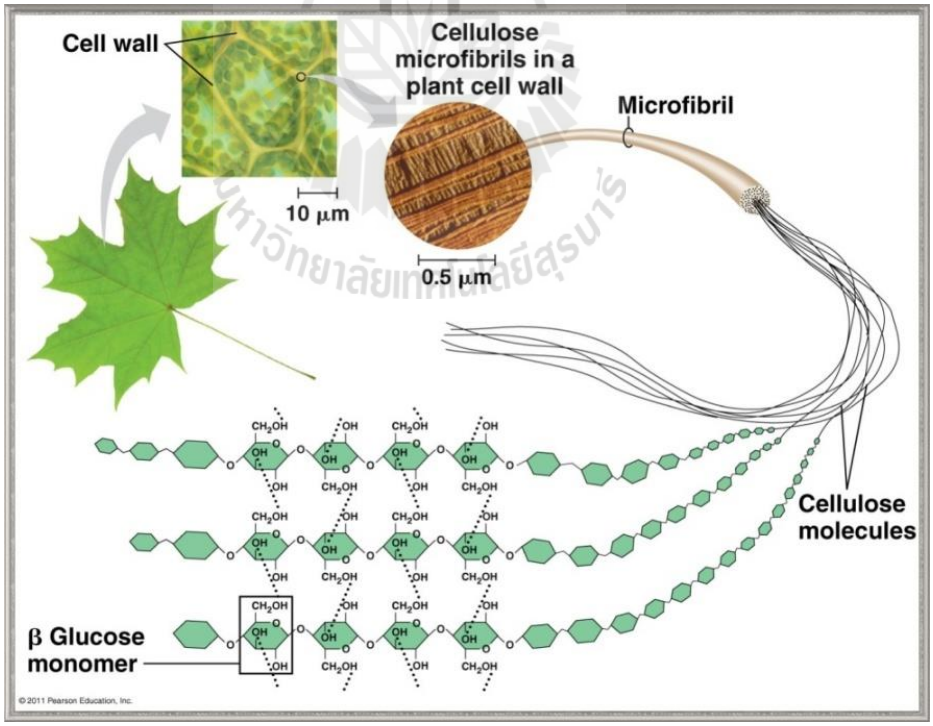

Figure 2.3 Schematic presentation of cellulose structure.

From : http://www.bio1151.nicerweb.com/Locked/media/ch05/cellulose.html.

2.2.2 Hemicellulose

Hemicellulose polysaccharides are found in all terrestrial plants, from woods, grasses and cereals. They were originally defined as those plant polysaccharides that could be separated from cellulose by extraction with alkali-water solutions. Hemicelluloses are closely associated in plant tissues with cellulose and lignin, and they are most often structural polysaccharides in these tissues. Hemicellulose in plants is a mixture of polysaccharides that are soluble in dilute acid. In secondary walls of plant cells, it is characterized by a linear xylan core polymer that consists of repeating units of β-1,4 linked xylose residues. Hemicelluloses are named according to the main sugar monomer unit in their backbone structure. Hemicelluloses are generally classified according to the main sugar residue in the backbone, e.g., xylans, mannans, and glucans, with xylans and mannans being the most prevalent (Figure 2.4). Thus, xylans are polymers with D-xylose units in the main chain and those with D-mannose, L-arabinose and D-galactose are referred to as mannans, arabinans and galactans, respectively. Xylan is the major component of hemicellulose and is, after cellulose, the second most abundant polysaccharide in nature. Xylans account for 30-35% of the cell wall material of annual plants (grasses and cereals), 15-30% of hardwoods and 7-10% of softwoods (Wilkie, 1979; Ladisch, Lin, Voloch, and Tsao, 1983). Due to the significant presence of xylans in plants it serves as a major constituent of animal feed.

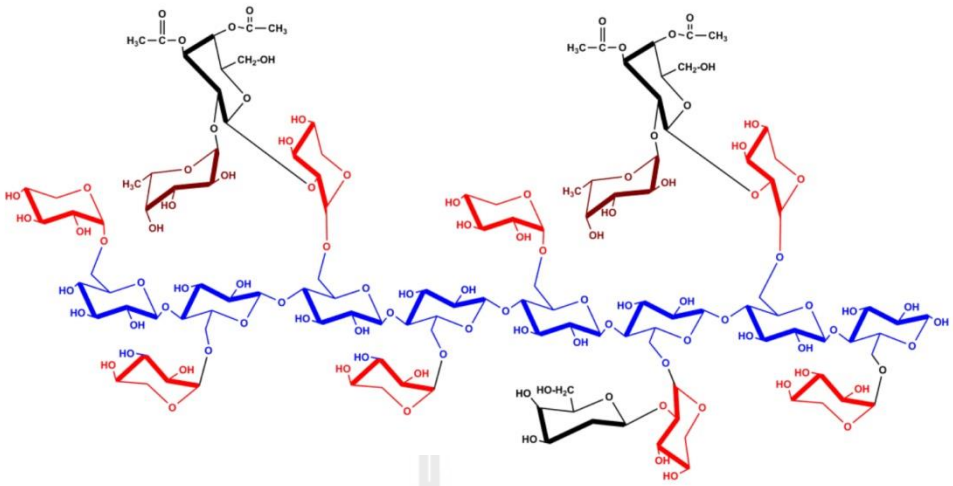

Figure 2.4 Hemicellulose structure.

From : Ochoa-Villarreal et al., 2012.

2.2.3 Pectin

Pectins are important both as cell wall components and as industrial gelling agents. Pectic polysaccharides are structurally complex and heterogeneous, they consist of a backbone of (1→4) α-D-galacturonosyl residues interrupted with typically a 10% substitution of (1→2)-α-L-rhamnopyranosyl residues. A fraction of the rhamnosyl residues are branch points for neutral sugar side-chains that contain L-arabinose and D-galactose. Pectins are noncellulosic acidic cell wall polysaccharides and are divided into three classes : homogalacturonan, rhamnogalacturonan I, and rhamnogalacturonan II. Pectins function as a sol-like matrix, providing water and ion retention, support and facilitation of cell wall modifying enzymes, cell wall porosity, cell-tocell adhesion, cell expansion, cell signaling, developmental regulation, and defense.

2.2.4 Lignin

Lignin is the second most abundant biological material on the planet, exceeded only by cellulose and hemicellulose, and comprises 15-25% of the dry weight of woody plants. This macromolecule plays a vital role in providing mechanical support to bind plant fibers together. Lignin also decreases the permeation of water through the cell walls of the xylem, thereby playing an intricate role in the transport of water and nutrients. Finally, lignin plays an important function in a plant's natural defense against degradation by impeding penetration of destructive enzymes through the cell wall.

2.3 Fibrolytic feed enzymes in ruminant animals

Feed enzymes for ruminants contain mainly cellulase and hemicellulase activities. Enzyme products are produced by batch fermentation process, beginning with a seed culture and growth media (usually containing nitrogen, carbohydrates, minerals, and surface active agents). Industrial fermentations usually take 3 to 7 d depending upon the microorganism and conditions for growth (Table 2.2). The types and activity of enzymes produced can vary widely depending on the strain selected and the growth substrate and culture conditions employed for enzyme production the focus of most enzyme-related research for ruminants has been on polysaccharidases that degrade plant cell wall. Cellulose and hemicellulose, the major structural polysaccharides in plants are converted to soluble sugars by enzymes collectively referred to as cellulase and hemicellulase as review by Beauchemin et al. (2004).

Table 2.1 Partial list of approved source organisms acceptable for use in ruminant feeds.

Activity	Source organism
Cellulase	*Aspergillus niger*, *Trichoderma longibrachiatum (T. reesei*, *T. viride), Humicola insolens*
Beta-glucanase	*Aspergillus niger*, *Aspergillus aculeatus*, *Bacillus lentus*, *Bacillus subtilis*, *Humicola insolens*, *Penicillum funiculosum*, *Trichoderma longibrachiatum (T. reesei*, *T. viride)*
Hemicellulase	*Aspergillus niger*, *Trichoderma longibrachiatum (T. reesei*, *T. viride), Bacillus lentus*, *Bacillus subtillis*, *Humicola insolens*, *Aspergillus aculeatus*
Xylanase	*Aspergillus niger*, *Bacillus lentus*, *Bacillus subtilis*, *Trichoderma longibrachiatum (T. reesei*, *T. viride)*, *Penicillum funiculosum*, *Humicola insolens*

From : Beauchemin et al., 2004.

2.3.1 Cellulases

Cellulases are a group of enzymes that hydrolyse cellulose or β-(1,4)-glucan. The major enzymes involved in cellulose hydrolysis are : endocellulase (endoglucanase, endo-β-1,4-glucanase, carboxymethyl cellulase or β-1,4-glucan glucanohydrolase; EC 3.2.1.4), exocellulase (exoglucanase, exo-β-1,4-glucanase, cellulose β-1,4-cellobiosidase; EC 3.2.1.91), and β-glucosidase (cellobiase or glucohydrolase, EC 3.2.1.21) (Table 2.2). In general, endoglucanases hydrolyze cellulose chains at random to produce cellulose oligomers of varying degree of

polymerization; the mode of action of each of these is : (1) endo-β-glucanase, 1,4-β-D-glucan glucanohydrolase, carboxymethyl cellulose : 'random' scission of cellulose chains yielding glucose and cello-oligosaccharides. (2) Exo-β-glucanase, 1,4-β-D-glucan cellobiohydrolases : exo-attack on the non-reducing end of cellulase with cellobiose as the primary structure. (3) L-Glucosidase, cellobiase : hydrolysis of cellobiose to glucose. The endo and exoglucanases are generally inhibited by cellobiose, and β-glucosidases are inhibited by glucose. The sites of cleavage by the major enzymes involved in cellulose hydrolysis are shown in Figure 2.5.

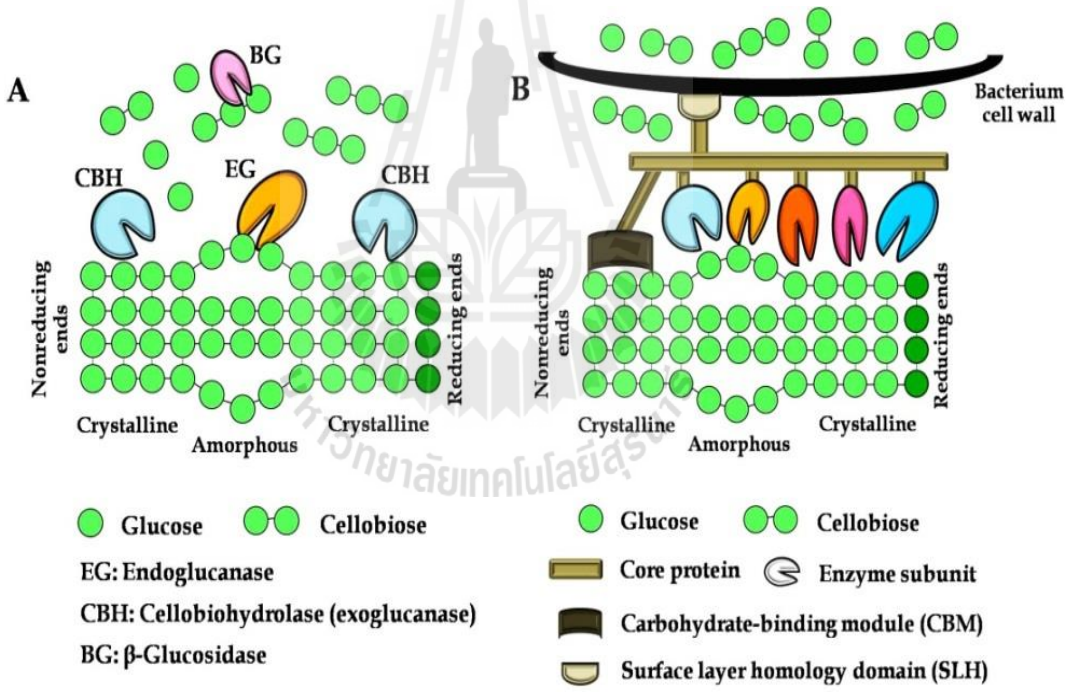

Figure 2.5 Simplified schematic of the hydrolysis of amorphous and microcrystalline celluloses by non-complexed (A) and complexed (B) cellulase systems.

From : Ratanakhanokchai et al., 2013.

2.3.2 Xylanase

Xylanases are produced by free-living and gut microorganisms and have also been found from algae, protozoa, snails, crustaceans and seeds of terrestrial plants. The main enzymes involved in degrading the xylan core polymer to soluble sugars are xylanases (EC 3.2.1.8) and β-1,4 xylosidase (EC 3.2.1.37) (Bhat and Hazlewood 2001). The xylanases include endoxylanases, which yield xylooligomers, and β-1,4-xylosidases, which yield xylose. Other hemicellulase enzymes involved primarily in the digestion of side chains include β-mannosidase (EC 3.2.1.25), α-L-arabinofuranosidase (EC 3.2.1.55), α-D-glucuronidase (EC 3.2.1.139), α-D-galactosidase (EC 3.2.1.22), acetyl-xylan esterases (3.1.1.72) and ferulic acid esterase (EC 3.1.1.73) (Table 2.2). A schematic representation of the enzymes involved in the degradation of arabinoxylan is given in Figure 2.6

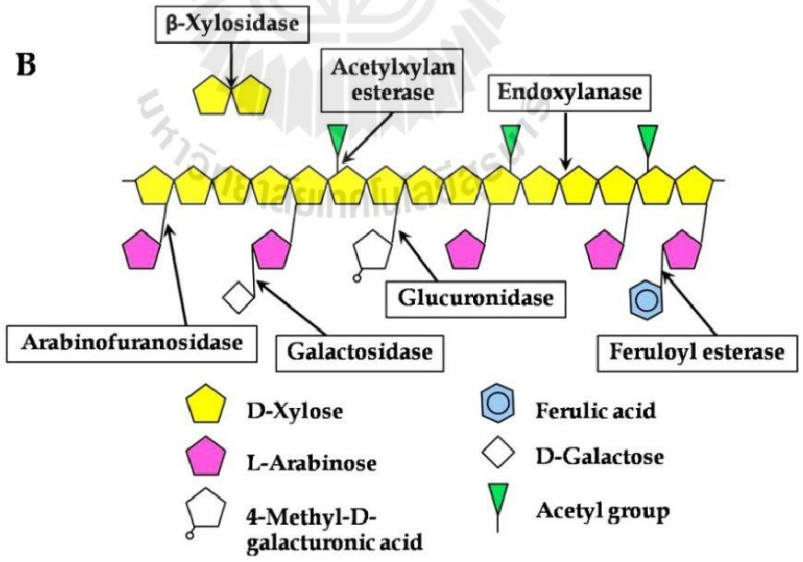

Figure 2.6 Schematic representation of the enzymes involved in the degradation of xylan.

From : Ratanakhanokchai et al., 2013.

Table 2.2 Major enzymes of plant cell wall hydrolysis.

Plant cell wall polymer	Enzyme	EC number	Substrate	Bonds hydrolyzed	Endproducts
Cellulose	Endo-β-1,4-glucanase	3.2.1.4	Cellulose	β-1,4-glucose linkages	Cellooligomers
	Exo-β-1,4-glucanase	3.2.1.91	Cellulose at Reducing end	β-1,4-glucose linkages	Cellobiose
	β -glucosidase	3.2.1.21	Cellobiose	β-1,4- glucose linkages	Glucose
Hemicellulose	Endo- β-1,4-xylanase	3.2.1.8	Xylan	β-1,4-xylose linkages	Xylooligomers
	β -1,4-xylosidase	3.2.1.37	Xylobiose	β-1,4-xylose linkages	Xylose
	α -L-arabinofuranosidase	3.2.1.55	Arabinoxylan	α-1-3 linkage	Arabinoxylan and arabinose
	α -glucuronidase	3.2.1.139	Glucuronoxylan	α-1-3 or α-1-2 linkage	Glucuronic acid and xylan
	Acetyl-xylan esterase	3.1.1.72	Acetylxylan Ferulic acid	acetylester bond	Acetate and xylan
	Ferulic acid esterase	3.1.1.73	Cross bridge or Linkage	feruloylester bond	Ferulic acid and xylan

Table 2.2 Major enzymes of plant cell wall hydrolysis (Continued).

Plant cell wall polymer	Enzyme	EC number	Substrate	Bonds hydrolyzed	Endproducts
Hemicellulose	Endo-1,3(4)- β-glucanase	3.2.1.6	Laminarin,β-D-Glucan	β-1,3- 1,4-hexose linkages in β-D-glucans	Laminarin oligomers β-D-glucan,oligomers
	Lichenase	3.2.1.73	Lichenan, β-D-Glucan	β-1,4-hexose linkages in β-D-glucans containing 1,3- and 1,4-bonds	Lichenan oligomers, β-D-glucan oligomers

From : Beauchemin et al., 2004.

2.4 Utilization of fiber in ruminant animals

Ruminants make up a significant proportion of the domesticated animal species worldwide, and among farmed livestock are the best adapted to utilization of plant cell walls. The degradation of plant cell walls by ruminants is of major economic importance in the developed as well as developing world. Rumen fermentation is unique in that efficient plant cell wall degradation relies on the cooperation between microorganisms that produce fibrolytic enzymes and the host animal that provides an anaerobic fermentation chamber. However, the utilization of fiber in mature forages by rumen microbes is slow and incomplete because of high cell wall and lignin content, that physical and structural barriers such as waxes and the cuticle of the epidermis limit rumen microbes and enzymes access to tissues of mature forages (Wilson and Kennedy, 1996). Increasing the efficiency with which the ruminal microorganisms degrades fiber has been the subject of extensive research. Therefore, the use of exogenous fibrolytic enzymes is an emerging technology that shows potential in terms of improving the utilization of forages by ruminants (Beauchemin et al., 2004).

2.4.1 Rumen fermentations

Carbohydrate polymers in plants are indigestible to most animals but can be hydrolyzed and fermented by a range of microorganisms in the rumen. Major end-products from the microbial fermentation are made available in return to the animal host (Weimer, 1998; Krause, Denman, Mackie, Morrison, Rae, Attwood, and McSweeney, 2003). The end products of this fermentation are volatile fatty acids (VFA; acetic, propionic, butyric), which form a major metabolic energy for the ruminant, and microbial protein synthesis (MPS) that are a major source of protein

and amino acids when absorbed in the lower digestive tract of the animal. The VFA are absorbed through the rumen wall, and serves as the primary source of energy for mucosal tissue and the host animal. The absorption of VFA may account up to 75 to 80% of the digestible energy requirement of the animal host, while MPS leaving the rumen may represent about 64% of metabolizable protein absorbed in its small intestine (NRC, 2001). Ruminants consume high fiber diets result in high levels of acetate and butyrate, while high cereal diets promote high levels of propionate (Beever and Mould, 2000). Acetate and butyrate are essential as energy and precursor source in milk fat synthesis in dairy rations (Mertens, 1997; Van Soest, 1994); and propionate is the primary precursor for synthesis of glucose.

2.5 Exogenous fibrolytic enzymes in an *in vitro* study

Cellulases and xylanases are respectively two major fibrolytic enzyme groups that specified to break ß-1-4 linkages joining sugar molecules of cellulose and xylan found in plant cell wall components (Beauchemin et al., 2004). A very useful *in vitro* technique to measure effects of exogenous enzyme treatment of forages is the i*n vitro* gas production technique, a summary of the most important findings thereof is given in Table (2.3). Eun and Beauchemin (2007a) evaluated the effects of 4 feed enzyme products that varied in enzymatic activities on the degradation of alfalfa hay and corn silage. They found that for all fibrolytic enzymes increased gas production and degradation of dry matter and fiber of alfalfa hay after 24 h incubation. For corn silage, after 24 h incubation none of the fibrolytic enzyme increased gas production or DM degradation, but all fibrolytic enzymes increased NDF degradation. Similarly, Eun et al. (2007a) reported that *in vitro* degradability of NDF and ADF from alfalfa

hay and corn silage were increased by exogenous fibrolytic enzymes after 24 h incubation, but the response depended upon the enzyme and its dose, with some additives effective for both forages when added at 1.4 mg/g of DM. In addition, Giraldo, Tejido, Ranilla, and Carro (2008) evaluated the effects of three fibrolytic enzymes (xylanase from *Trichoderma viride* (XYL) and fibrolytic enzymes from *Aspergillus niger* (ASP) and *Trichoderma longibrachiatum* (TR) on the fermentation of three substrates composed of grass hay : concentrate in the proportions (DM) basis of 0.7 : 0.3, 0.5 : 0.5, and 0.3 : 0.7. They found that all enzymes increased the true degradability of substrate DM and the production of acetate, propionate, total VFA and gas after 8 h incubations, and after 24 h incubation some of the observed effects disappeared, but all enzymes still increased the degradability of substrate acid detergent fiber. In other studies, also reported supplementation of fibrolytic enzyme feed additives that showed potential in terms of improving the *in vitro* rumen degradability of alfalfa hay (Eun et al., 2007a; Eun and Beauchemin, 2007b; Holtshausen et al., 2011), grass hay (Krueger and Adesogan, 2008; Yang, Son, and Beauchemin, 2011), corn stalks (Yang and Xie, 2010), corn silage (Eun and Beauchemin, 2007a), total mixed ration (TMR) (Bowman, Beauchemin, Shelford, 2002) and rice straw (Yang et al., 2011).

Table 2.3 Summary of the most important findings the effects of exogenous fibrolytic enzyme on *in vitro* ruminal fermentation of forage.

Reference	Enzyme	Substrate	Incubation Time (h)	Result
Bowman et al. (2002)	fibrolytic enzyme	TMR	12	Increased degradation of DM
Eun and Beauchemin (2007a)	4 fibrolytic enzyme (endoglucanase, exoglucanase, xylanase, and protease)	Corn silage and alfafal hay	24	Increased gas production, degradation of DM and fiber depended upon enzyme
Eun and Beauchemin (2007b)	13 endoglucanases 10 xylanases	Alfalfa hay	18	Increased gas production and organic matter degradation, total VFA was not affected
Eun and Beauchemin (2007c)	8 fibrolytic enzyme (endoglucanase and xylanase)	Corn silage	24	Only one product improved fiber degradability, enzyme high in endoglucanase and low xylanase have the potential for corn silage
Eun at al. (2007a)	5 fibrolytic enzyme (endoglucanase and xylanase)	Alfalfa hay, Corn silage	24	Increased GP, degradation of fiber, optimum Dose rate was 1.4 mg/ g of DM

Table 2.3 Summary of the most important findings the effects of exogenous fibrolytic enzyme on *in vitro* ruminal fermentation of forage (Continued).

Reference	Enzyme	Substrate	Incubation Time (h)	Result
Eun at al. (2007b)	2 proteases, 3 proportion of endoglucanase and xylanase	Alfalfa hay	24	Increased degradation of fiber depended upon enzyme
Giraldo et al. (2008)	Fibrolytic enzymes from *Trichoderma viride*, *Aspergillus niger* and *Trichoderma longibrachiatum*	Grass hay	24	Increased degradation of DM, total VFA, acetate and propionate.
Holtshausen et al. (2011)	Developmental fibrolytic enzyme additive (AB Vista, Marlborough, UK)	Alfalfa hay, alfalfa silage, corn silage	24	Increased degradation of DM, NDF and ADF depended upon dose rate
Krueger and Adesogan (2008)	Combinations of Ferulic acid esterase, cellulase and xylanase	Grass hay	24	Increased degradation of DM, propionate, butyrate. Decreased acetate
Yang and Xie (2010)	18 commercial enzyme products	Corn stalks	24	Increased degradation of DM

Table 2.3 Summary of the most important findings the effects of exogenous fibrolytic enzyme on *in vitro* ruminal fermentation of forage (Continued).

Reference	Enzyme	Substrate	Incubation time (h)	Result
Yang et al. (2011)	26 enzyme additives (endoglucanase, xylanase, β-glucanase, α- amylase, and protease activities)	Alfalfa hay, rice straw	48	Increased degradation of DM, NDF and ADF depended upon enzyme

2.6 Exogenous fibrolytic enzymes in an *in vivo* study

The principal rationale for the use of enzymes is to improve the nutritive value of feedstuffs by increasing the efficiency of feed utilization in ruminants and reduce waste production (Beauchemin et al., 2004). Not all exogenous fibrolytic enzymes are effective to improve the nutritive value of feedstuffs depended upon type of enzyme additives. Alvarez, Pinos-Rodriguez, Herrera, Garcia, Gonzalez, and Barcena (2009) studied the effect of two commercial fibrolytic enzyme products on rumen digestibility in steers fed high fiber diets. They found that NDF disappearance rate, ADF potential disappearance and ADF disappearance rate of oat straw were increased by supplementation of two enzyme additives. However, dry matter intake (DMI) was not affected by enzyme additives. Chung, Zhou, Holtshausen, Alexander, McAllister, Guan, Oba, and Beauchemin (2012) also reported that DMI was not affected by enzyme, averaging 25.6, 24.4, and 25.5 kg/d for the control, low, and high enzyme diet, respectively. However, Lewis et al. (1999) reported that supplementation of fibrolytic enzyme increased DMI when enzyme was applied to forage. In contrast, Holtshausen et al., (2011) reported that 60 dairy cows in early lactation were fed diets containing no enzyme, low enzyme (Econase RDE; 0.5 ml of enzyme/kg of diet DM), and high enzyme (Econase RDE; 1.0 ml of enzyme/kg of diet DM). They found that DMI was decreased by supplementation of enzyme to the diet, with the decline in intake proportional to the dose of the enzyme. Supplementation of exogenous fibrolytic enzymes has improvement of microbial activities in the rumen with positive enhancement on the animal performance (Lewis et al., 1999; Rode et al., 1999). Gado, Salem, Robinson, and Hassan (2009) studied effects of a mixture of exogenous enzymes (ZADO®) on ruminal fermentation, feed intake, digestibility, as well as milk

production and composition in cows fed total mixed rations. They found that enzyme supplementation increased DIM and digestibility of DM, NDF and ADF in the total tract of supplemented cows. Supplementation of enzymes also increased milk and milk protein production (12.8-15.7 and 0.45-0.57 kg/d respectively. Similarly, Nowak, Kruczynska, and Grochowska (2003) also reported that exogenous fibrolytic enzymes increased DM, NDF and ADF disappearances of wheat straw and TMR. Some studies have shown substantial to increased weight gain of steers up to 30% (Beauchemin, Rode, and Sewalt, 1995). Beauchemin, Rode, and Karren (1999) also reported that enzyme supplementation had no effect on dry matter intake but increased average daily gain by 9% (1.40 to 1.53 kg d^{-1}) and numerically improved feed-to-gain ratio by 10% (7.72 to 6.95 kg dry matter kg^{-1} gain). In other studies, also reported supplementation of fibrolytic enzyme feed additives that showed potential in terms of increased the DMI (Beauchemin, Rode, Maekawa, Morgavi, and Kampen, 2000), numbers of total viable bacteria in (Nsereko, Beauchemin, Morgavi, Rode, Furtado, McAllister, Iwaasa, Yang, and Wang, 2002), milk production efficiency (Holtshausen et al., 2011), milk production (Kung, Treacher, Nauman, Smagala, Endres, and Cohen, 2000; Lewis et al., 1999; Rode et al., 1999; Yang, Beauchemin, and Rode, 1999). However, the effects were inconsistent with some studies reporting no effects on *in vivo* digestibility or animal performance (Lewis et al., 1999; Knowlton et al., 2002; Vicini, Bateman, Bhat, Clark, Erdman, Phipps, Van Amburgh, Hartnell, Hintz, and Hard, 2003). The use of exogenous fibrolytic enzymes as an emerging technology has potential for improving fiber digestion (*in vitro*), forage utilization, and production by ruminants. However, the effective of enzyme depending upon several factors such as enzyme activity, type and dose of enzyme, type of diet, enzyme application

method, and animal physiological status. Therefore, that factor is needed to be considered before use in ruminant animals.

2.7 References

Alvarez, G., Pinos-Rodriguez, J.M., Herrera, J.G., Garcia, J.C., Gonzalez, S.S., and Barcena, R. (2009). Effects of exogenous fibrolytic enzymes on ruminal digestibility in steers fed high fiber rations. **Livest. Sci.** 121: 150-154.

Beauchemin, K.A., Colombatto, D., and Morgavi, D.P. (2004). A rationale for the development of feed enzyme products for ruminants. **Can. J. Anim. Sci.** 84: 23-36.

Beauchemin, K.A., Rode, L.M., and Karren, D. (1999). Use of feed enzymes in feedlot finishing diets. **Can. J. Anim. Sci.** 79: 243-246.

Beauchemin, K.A., Rode, L.M., Maekawa, M., Morgavi D.P., and Kampen, R. (2000). Evaluation of a nonstarch polysaccharidase feed enzyme in dairy cow diets. **J. Dairy Sci.** 83: 543-553.

Beauchemin, K.A., Rode, L.M., and Sewalt, V.J.H. (1995). Fibrolytic enzymes increase fiber digestibility and growth rate of steers fed dry forages. **Can. J. Anim. Sci.** 75: 641-644.

Beever, D.E, and Mould, F.L. (2000). Forage evaluation for efficient ruminant livestock production. In: D.L. Givens, E. Owen, R.F.E. Axford, and H.M. Omed (eds.). **Forage evaluation in ruminant nutrition** (pp 15-42). CAB inter.

Bhat, M.K., and Hazlewood, G.P. (2001). Enzymology and other characteristics of cellulases and xylanases. In: M.R. Bedford and G.G. Partridge (eds.).

Enzymes in farm animal nutrition (pp 11-60). UK, CABI Publishing.

Bowman, G.R., Beauchemin, K.A., and Shelford, J.A. (2002). The proportion of the diet to which firbolytic enzyme are added affects nutrient digestion by lactating dairy cows. **J. Dairy Sci.** 85: 3420-3429.

Chung, Y.H., Zhou, M., Holtshausen, L., Alexander, T.W., McAllister, T.A., Guan, L.L., Oba, M., and Beauchemin, K.A. (2012). A fibrolytic enzyme additive for lactating Holstein cow diets: Ruminal fermentation, rumen microbial populations and enteric methane emissions. **J. Dairy Sci.** 95: 1419-1427.

Eun, J.S., and Beauchemin, K.A. (2007a). Enhancing *in vitro* degradation of alfalfa hay and corn silage using feed enzymes. **J. Dairy Sci.** 90: 2839-2851.

Eun, J.S., and Beauchemin, K.A. (2007b). Assessment of the efficacy of varying experimental exogenous fibrolytic enzymes using *in vitro* fermentation characteristics. **Anim. Feed Sci. Technol.** 132: 298-315.

Eun, J.S., and Beauchemin, K.A. (2007c). Assessment of the potential of feed enzyme additives to enhance utilization of corn silage fibre by ruminants. **Can J. Anim. Sci.** 88: 97-106.

Eun, J.S., Beauchemin, K.A. and Schulze, H. (2007a). Use of exogenous fibrolytic enzymes to enhance *in vitro* fermentation of alfalfa hay and corn silage. **J. Dairy Sci.** 90: 1440-1451.

Eun, J.S., Beauchemin, K.A., and Schulze, H. (2007b). Use of an *in vitro* fermentation bioassay to evaluate improvements in degradation of alfalfa hay due to exogenous feed enzymes. **Anim. Feed Sci. Technol.** 135: 315-328.

Fisher, D.S., Burns, J.C., and Moore, J.E. (1995). The nutritive evaluation of forage. In: R.F. Barnes, D.A. Miller, and C.J. Nelson (eds.). **Forages: The science of grassland** (5 th ed., pp 105-116).

Gado, H.M., Salem, A.Z.M., Robinson, P.H., and Hassan, M. (2009). Influence of exogenous enzymes on nutrient digestibility, extent of ruminal fermentation as well as milk production and composition in dairy cows. **Anim. Feed Sci. Technol.** 154: 36-46.

Giraldo, L.A., Tejido, M.L., Ranilla, M.J., and Carro, M.D. (2008). Effects of exogenous fibrolytic enzymes on *in vitro* ruminal fermentation of substrates with different forage:concentrate ratios. **Anim. Feed Sci. Technol.** 141: 306-325.

Holtshausen, L., Chung, Y.H., Gerardo-Cuervo, H., Oba, M., and Beauchemin, K.A. (2011). Improved milk production efficiency in early lactation dairy cattle with dietary addition of a developmental fibrolytic enzyme additive. **J. Dairy Sci.** 94: 89-907.

Knowlton, K.F., McKinney, J.M., and Cobb, C. (2002). Effect of a direct-fed fibrolytic enzyme formulation on nutrient intake, partitioning, and excretion in early and late lactation Holstein cows. **J. Dairy Sci.** 85: 3328-3335.

Krause, D.O., Denman, S.E., Mackie, R.I., Morrison, M., Rae, A.L., Attwood, G.T., and McSweeney, C.S. (2003). Opportunities to improve fiber degradation in the rumen: Microbiology, ecology and genomics. **FEMS Microbiology Reviews.** 27: 663-693.

Krueger, N.A., and Adesogan, A.T. (2008). Effects of different mixtures of fibrolytic enzymes on digestion and fermentation of bahiagrass hay. **Anim. Feed Sci. Technol.** 145: 84-94.

Kung, L.Jr., Treacher, R.J., Nauman, G.A., Smagala, A.M., Endres, K.M., and Cohen, M.A. (2000). The effect of treating forages with fibrolytic enzymes on its nutritive value and lactation performance of dairy cows. **J. Dairy Sci.** 83:

115-122.

Ladisch, M.R., Lin, K.W., Voloch, M., and Tsao, G.T. (1983). Process considerations in the enzymatic hydrolysis of biomass. **Enzyme and Microbial Technology.** 5: 82-102.

Lewis, G.E., Sanchez, W.K., Hunt, C.W., Guy, M.A., Pritchard, G.T., Swanson, B.I., and Treacher, R.J. (1999). Effect of direct-fed fibrolytic enzymes on the lactational performance of dairy cows. **J. Dairy Sci.** 82: 611-617.

McDougall, G.J., Morrison, I.M., Stewart, D., and Hillman, J.R. (1996). Plant cell walls as dietary fibre: Range, structure, processing and function. **J. Sci. Food. Agric.** 70: 133-150.

Mertens, D.R. (1997). Creating a system for meeting the fibre requirements of dairy cows. **J. Dairy Sci.** 80: 1463-1481.

Nowak, W., Kruczynska, H., and Grochowska, S. (2003). The effect of fibrolytic enzymes on dry matter, ADF, and NDF ruminal disappearance and intestinal digestibility. **Czech J. Amin. Sci.** 48: 191-196.

NRC. (2001). **Nutrient requirement of dairy cattle** (7th ed.). Washington DC: Natl. Acad. Press.

Nsereko, V.L., Beauchemin, K.A., Morgavi, D.P., Rode, L.M., Furtado, A.F., McAllister, T.A., Iwaasa, A.D., Yang, W.Z., and Wang, Y. (2002). Effect of a fibrolytic enzyme preparation from *Trichoderma longibrachiatum* on the rumen microbial population of dairy cows. **Can. J. Microbiol.** 48: 14-20.

Ochoa-Villarreal, M., Aispuro-Hernández, E., Martínez-Téllez, M.A., and Irasema Vargas-Arispuro, I. (2012). **Plant cell wall polymers: Function, structure and biological activity of their derivatives** [on-line]. Available:

http://www.intechopen.com/books/polymerization/plant-cell-wall-polymers-function-structure-and-biological-activity-of-their-derivatives.

Ratanakhanokchai, K., Waeonukul, R., Pason, P., Tachaapaikoon, Ch., Kyu, K.L., Sakka, K., Kosugi, A., and Mori, Y. (2013). Paenibacillus curdlanolyticus Strain B-6 Multienzyme Complex: A Novel System for Biomass Utilization. **Biomass Now - Cultivation and Utilization.**

Rode, L.M., Yang, W.Z., and Beauchemin, K.A. (1999). Fibrolytic enzyme supplements for dairy cows in early lactation. **J. Dairy Sci.** 82: 2121-2126.

Sticklen, M.B. (2008) Plant genetic engineering for biofuel production: towards affordable cellulosic ethanol. **Nat. Rev. Genet.** 9: 433-443.

Van Soest, P.J. (1994). **Nutritional ecology of the ruminant** (2nd ed.). New York: Cornell University Press.

Vicini, J.L., Bateman, H.G., Bhat, M.K., Clark, J.H., Erdman, R.A., Phipps, R.H., Van Amburgh, M.E., Hartnell, G.F., Hintz, R.L., and Hard, D.L. (2003). Effect of feeding supplemental fibrolytic enzymes or soluble sugars with malic acid on milk production. **J. Dairy Sci.** 86: 576-585.

Weimer, P.J. (1998). Manipulating ruminal fermentation: A microbial ecological perspective. **J. Anim. Sci.** 76: 3114-3122.

Wilkie, K.C.B. (1979). The hemicelluloses of grasses and cereals. **Adv. Carbohydr. Chem. Biochem.** 36: 215-264.

Wilson, J.R., and Kennedy, P.M. (1996). Plant and animal constraint to voluntary feed intake associated with fiber characteristics and particle breakdown and passage in ruminants. **Austr. J. Agric. Res.** 47: 199-225.

Yang, H.J., and Xie, C.Y. (2010). Assessment of fibrolytic activities of 18 commercial enzyme products and their abilities to degrade the cell wall fraction of corn

stalks in in vitro enzymatic and ruminal batch cultures. **Anim. Feed Sci. Technol.** 159: 110-121.

Yang, W.Z., Beauchemin, K.A., and Rode, L. (1999). Effects of an enzyme feed additive on extent of digestion and milk production of lactating dairy cows. **J. Dairy Sci.** 82: 391-403.

Yang, H.E., Son, Y.S., and Beauchemin, K.A. (2011). Effects of exogenous Enzymes on ruminal fermentation and degradability of alfalfa hay and rice straw. **Asian-Aust. J. Anim. Sci.** 24: 56-64.

http://www.bio1151.nicerweb.com/Locked/media/ch05/cellulose.html.

http://www.phschool.com/science/biology_place/biocoach/plants/walls.html.

CHAPTER III

USE OF FIBROLYTIC ENZYMES ADDITIVES TO ENHANCE *IN VITRO* RUMINAL FERMENTATION OF CORN SILAGE

3.1 Abstract

Two experiments were conducted to evaluate the effect of four enzyme additives on ruminal fermentation of corn silage using a 48 h batch culture *in vitro* assay with buffer and ruminal fluid. Experiment 1 (Exp. 1) and Experiment 2 (Exp. 2) were conducted as completely randomized designs each with two runs and four replicates. The enzyme additives (E1, E2, E3, and E4) were commercial products that provided a range in endoglucanase, exoglucanase, and xylanase activities. For xylanase (birch wood and oat spelt substrate) and endoglucanase (carboxymethylcellulose substrate), the enzyme products (per ml) were ranked E4>E1>E2>E3. In Exp. 1, the four enzymes were added at 0, 2, 4, and 8 μl/g of corn silage dry matter (DM), whereas in Exp. 2 enzymes were added at 0, 0.5, 1, 2, and 4 μl/g DM. Gas production (GP) was measured at 3, 6, 12, 18, 24, and 48 h after incubation. Degradability of DM (DMD), neutral detergent fiber (NDFD), and acid detergent fiber (ADFD), and volatile fatty acid concentrations (VFA; total and individual molar proportions) were determined after 24 and 48 h. In Exp. 1, E1 and E2 had higher NDFD and ADFD at 24 and 48 h of incubation ($P<0.001$) compared with

E3 and E4. Increasing dose rate increased NDFD and ADFD for all enzymes (except ADFD for E4 at 48 h), with the optimum dose rate dependant on the enzyme additive (dose enzyme; $P<0.01$). There were some treatment effects on DMD and total GP at 24 and 48 h, but these responses were not consistent with responses in NDFD and ADFD. Experiment 2 was conducted to confirm the effects and optimum dose rate of each enzyme additive. In Exp. 2, DMD was not affected by enzyme after 24 and 48 h incubation. There were no enzyme dose interactions for DMD, NDFD, or ADFD after 24 or 48 h of incubation (except for ADFD at 48 h). After 24 h, DMD, NDFD, and ADFD increased linearly with increasing dose ($P<0.05$); after 48 h DMD increased linearly, whereas NDFD increased quadratically with increasing enzyme dose ($P<0.05$). The ADFD increased linearly after 48 h for E3 and E4, but after 48 h ADFD increased quadratically for E1 and E2. Total GP was consistently lowest for E4 at both incubation times ($P<0.05$). There were no enzyme dose interactions ($P>0.05$) for any of the fermentation variables at either 24 or 48 h of incubation in Exp. 2. There were differences amongst the additives for total VFA at 24 and 48 h ($P<0.05$); increasing enzyme dose decreased total VFA after 24 h but increased total VFA at 48 h, such that all doses were higher than the control ($P<0.001$). Overall, the enzyme additives increased NDFD and ADFD of corn silage *in vitro*; however, E1 and E2 were more effective than E3 or E4. Responses to increasing dose of enzyme were generally linear or curvilinear, and the optimum dose rate differed amongst the products evaluated. Evaluation of the enzymes at 24 and 48 h generally led to the same ranking of the additives, and the degradation of NDF and ADF was more useful in differentiating the enzymes compared with DM and total GP.

3.2 Introduction

Supplementation of enzyme feed additives in ruminant diets have potential to improve forage utilization. Enzyme products containing mainly cellulase and xylanase activities degrade cellulose and hemicellulose, that the major constituents of plant cell walls. Some studies have shown that enzyme additives increase *in vitro* (Yang and Xie, 2010; Eun et al., 2007) and *in vivo* (Yang et al., 2000) fiber digestion. It is important to establish optimum dose rate of specific enzyme products because dose rate directly affects the cost : benefit ratio of feeding enzymes. Therefore, the effects of enzyme products and optimum dose rates need to screen using *in vitro* methods before enzyme products can be used in commercial ruminant farm, because *in vitro* method can screen large number of samples or treatments and low cost. This part of our study focused on *in vitro* screening method with corn silage as substrate, because corn silage is the major forages fed to dairy cattle in many parts of the world.

3.3 Objectives

The objective of the present study was to evaluate the potential of various enzyme additives with different enzyme activities to enhance *in vitro* ruminal fiber degradation and fermentation profile of corn silage, and to determine the optimum dose rate of individual enzyme product.

3.4 Materials and methods

Experiment 1

The experiment was conducted as a completely randomized design with 2 runs (batches) and 4 replicates per run with 16 treatments arranged as a factorial (4 enzyme

additives × 4 doses). The runs were conducted on separate days and the corn silage was used as substrate. 4 commercial enzyme feed additives (E1, E2, E3, and E4) were used in this study, and 4 different doses were used for each enzyme : 0, 2, 4, and 8 µl/g DM of substrate.

3.4.1 Substrate and enzyme preparation

The corn silage was dried at 55°C until dry (48 h) and was ground with mill (Wiley mill standard model 4, Arthur H. Thomas, Philadelphia, PA, USA) through 1 mm screen. Approximately 0.9 g DM of the ground corn silage was weighed into an acetone washed and preweighed filter bag (F57, Ankom Technology, Macedon, NY). Four replications were prepared for each treatment for each batch culture incubation time. The enzyme products were diluted with water and then added (200 µl) directly onto substrates (corn silage) in the filter bags (before sealing) at 4 doses of each enzyme : 0 (control), 2, 4, and 8 µl/g of substrate DM. The bag was heat-sealed, and then placed into a 125 ml bottle and incubated at room temperature for 3 h.

3.4.2 *In vitro* fermentations

Rumen fluid was collected from 3 cannulated cows approximately 3 h after the morning feeding, and strained through 4 layers of cheesecloth into a flask and flushed with oxygen-free CO_2. Rumen fluid was transported in insulated flasks to the laboratory within less than 1 h of collection. Anaerobic buffer medium (60 ml; Goering and Van Soest, 1970) containing tryptone, buffer, macro and micro mineral solution, resazurin and water (see Appendix) was adjusted to pH 6.0 using 1 *M* trans-aconitic acid (Sigma Chemicals, St. Louis, MO), and then added to each bottle. In addition to buffer, rumen fluid (15 ml) was added to each bottle in a ratio of 1 : 4 (rumen fluid : anaerobic buffer medium) under continuous flushing with CO_2. The bottles were closed with rubber stoppers and aluminium seal caps immediately after

loading and the bottles were incubated at 39°C on a rotary shaker for 24 and 48 h. Negative control (rumen fluid plus anaerobic buffer medium) and blanks (filter bags plus anaerobic buffer medium and rumen fluid) were also incubated using 4 replications for correction of gas production and degradability, respectively. Head space gas production (GP) resultant of substrate fermentation was measured at 3, 6, 12, 18, 24, and 48 h post incubation. The GP was measured by inserting a 23 gauge (0.6 mm) needle attached to a pressure transducer connected to a visual display. After 24 and 48 h of incubation, 4 bottles for each treatment were removed from the incubator, gas pressure was measured, and then bottles were placed on cold water to stop the fermentation. The gas pressure was converted to gas volume using the equation reported by Mauricio et al. (1999).

$$\text{Gas volume} = 0.18 + (3.697 \times \text{gas pressure}) + (0.0824 \times \text{gas pressure}^2)$$

Total cumulative gas production at 24 and 48 h were calculated by summing the gas volumes at each previous measurement time.

3.4.3 Determination of VFA at 24 and 48 h

Concentration of VFA was measured at 24 and 48 h incubation after measuring gas. A 5 ml sample of fluid was added to 1 ml of 25-% meta-phosphoric acid for measurement of VFA concentrations. The VFA concentration was analyzed by using a gas chromatograph (model 5890, Hewlett-Packard Lab, Palo Alto, CA) with a capillary column (30 m 0.32 mm i.d., 1μm phasethickness, Zebron ZB-FAAP, Phenomenex, Torrance, CA), and flame ionization detection. The oven temperature was 150°C (no hold time), which was then increased by 20°C/min to 210°C, and held at this temperature for 2 min. The injector temperature was 225°C, the detector temperature was 250°C, and the carrier gas was helium.

3.4.4 DM, NDF, and ADF degradability

After 24 h and 48 h of incubation, the filter bags were removed (4 filter bags for each time and treatment) from bottles and washed under a stream of cold water until the water ran clear. The bags were dried in an oven at 55°C for 48 h to completely dry and DM degradability was determined by the loss of DM from the bags. The contents of the bags were then assayed for NDF and ADF content, and NDF and ADF degradability were calculated (NDFD and ADFD). The NDF and ADF analyses were conducted sequentially using an ANKOM200 Fiber analyzer unit based on the procedure described by Van Soest et al. (1991). Sodium sulfite (10 g/l NDF solution) and heat-stable bacterial amylase (2 ml/l NDF solution) were used in the analysis of NDF.

3.4.5 Enzyme activity

The enzyme additives were analyzed for their endoglucanase, exoglucanase, xylanase and α-amylase activities, according to procedures recommended by Colombatto and Beauchemin (2003). The substrates used respectively were medium-viscosity carboxymethylcellulose (Catalog no. C-5678), cellulose (Sigmacell Cellulose; Catalog no. S-3504), xylan (oat spelt, Catalog no. X-0627; birch wood, Catalog no. X-0502), and starch (Catalog no. S-3504) with all substrates sourced from Sigma Chemical (St. Louis,MO, USA). The assay conditions were 39°C and pH 6.0 to reflect the average pH conditions in the rumen of a dairy cow. A 1% diluted substrate solution (1.0 ml) and 0.1 M citrate phosphate buffer (0.9 ml) were added to test tubes in triplicate and allowed to pre-warm in a water bath at 39°C. The reaction was initiated by adding 0.1 ml of prewarmed diluted enzyme solution (diluted in buffer). Incubations were allowed to continue for exactly 15, 120, 5, and 10 min for endoglucanase, exoglucanase, xylanase, and α-amylase, respectively. The reaction

was terminated by adding 3.0 ml of 3,5-dinitrosalicylic acid solution. Substrate blanks (triplicate) were prepared by adding 1 ml of diluted substrate, 0.9 ml of buffer and 0.1 ml of distilled water. Enzyme blanks were prepared by adding 0.1 ml of diluted enzyme, 0.9 ml of buffer and 1.0 ml of distilled water. After termination of the reaction with dinitrosalicylic acid, tubes were capped with marbles and boiled for 5 min in a water bath. To determine enzymatic activity, 200 µl of the reaction contents were transferred in duplicate into a microtiter plate and absorbance was read at 544 nm against glucose or xylose standards (from 0 to1 mg) processed under identical conditions. Enzyme activities were expressed as µmol of reducing sugar released/min ml^{-1}.

Experiment 2

The objective of this study was to confirm the optimum dose rate of each enzyme additive determined in Exp. 1. Thus, the same enzyme products and the same corn silage were used; however, a narrower range of dose rates were applied. The experiment was conducted as a completely randomized design with 2 runs (batches) and 4 replicates per run. The runs were conducted on separate days, and the experimental procedure was the same as described for Exp. 1.

3.4.6 Statistical analysis

Data analyses were conducted using the mixed model procedure of SAS (SAS Institute Inc., Cary, NC). Data from Exp. 1 and 2 were analyzed separately as a completely randomized design with enzyme additive, dose and their interaction included in the model as fixed effects. Within experiment, run was considered a random effect. When the interaction between enzyme and dose was significant ($P<0.05$), contrasts and orthogonal polynomial contrasts were performed to determine linear, quadratic and cubic responses to dose within enzyme. When the main effect of

dose was significant (P<0.05), contrasts and orthogonal polynomial contrasts were performed to determine overall linear, quadratic and cubic responses to dose. Significance was declared at P<0.05.

3.4.7 Experimental site

The experiment was conducted at Agriculture and Agri-Food Canada, Lethbridge Research Center, Lethbridge, AB, Canada.

3.4.8 Duration

The duration of the present experiment was from January to December 2012.

3.5 Results

Enzyme activity

All enzyme additives supplied xylanase, endoglucanaseand exoglucanase activity, but only E2 and E4 supplied amylase activity (Table 3.1). For both endoglucanase and xylanase activity, the enzyme products (per ml) were ranked E4>E1>E2>E3, regardless of the xylan substrate used (i.e., oat spelt versus birch wood). Thus, E4 was the most concentrated source of xylanase and endoglucanase, while E3 was the least concentrated product. The relationship between xylanase determined using either oat spelt or birchwood was strong (Pearson correlation coefficient = 0.98), but E1, E2 and E3 had higher xylanase activity when oat spelt was the substrate whereas E4 had higher xylanase activity when birch wood was used. Additive E1 had highest exoglucanase activity, while E3 had the least exoglucanase activity.

Experiment 1

After 24 h incubation, there was no difference ($P>0.05$) between enzyme additives in terms of their effects on DMD or TGP, but enzyme additives differed ($P<0.05$) in their effects on NDFD and ADFD (Table 3.2). After 48 h of incubation, in addition to effects on NDFD and ADFD, the enzyme additives also differed ($P=0.04$) in their effects on DMD, although TGP remained similar for all additives ($P>0.05$).

At both time points, E1 and E2 had higher NDFD and ADFD than E3 and E4 ($P<0.05$). Effects of enzyme and dose were more prominent for the fiber fractions than for DM. At both incubation times, the effect of enzyme dose on NDFD and ADFD depended upon the additive (enzyme × dose interactions, $P\leq0.01$). For E1, the response to dose was linear and quadratic (linear only for ADFD after 48 h), with highest NDFD and ADFD at the highest dose for 24 and 48 h. For E2, response to dose was linear, quadratic and cubic (only linear and quadratic for NDFD after 24 h), such that at both incubation times all doses increased NDFD and ADFD compared to the control ($P<0.05$), with no differences amongst the levels of enzyme applied ($P>0.05$).

Table 3.1 Enzyme activity of the four enzyme additives used.

Product	Enzymatic activity[2]				
	Xylanase		Endo-glucanase	Exo-glucanase	Amylase
	Oat spelt	Birch wood			
E1	1804±26	1721±21	352±5.9	13.9±0.74	-
E2	1372±70	1172±31	159±5.2	8.9±0.05	0.29
E3	616±51	575±11	59±2.5	3.3±0.10	-
E4	3034±41	3979±10	360±16.3	9.3±0.16	0.37

[1] E1 : 75 : 25 combination of Cellulase PLUS and Xylanase PLUS (Dyadic International, Florida, USA); E2 : Rovabio Excel LC2 (Adisseo USA Inc, Georgia, USA); E3 : Cinabio (Adisseo USA Inc, Georgia, USA), and E4 : Econase RDE (AB Vista, Marlborough, UK).

[2] Endoglucanase, exoglucanase and amylase activity were expressed as μmoles of glucose released per minute per milliliter enzyme. Xylanase activity was expressed as μmoles of xylose released per minute per milliliter enzyme.

For E3, NDFD and ADFD response to dose was linear and quadratic after 24 h of incubation, and just linear after 48 h. As a result, at both incubation times, all doses increased NDFD compared to the control, with no differentiation amongst the doses, but ADFD was only increased with the 4 and 8 μl/g DM doses compared with control. For E4, NDFD, and ADFD response to dose was linear (and cubic for NDFD) after 24 h of incubation, such that all doses increased NDFD compared with control with no differentiation amongst the doses ($P>0.05$).

Table 3.2 Effect of enzyme (E) and dose (D) on the degradability (%) of dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF), and total gas production (GP) from corn silage after 24 and 48 h of incubation with ruminal fluid (Experiment 1) (N = 8).

Enzyme[1]	Dose (µl/g DM)	24 h				48 h			
		Degradability (%)			Total GP (ml/ g DM)	Degradability (%)			Total GP (ml/ g DM)
		DM	NDF	ADF		DM	NDF	ADF	
E1	**Mean**	**48.8**	**19.5^{A}**	**12.6^{A}**	**84.7**	**57.4^{A}**	**25.6^{A}**	**18.0^{A}**	**118.7**
	0	46.9	16.7^{c}	8.9^{c}	79.6	56.6	22.7^{c}	14.5^{c}	107.5
	2	48.7	19.5^{b}	13.0^{b}	84.8	57.0	25.6^{b}	17.4^{b}	119.4
	4	50.3	20.2^{b}	13.4^{b}	86.8	57.1	26.2^{b}	18.1^{b}	121.0
	8	49.4	21.7^{a}	15.3^{a}	87.6	58.7	28.1^{a}	22.0^{a}	126.8
	Contrast		l, q	l, q			l, q	l	
E2	**Mean**	**49.3**	**20.1^{A}**	**13.0^{A}**	**83.2**	**57.2^{A}**	**26.3^{A}**	**18.6^{A}**	**119.7**
	0	46.9	16.7^{b}	8.9^{b}	79.6	56.6	22.7^{b}	14.5^{b}	107.5
	2	49.8	20.5^{a}	14.1^{a}	83.0	57.1	27.4^{a}	20.0^{a}	121.4
	4	49.8	21.5^{a}	14.6^{a}	85.3	56.8	27.2^{a}	19.7^{a}	123.7
	8	50.6	21.5^{a}	14.7^{a}	84.7	58.3	27.8^{a}	20.1^{a}	126.1
	Contrast		l, q	l, q, c			l, q, c	l, q, c	

Table 3.2 Effect of enzyme (E) and dose (D) on the degradability (%) of dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF), and total gas production (GP) from corn silage after 24 and 48 h of incubation with ruminal fluid (Experiment 1) (*N* = 8) (Continued).

Enzyme[1]	Dose (µl/g DM)	24 h				48 h			
		Degradability (%)			Total GP (ml/ g DM)	Degradability (%)			Total GP (ml/ g DM)
		DM	NDF	ADF		DM	NDF	ADF	
E3	**Mean**	**48.1**	**18.3^{B}**	**11.2^{B}**	**81.8**	**56.8^{AB}**	**24.2^{B}**	**15.8^{B}**	**117.7**
	0	46.9	16.7^{b}	8.9^{b}	79.6	56.6	22.7^{b}	14.5^{b}	107.5
	2	48.1	18.3 ^{a}	10.6^{b}	82.5	56.7	24.2^{a}	14.7^{b}	118.3
	4	47.9	19.1 ^{a}	12.8^{a}	82.2	57.0	24.9^{a}	17.0^{a}	122.0
	8	49.2	18.9 ^{a}	12.4^{a}	82.9	56.9	25.2^{a}	17.2^{a}	122.9
	Contrast		l, q	l, q			l	l	
E4	**Mean**	**47.9**	**18.4^{B}**	**10.5^{B}**	**83.1**	**56.5^{B}**	**23.9^{B}**	**15.2^{B}**	**116.0**
	0	46.9	16.7^{b}	8.9^{b}	79.6	56.6	22.7^{b}	14.5	107.5
	2	48.1	18.9 ^{a}	10.5^{b}	83.6	56.5	24.6^{a}	15.3	115.7
	4	47.5	18.5 ^{a}	10.3^{b}	83.8	56.9	24.7^{a}	15.6	119.3
	8	48.9	19.5 ^{a}	12.4^{a}	84.4	55.8	23.8^{ab}	15.2	121.4
	Contrast		l, c	l			q		

Table 3.2 Effect of enzyme (E) and dose (D) on the degradability (%) of dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF), and total gas production (GP) from corn silage after 24 and 48 h of incubation with ruminal fluid (Experiment 1) (N = 8) (Continued).

Enzyme[1]	Dose (µl/g DM)	24 h				48 h			
		Degradability (%)			Total GP (ml/ g DM)	Degradability (%)			Total GP (ml/ g DM)
		DM	NDF	ADF		DM	NDF	ADF	
Dose	0	46.9^{B}	16.7	8.9	79.7^{B}	56.6	22.7	14.5	107.5^{C}
	2	48.7^{A}	19.3	12.1	83.5^{A}	56.8	25.4	16.9	118.7^{B}
	4	48.9^{A}	19.8	12.8	84.5^{A}	57.0	25.7	17.6	121.5^{AB}
	8	49.7^{A}	20.4	13.7	85.0^{A}	57.4	26.2	18.6	124.3^{A}
SEM									
Enzyme		2.08	0.27	0.31	7.43	1.32	0.24	0.41	8.12
Dose		2.08	0.27	0.31	7.43	1.32	0.24	0.41	8.12
Interaction		2.23	0.55	0.61	7.59	1.38	0.49	0.82	8.52
P value									
Enzyme		0.15	<0.001	<0.001	0.14	0.04	<0.001	<0.001	0.36

Table 3.2 Effect of enzyme (E) and dose (D) on the degradability (%) of dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF), and total gas production (GP) from corn silage after 24 and 48 h of incubation with ruminal fluid (Experiment 1) (*N* = 8) (Continued).

Enzyme[1]	Dose (µl/g DM)	24 h				48 h			
		Degradability (%)			Total GP (ml/ g DM)	Degradability (%)			Total GP (ml/ g DM)
		DM	NDF	ADF		DM	NDF	ADF	
Dose		<0.001	<0.001	<0.001	<0.001	0.09	<0.001	<0.001	<0.001
Enzyme*Dose		0.84	0.01	0.009	0.97	0.08	<0.001	<0.001	0.99

l, q, c : Within a column, the effect of dose for individual enzyme products is linear, quadratic, and cubic, respectively, at P<0.05.

[1]Enzyme E1, E2, E3, and E4 are identified in Table 3.1.

[a,b,c]Means within the same column within enzyme having different superscript letters are different at P<0.05

[A,B,C]Mean within the same column for the main effects of enzyme or does having different superscript letters are different at P<0.05.

However, only the highest dose increased ADFD after 24 h of incubation ($P<0.05$). After 48 h of incubation, the response to dose of E4 for NDFD was quadratic, with the 2 and 4 μl/g DM doses higher ($P<0.05$) than the control, but the highest dose (8 μl/g DM) similar to the control ($P>0.05$). After 48 h of incubation, ADFD was similar ($P>0.05$) for all doses of E4 compared with the control.

For TGP, there was no enzyme dose interaction ($P\geq0.97$) after either 24 or 48 h of incubation (Table 3.2). After 24 h, all doses had higher TGP than the control, with similar TGP for 2, 4, and 8μl/g DM. After 48 h, there was a linear and quadratic response in TGP to dose, with greater TGP with 4 and 8 μl/g DM. All enzyme additives showed a similar pattern of GP rate (ml/h) over the 48 h of incubation; GP rate was highest at the beginning of fermentation with peak GP rate at 3 h (data not shown). The rate of GP was lowest for the control at the peak, and at the end of incubation.

After 24 h, neither enzyme nor dose affected ($P>0.05$) total VFA (Table 4.3). The effect of dose on molar proportion of acetate depended upon the enzyme additive (enzyme × dose interaction, $P=0.04$). Compared with the control, added enzymes had no effect on acetate proportion for E1, E2, and E3, but for E4, 4 and 8 μl/g DM lowered acetate proportion compared with 0 and 2 μl/g DM. Thus, mean acetate proportions were lower for E4 than for the other enzyme additives. Also after 24 h, molar proportion of propionate was higher ($P<0.05$) for E4 and E2 than E1, with E3 being intermediate. Thus, acetate to propionate ratio ($P<0.05$) was highest for E1, intermediate for E3, followed by E2, and lowest for E4. The effect of dose on acetate to propionate ratio after 24 h was enzyme dependent (enzyme × dose interaction, $P=0.008$); acetate to propionate ratio decreased linearly with dose for E3 and E4, whereas the response for E1 was cubic and there was no response for E2.

After 48 h, enzyme additive affected (P<0.05) total VFA, but only tended to affect propionate concentration (P=0.07) and acetate to propionate ratio (P=0.08) (Table 3.3). Thus, effects of enzymes on total VFA were more pronounced after 48 h than after 24 h, but the opposite was true for molar proportions of VFA. Total VFA were higher (P<0.05) for E3 and E4 than for E2, with E1 being intermediate (P>0.05). There were no effects (P>0.05) of dose on total VFA, molar proportion of individual VFA, or acetate to propionate ratio (P>0.05), although there was trend (P=0.08) for propionate concentration to increase with increasing dose rate.

Experiment 2

After 24 and 48 h of incubation, DMD was not affected (P=0.22) by enzyme, but NDFD and ADFD differed (P<0.01) amongst enzymes (Table 4.4). After 24 h, NDFD was lower for E3 than the other enzymes, and ADFD was lower for E3 and E4 compared with E1 and E2. After 48 h, NDFD was lower for E3 and E4 compared with E2, with E1 being intermediate, and ADFD was lower for E3 and E4 compared with E1 and E2. After 24 h, all enzyme doses increased (P<0.05) NDFD and ADFD linearly, such that the highest dose (4 μl/g DM) differed from the intermediate doses (0.5-2.0 μl/g DM), which all differed from the control dose.

Table 3.3 Effect of enzyme additive (E) and dose (D) on the volatile fatty acid (VFA) concentrations from corn silage after 24 and 48 h of incubation with ruminal fluid (Experiment 1) (*N* = 8).

Enzyme[1]	Dose (µl/g DM)	24					48				
		Total VFA (m*M*)	Molar proportions[2]			Ac : Pr	Total VFA (m*M*)	Molar proportions[2]			Ac : Pr
			Ac	Pr	Bu			Ac	Pr	Bu	
E1	**Mean**	**123.5**	**63.9^{A}**	**15.9^{C}**	**12.3^{AB}**	**4.02^{A}**	**128.5^{AB}**	**60.1**	**17.8**	**13.5**	**3.37**
	0	122.8	63.9^{ab}	15.9	12.4	4.01^{ab}	131.3	60.5	17.6	13.5	3.44
	2	128.6	64.1^{a}	15.7	12.3	4.10^{ab}	131.0	59.9	17.9	13.6	3.35
	4	119.7	63.4^{b}	16.1	12.6	3.92^{b}	126.9	60.1	17.8	13.5	3.37
	8	122.9	64.1^{a}	15.8	12.2	4.05^{a}	124.9	59.9	18.0	13.5	3.34
	Contrast		c			C					
E2	**Mean**	**118.8**	**63.9^{A}**	**16.1^{AB}**	**12.2^{BC}**	**3.96^{B}**	**125.4^{B}**	**59.9**	**18.0**	**13.6**	**3.34**
	0	122.8	63.9	15.9	12.4	4.01	131.3	60.5	17.6	13.5	3.44
	2	118.5	64.0	16.1	12.2	3.93	126.5	59.8	18.1	13.5	3.31
	4	116.4	63.9	16.1	12.2	3.96	120.4	59.9	18.1	13.5	3.31
	8	117.3	63.7	16.3	12.2	3.95	123.2	59.5	18.1	13.7	3.29
	Contrast	ns				Ns					

Table 3.3 Effect of enzyme additive (E) and dose (D) on the volatile fatty acid (VFA) concentrations from corn silage after 24 and 48 h of incubation with ruminal fluid (Experiment 1) (*N* = 8) (Continued).

Enzyme[1]	Dose (µl/g DM)	24					48				
		Total VFA (m*M*)	Molar proportions[2]			Ac : Pr	Total VFA (m*M*)	Molar proportions[2]			Ac : Pr
			Ac	Pr	Bu			Ac	Pr	Bu	
	Mean	**114.2**	**63.9^{A}**	**16.0^{BC}**	**12.1^{C}**	**3.99^{AB}**	**131.7^{A}**	**60.1**	**17.8**	**13.5**	**3.38**
	0	122.8	63.9	15.9	12.4	4.01^{ab}	131.3	60.5	17.6	13.5	3.44
E3	2	110.4	64.2	15.9	12.1	4.03^{a}	125.6	60.2	18.0	13.2	3.36
	4	108.4	63.9	16.0	12.2	3.99^{ab}	136.8	59.9	17.9	13.6	3.35
	8	117.2	63.7	16.2	12.2	3.92^{b}	133.3	60.2	17.9	13.4	3.36
	Contrast		ns			l					
	Mean	**116.6**	**63.4^{B}**	**16.2^{A}**	**12.4^{A}**	**3.88^{C}**	**133.6^{A}**	**60.4**	**17.6**	**13.4**	**3.43**
	0	122.8	63.9^{a}	15.9	12.4	4.01^{a}	131.3	60.5	17.6	13.5	3.44
E4	2	113.1	63.9^{a}	16.1	12.2	3.89^{a}	136.0	60.1	17.8	13.5	3.38
	4	110.1	62.8^{b}	16.4	12.6	3.83^{ab}	133.7	60.1	17.6	13.5	3.42
	8	120.1	62.9^{b}	16.5	12.5	3.79^{b}	133.3	60.9	17.5	13.2	3.50
	Contrast		l, c			l					

Table 3.3 Effect of enzyme additive (E) and dose (D) on the volatile fatty acid (VFA) concentrations from corn silage after 24 and 48 h of incubation with ruminal fluid (Experiment 1) (*N* = 8) (Continued).

Enzyme[1]	Dose (µl/g DM)	24					48				
		Total VFA (m*M*)	Molar proportions[2]			Ac : Pr	Total VFA (m*M*)	Molar proportions[2]			Ac : Pr
			Ac	Pr	Bu			Ac	Pr	Bu	
Dose	0	122.3	63.8^{A}	15.9^{B}	12.4^{A}	4.01^{A}	131.3	60.5	17.6	13.5	3.44
	2	117.6	64.0^{A}	15.9^{B}	12.2^{B}	3.99^{B}	129.8	60.0	17.9	13.5	3.35
	4	113.7	63.5^{A}	16.2^{A}	12.4^{A}	3.93^{AB}	129.4	60.0	17.9	13.5	3.36
	8	119.4	63.6^{B}	16.2^{A}	12.3^{AB}	3.93^{C}	128.7	60.1	17.9	13.7	3.37
SEM											
Enzyme		2.87	0.10	0.06	0.05	0.018	2.03	0.19	0.09	0.08	0.026
Dose		2.87	0.10	0.06	0.05	0.018	2.03	0.19	0.09	0.08	0.026
Interaction		5.75	0.20	0.11	0.09	0.036	4.05	0.38	0.19	0.15	0.052
P											
Enzyme		0.14	0.001	0.001	0.007	<0.001	0.03	0.34	0.07	0.62	0.08

Table 3.3 Effect of enzyme additive (E) and dose (D) on the volatile fatty acid (VFA) concentrations from corn silage after 24 and 48 h of incubation with ruminal fluid (Experiment 1) (*N* = 8) (Continued).

Enzyme[1]	Dose (µl/g DM)	24					48				
		Total VFA (m*M*)	Molar proportions[2]			Ac:Pr	Total VFA (m*M*)	Molar proportions[2]			Ac:Pr
			Ac	Pr	Bu			Ac	Pr	Bu	
Dose		0.20	0.002	<0.001	0.02	0.003	0.83	0.30	0.08	0.85	0.11
Enzyme*Dose		0.93	0.04	0.14	0.11	0.008	0.40	0.80	0.91	0.73	0.85

l, c : Within a column, the effect of dose for individual enzyme products is linear, quadratic, and cubic, respectively, at P<0.05.

[a,b,c]Means within the same columns within enzyme having different letters are different at P<0.05.

[A,B,C]Mean within the same column for the main effects of enzyme or does having different letters are different at P<0.05.

[1]Enzyme E1, E2, E3, and E4 are identified in Table 3.1.

[2]Expressed as individual VFA, mol/100 mol; Ac=acetate, Pr=propionate, and Bu=butyrate, not included isobutyrate, isovalerate, valerate, and caproate.

After 48 h, NDFD increased (P<0.001) quadratically with increasing dose, but for ADFD, the effects of dose depended on the enzyme (enzyme × dose interaction, P=0.005). For E1 and E2, ADFD responded linearly and quadratically to dose, whereas E3 and E4 responded linearly to dose.

Total GP after 24 h was highest for E1 and E2, and lowest for E4 (P<0.05), with all doses equally increasing TGP compared with the control (P<0.05). After 48 h, TGP was higher (P<0.05) for E1, E2, and E3 compared with E4, and all doses increased TGP compared with the control dose (P<0.05). All 4 enzymes showed a similar pattern of GP rate (ml/h) with the highest rate after 18 h of incubation (data not shown). There were no enzyme dose interactions (P>0.05) for any of the fermentation variables after either 24 or 48 h of incubation (Table 3.5). After 24 h, total VFA were higher (P<0.05) for E1 than the other enzymes, and there was a linear and cubic response to dose (P=0.008). By 48 h, total VFA were higher (P=0.05) for E2 compared with E1, with the others being intermediate (P>0.05). All doses increased (P<0.001) total VFA in a quadratic manner at 48 h. After 24 h, there were no treatment differences (P>0.05) for molar proportions of VFA or acetate to propionate ratio, but after 48 h, propionate proportion was lower (P<0.05) for E4 compared with E1 and E3 and acetate to propionate ratio was higher for E4 and E2 compared with E3. However, there was no effect of dose rate on molar proportions of acetate or propionate or acetate to propionate ratio after 48 h of incubation.

3.6 Discussion

The enzyme additives evaluated were commercial products, each with a unique range in endoglucanase, exoglucanase and xylanase activities. Although not

assayed in our study, other minor fibrolytic enzymic activities likely also varied amongst these additives. Enzyme additives typically have a wide range of fibrolytic activities depending on the organism used to produce the enzyme, the growth substrate, and the culture conditions employed for enzyme production as reviewed by Beauchemin et al. (2004). There is a lack of standardization of methodology used to assay enzyme activity of ruminant feed enzymes. As indicated by Colombatto and Beauchemin (2003), the resulting enzymic activity is a function of the conditions of the enzyme assay, particularly the substrate used, temperature, and pH. In our study both oat spelt and birch wood xylan were used in the determination of xylanase.

There was a strong correlation between the xylanase activities determined using these two substrates, and thus the enzyme additives ranked similarly using either xylan substrate. However, E1, E2 and E3 had higher xylanase activity when oat spelt was used, whereas E4 had higher xylanase activity when birch wood was used. Birch wood and oat spelt differ in their composition, and hence result in different xylanase activity when used as xylan substrates. The structure of xylan from different sources depends on extraction procedures and degree of substitution of the xylan backbone with other residues (Ghatora, Chadha, Badhan, Saini, and Bhat, 2006).

Table 3.4 Effect of enzyme additives (E) and dose (D) on the degradability (%) of dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF), and total gas production (GP) from corn silage after 24 and 48 h of incubation with ruminal fluid (Experiment 2) (N = 8).

Enzyme[1]	Dose (µl/g DM)	24 h				48 h			
		Degradability (%)			Total GP	Degradability (%)			Total GP
		DM	NDF	ADF	ml/ g DM	DM	NDF	ADF	ml/ g DM
E1	**Mean**	**44.1**	**17.9**	**9.2^{A}**	**72.8^{AB}**	**56.0**	**24.5^{AB}**	**14.9^{A}**	**100.8^{A}**
	0	42.5	16.2	7.0	67.7	55.1	22.9	12.5^{c}	96.2
	0.5	44.5	17.6	9.0	70.7	55.9	23.9	12.9^{c}	101.1
	1.0	45.0	17.6	9.0	74.8	56.5	24.7	15.2^{b}	103.4
	2.0	44.2	18.3	9.5	74.4	55.9	25.0	15.8^{b}	100.7
	4.0	44.2	19.7	11.5	76.5	56.4	26.1	18.0^{a}	102.4
	Contrast							l, q	
E2	**Mean**	**43.5**	**17.8**	**8.9^{A}**	**74.3^{A}**	**56.1**	**24.9^{A}**	**15.1^{A}**	**100.0^{A}**
	0	42.5	16.2	7.0	67.7	55.1	22.9	12.5^{c}	96.2
	0.5	43.1	17.8	8.3	76.0	56.5	24.7	14.8^{b}	101.6
	1.0	42.6	17.7	8.8	77.3	56.2	24.9	15.2^{ab}	100.3
	2.0	44.8	18.3	9.9	74.9	56.3	26.0	16.6^{a}	99.2

Table 3.4 Effect of enzyme additives (E) and dose (D) on the degradability (%) of dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF), and total gas production (GP) from corn silage after 24 and 48 h of incubation with ruminal fluid (Experiment 2) (*N* = 8) (Continued).

Enzyme[1]	Dose (µl/g DM)	24 h				48 h			
		Degradability (%)			Total GP	Degradability (%)			Total GP
		DM	NDF	ADF	ml/ g DM	DM	NDF	ADF	ml/ g DM
E2	4.0	44.4	19.0	10.6	76.3	56.5	25.8	16.3^{ab}	102.8
	Contrast							l, q	
E3	**Mean**	**43.8**	**16.9**	**7.8^{B}**	**72.3^{BC}**	**55.7**	**24.0^{BC}**	**13.5^{B}**	**100.0^{A}**
	0	42.5	16.2	7.0	67.7	55.1	22.9	12.5^{c}	96.2
	0.5	44.0	16.9	7.9	74.5	55.6	23.9	13.1^{c}	102.3
	1.0	42.9	17.3	7.6	73.1	55.7	23.6	13.3^{c}	102.2
	2.0	44.5	16.4	7.4	73.3	56.0	24.3	13.9^{bc}	101.5
	4.0	45.0	17.5	9.1	73.0	56.2	25.3	15.0^{b}	97.7
	Contrast							l	
E4	**Mean**	**43.0**	**17.5**	**7.8^{B}**	**70.4^{C}**	**55.6**	**23.6^{C}**	**13.1^{B}**	**96.1^{B}**
	0	42.5	16.2	7.0	67.7	55.1	22.9	12.5^{ab}	96.2
	0.5	43.8	17.3	7.2	70.6	55.2	22.7	12.3^{b}	93.0

Table 3.4 Effect of enzyme additives (E) and dose (D) on the degradability (%) of dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF), and total gas production (GP) from corn silage after 24 and 48 h of incubation with ruminal fluid (Experiment 2) (N = 8) (Continued).

Enzyme[1]	Dose (µl/g DM)	24 h				48 h			
		Degradability (%)			Total GP	Degradability (%)			Total GP
		DM	NDF	ADF	ml/ g DM	DM	NDF	ADF	ml/ g DM
E4	1.0	42.2	17.1	7.4	71.0	55.7	23.7	13.1^{ab}	96.2
	2.0	42.7	18.0	8.3	71.1	56.0	24.0	13.8^{a}	96.4
	4.0	43.7	18.74	9.0	71.5	56.0	25.2	13.9^{a}	98.7
	Contrast							l	
Dose	0	42.5	16.2^{C}	7.0^{C}	67.7^{B}	55.1	22.9^{D}	12.5	96.2
	0.5	43.8	17.4^{B}	8.1^{B}	73.0^{A}	55.8	23.8^{C}	13.3	99.5
	1.0	43.2	17.5^{B}	8.2^{B}	74.0^{A}	56.0	24.2^{BC}	14.2	100.6
	2.0	44.0	17.7^{AB}	8.8^{B}	73.4^{A}	56.0	24.8^{B}	15.0	99.5
	4.0	44.3	18.7^{A}	10.0^{A}	74.3^{A}	56.3	25.6^{A}	15.8	100.4
SEM									
Enzyme		0.85	0.35	0.35	0.70	0.29	0.26	0.25	1.16
Dose		0.95	0.40	0.39	0.78	0.33	0.29	0.28	1.30
Enzyme*Dose		1.90	0.79	0.77	1.57	0.66	0.57	0.56	2.60

Table 3.4 Effect of enzyme additives (E) and dose (D) on the degradability (%) of dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF), and total gas production (GP) from corn silage after 24 and 48 h of incubation with ruminal fluid (Experiment 2) (*N* = 8) (Continued).

Enzyme[1]	Dose (µl/g DM)	24 h				48 h			
		Degradability (%)			Total GP	Degradability (%)			Total GP
		DM	NDF	ADF	ml/ g DM	DM	NDF	ADF	ml/ g DM
P-values									
Enzyme		0.83	0.16	0.004	<0.001	0.55	0.005	<0.001	0.02
Dose		0.64	<0.001	<0.001	<0.001	0.14	<0.001	<0.001	0.13
Enzyme*Dose		1.00	0.99	0.88	0.47	1.00	0.83	0.008	0.77

l : Within a column, the effect of dose for individual enzyme products is linear, quadratic, and cubic, respectively, at P<0.05.

[1]Enzyme E1, E2, E3, and E4 are identified in Table 3.1.

[a,b,c]Means within the same columns within enzyme having different letters are different at P<0.05.

[A,B,C,D]Mean within the same column for the main effects of enzyme or does having different letters are different at P<0.05.

Xylans from grasses and cereals (e.g., oat spelt) contain arabinofuranosyl and glucopyranosyluronic acid substituents, whereas xylans from hardwoods (e.g., birch wood) contain substantial amounts of glucopyranosyluronic acid and very small amounts of arabinofuranosyl substituents (Kormelink and Voragen, 1993). Thus, oat spelt xylan is usually considered to be a more representative substrate for ruminant feed enzymes. Kung, Cohen, Rode, and Treacher (2002) reported different activity profiles when enzyme additives were assayed at different pH values and suggested that if the additives are to be supplemented at the time of feeding, the most effective additives would have high activity at a pH range reflective of conditions in the rumen. The same rationale can be used for temperature. In our study, the enzyme assays were conducted at pH 6 and 39°C as suggested by Colombatto and Beauchemin (2003) to reflect the mean ruminal conditions of a typical dairy cow fed a diet containing forage and concentrate.

The present study evaluated several commercially produced fibrolytic enzyme additives for their potential to be used as ruminant feed additives for corn silage based diets. *In vitro* techniques are often used as a bioassay to predict *in vivo* response to exogenous enzymes because animal responses cannot be predicted from enzyme activities alone (Beauchemin et al., 2004). Furthermore, conducting animal feeding studies is very costly, thus it is important to conduct preliminary screening of enzymes to determine their potential for further evaluation.

Table 3.5 Effect of enzyme additive (E) and dose (D) on the volatile fatty acid (VFA) concentrations from corn silage after 24 and 48 h of incubation with ruminal fluid (Experiment 2) (*N* = 8).

Enzyme[1]	Dose (µl/g DM)	24 h					48 h				
		Total VFA (mM)	Molar proportions[2]			Ac : Pr	Total VFA (mM)	Molar proportions[2]			Ac : Pr
			Ac	Pr	Bu			Ac	Pr	Bu	
E1	**Mean**	**118.3^{A}**	**61.1**	**17.5**	**13.0**	**3.49**	**126.2^{B}**	**56.3**	**20.2^{AB}**	**14.4**	**2.79^{BC}**
	0	117.9	60.4	17.4	13.4	3.49	122.2	56.1	20.0	14.7	2.81
	0.5	116.9	61.0	17.5	13.1	3.49	126.8	56.2	20.4	14.5	2.76
	1.0	120.9	61.2	17.7	13.0	3.47	127.7	56.1	20.4	14.5	2.75
	2.0	119.9	61.4	17.6	12.7	3.49	124.3	56.6	20.2	14.3	2.80
	4.0	118.7	61.4	17.5	12.8	3.52	130.0	56.6	20.2	14.2	2.81
E2	**Mean**	**110.9^{B}**	**60.9**	**17.6**	**13.0**	**3.47**	**132.7^{A}**	**56.7**	**20.0^{BC}**	**14.3**	**2.85^{AB}**
	0	117.9	60.4	17.4	13.4	3.49	122.2	56.1	20.0	14.7	2.81
	0.5	105.8	61.1	17.5	12.9	3.50	130.2	56.8	20.1	14.1	2.84
	1.0	110.6	61.4	17.5	12.8	3.51	135.5	57.1	19.7	14.2	2.90
	2.0	111.4	60.8	17.8	13.1	3.42	140.8	57.3	20.0	13.9	2.88
	4.0	108.8	60.8	17.9	13.0	3.41	135.4	56.3	20.1	14.5	2.81

Table 3.5 Effect of enzyme additive (E) and dose (D) on the volatile fatty acid (VFA) concentrations from corn silage after 24 and 48 h of incubation with ruminal fluid (Experiment 2) (*N* = 8) (Continued).

Enzyme[1]	Dose (µl/g DM)	24 h					48 h				
		Total VFA (mM)	Molar proportions[2]			Ac : Pr	Total VFA (mM)	Molar proportions[2]			Ac : Pr
			Ac	Pr	Bu			Ac	Pr	Bu	
E3	**Mean**	**112.0^{B}**	**60.9**	**17.6**	**13.0**	**3.47**	**130.4^{AB}**	**55.9**	**20.3^{A}**	**14.6**	**2.76^{C}**
	0	117.9	60.4	17.4	13.4	3.49	122.2	56.1	20.0	14.7	2.81
	0.5	111.8	60.8	17.8	13.1	3.42	136.5	57.0	20.0	14.2	2.86
	1.0	106.6	60.9	17.6	13.0	3.47	133.3	55.3	20.4	14.8	2.71
	2.0	115.5	61.1	17.6	12.9	3.47	138.0	56.2	20.2	14.4	2.78
	4.0	108.4	61.3	17.6	12.9	3.50	125.0	54.8	20.8	14.9	2.63
E4	**Mean**	**111.3^{B}**	**60.9**	**17.6**	**12.9**	**3.49**	**127.8^{AB}**	**56.4**	**19.7^{C}**	**14.6**	**2.87^{A}**
	0	117.9	60.4	17.4	13.4	3.49	122.2	56.1	20.0	14.7	2.81
	0.5	116.7	61.6	17.4	12.6	3.55	127.1	55.9	19.8	14.7	2.82
	1.0	107.2	61.6	17.5	12.6	3.53	128.7	56.6	19.7	14.4	2.87
	2.0	106.9	60.4	17.8	13.1	3.40	132.9	57.1	19.4	14.2	2.94
	4.0	107.9	60.7	18.0	12.9	3.38	128.1	56.4	19.5	14.7	2.90

Table 3.5 Effect of enzyme additive (E) and dose (D) on the volatile fatty acid (VFA) concentrations from corn silage after 24 and 48 h of incubation with ruminal fluid (Experiment 2) (N = 8) (Continued).

Enzyme[1]	Dose (µl/g DM)	24 h					48 h				
		Total VFA (mM)	Molar proportions[2]			Ac : Pr	Total VFA (mM)	Molar proportions[2]			Ac : Pr
			Ac	Pr	Bu			Ac	Pr	Bu	
Dose	0	117.9	60.4	17.4	13.4	3.49	122.2^{B}	56.1	20.0	14.7	2.81
	0.5	112.8	61.2	17.6	12.9	3.49	130.1^{A}	56.6	20.1	14.4	2.82
	1.0	111.3	61.3	17.6	12.8	3.49	130.4^{A}	56.3	20.0	14.5	2.81
	2.0	113.5	60.9	17.7	12.9	3.45	134.0^{A}	56.8	20.0	14.2	2.85
	4.0	111.0	61.1	17.7	12.8	3.45	129.6^{A}	56.0	20.2	14.6	2.79
SEM											
Enzyme		2.10	0.33	0.09	0.22	0.035	1.77	0.29	0.10	0.17	0.027
Dose		2.35	0.37	0.10	0.24	0.039	1.98	0.33	0.12	0.20	0.030
Interaction		4.70	0.74	0.21	0.48	0.078	3.95	0.65	0.23	0.39	0.061
P-values											
Enzyme		0.03	0.98	0.93	0.98	0.96	0.05	0.24	<0.001	0.58	0.02

Table 3.5 Effect of enzyme additive (E) and dose (D) on the volatile fatty acid (VFA) concentrations from corn silage after 24 and 48 h of incubation with ruminal fluid (Experiment 2) (N = 8) (Continued).

Enzyme[1]	Dose (µl/g DM)	24 h					48 h				
		Total VFA (mM)	Molar proportions[2]			Ac : Pr	Total VFA (mM)	Molar proportions[2]			Ac : Pr
			Ac	Pr	Bu			Ac	Pr	Bu	
Dose		0.24	0.54	0.09	0.34	0.85	0.001	0.51	0.85	0.37	0.68
Enzyme*Dose		0.79	0.99	0.82	0.99	0.97	0.48	0.74	0.24	0.99	0.44

[A,B,C]Mean within the same column for the main effects of enzyme or does having different letters are different at P<0.05.

[1]Enzyme E1, E2, E3, and E4 are identified in Table 3.1.

[2]Expressed as individual VFA, mol/100 mol; Ac=acetate; Pr=propionate, and Bu=butyrate, not included isobutyrate, isovalerate, valerate, and caproate.

While an *in vitro* assay can be useful for identifying effective enzymes for feeding studies, factors such as scaling up the dose rate from *in vitro* to *in vivo*, method of adding the enzyme to feed, differences in composition of the diet, and animal variability can influence whether effects observed *in vitro* are also observed *in vivo* (Beauchemin et al., 2004). Because the *in vitro* assay was used in our study to recommend enzymes for future use in dairy cow feeding studies, the pH of the buffer used was adjusted to pH 6 to reflect the typical pH in the rumen of dairy cows. The final pH after 48 h of incubation ranged from 5.69 to 6.00 (data not shown). The relatively low pH of the batch culture assay in this study resulted in NDFD and ADFD values at 48 h that would be expected to be lower than had a buffer with higher pH been used in the *in vitro* assay.

To ensure our *in vitro* screening methodology was relevant to *in vivo* results, we used two (E1 and E4) additives that had been used previously in feeding studies with dairy cows where positive results had been reported. However, corn silage was only used in the study by Arriola et al. (2011), who allocated 60 dairy cows in early lactation to high (520 g/kg roughage, including 370 g/kg corn silage) and low (670 g/kg roughage, including 490 g/kg corn silage) concentrate diets with and without enzyme (E1) supplementation (DM basis; 3.4 mg of enzyme/g of ration DM). Milk production efficiency (kg of 3.5% fat-corrected milk/kg of DM intake) increased by 16% for the low concentrate diet, and by 6% for the high concentrate diet. In a companion metabolism using the same treatments, total tract digestibility of DM, crude protein, NDF and ADF were all increased with supplemental enzymes, regardless of level of roughage in the diet. Thus, E1 was considered a positive control in our *in vitro* study.

Although there were some small differences between the results for Exp. 1 and 2, generally Exp. 2 confirmed the results observed in Exp. 1. Based on improvements in NDFD and ADFD in both studies, E1 and E2 were more effective than E3 and E4 after both 24 and 48 h of incubations (Figure 3.1). Enzyme E1 increased NDFD respectively by up to 30% and 24% at 24 and 48 h, in Exp. 1 and by up to 22% and 14%, in Exp. 2. Similarly, E2 increased NDFD respectively by up to 29% and 22% at 24 and 48 h, in Exp. 1 and by up to 17% and 14%, in Exp. 2. For all enzymes, improvements in ADFD were greater than for NDFD. For E1, ADFD respectively increased by up to 72% and52% at 24 and 48 h, in Exp. 1 and by 65% and 44%, in Exp. 2. For E2, ADFD respectively increased by up to 65% and 39% at 24 and 48 h, in Exp. 1 and by 51% and 33%, in Exp. 2. Thus, the increases in NDFD and ADFD were fairly similar for E1 and E2. Given that E1 improved performance of dairy cows fed a diet containing corn silage (Arriola et al., 2011), it is recommended that E1 and E2 be further evaluated *in vivo* studies using diets based on corn silage.

Maximum improvements in NDFD for E3 were respectively 13% and 11% after 24 and 48 h, in Exp. 1, and 8% and 10%, in Exp. 2 (Figure 3.1). For ADFD, these were respectively 39% and 19%, in Exp. 1 and 30% and 20%, in Exp. 2. While positive, these improvements were lower than for E1 and E2, and thus if E3 is to be used as a feed additive for dairy cows, it would need to be priced significantly lower than E1 and E2 such that higher dose rates could be used. For E4, maximum improvements in NDFD and ADFD were of the same magnitude as observed for E3 (Figure 3.1).

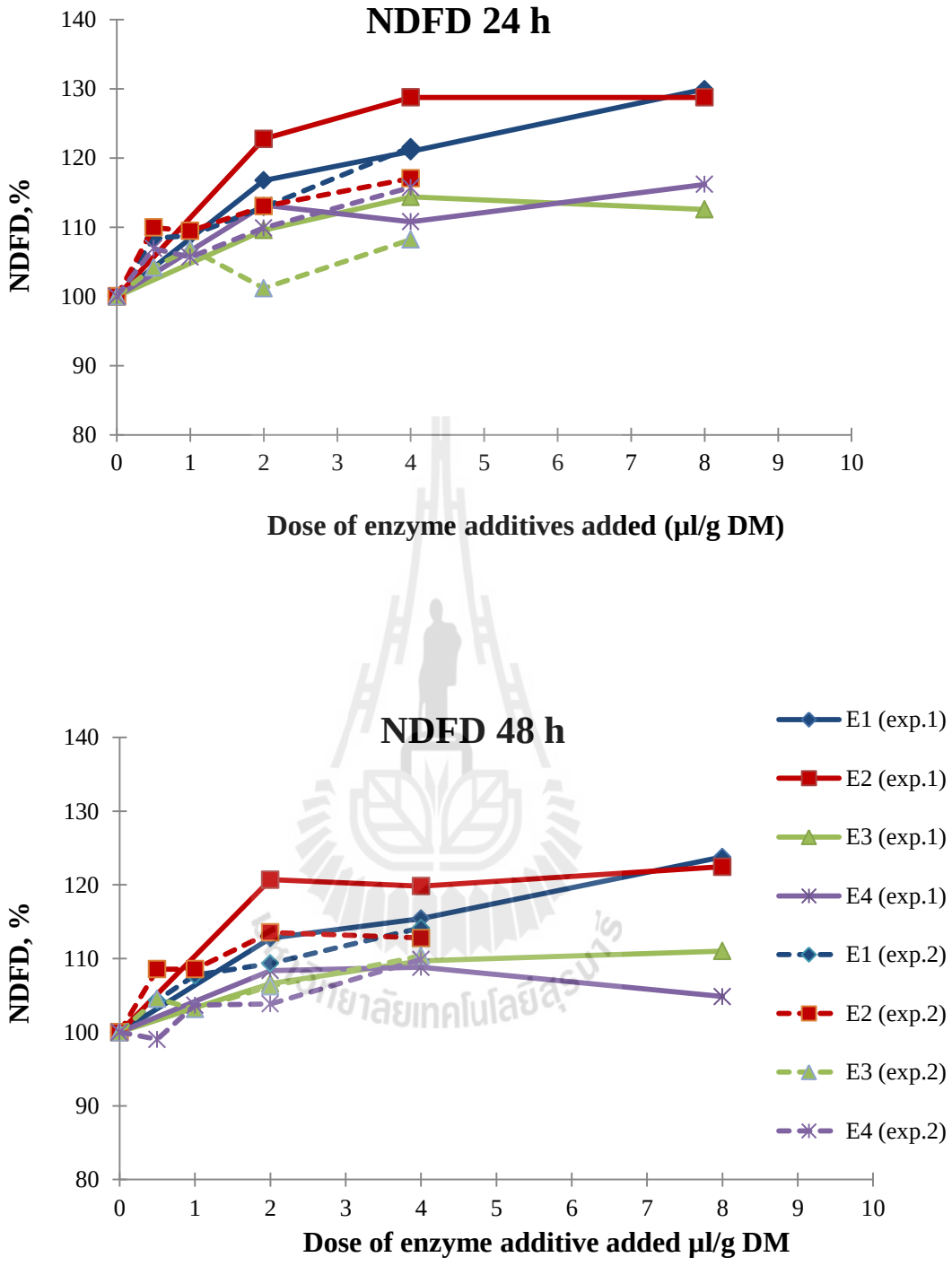

Figure 3.1 Percentage increase in neutral detergent fiber disappearance (NDFD) from corn silage after 24 and 48 h of incubation in Experiment 1 and Experiment 2. Enzyme E1, E2, E3, and E4 are identified in Table 3.1.

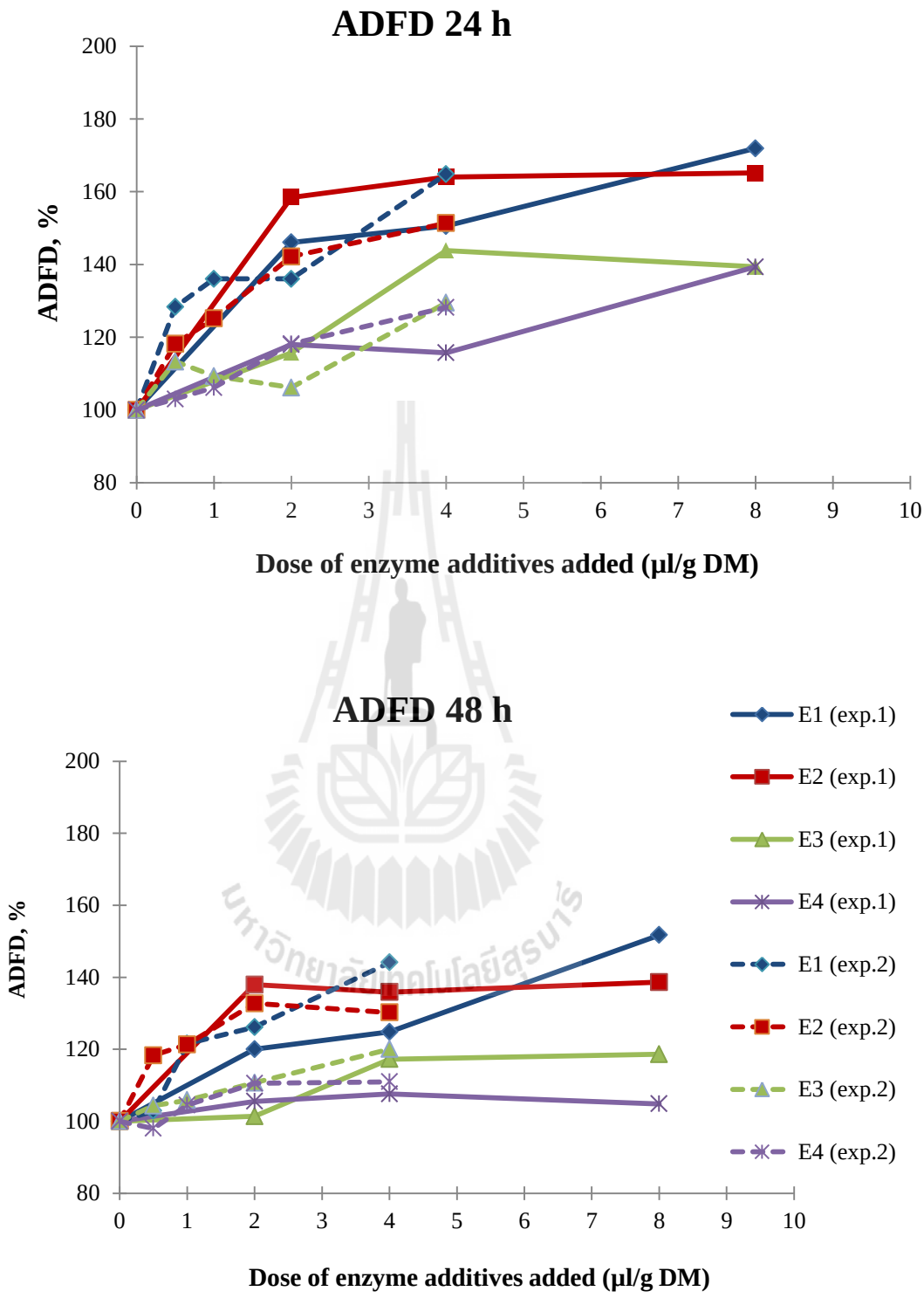

Figure 3.2 Percentage increase in acid detergent fiber disappearance (ADFD) from corn silage after 24 and 48 h of incubation in Experiment 1 and Experiment 2. Enzyme E1, E2, E3, and E4 are identified in Table 3.1.

In Exp. 1, maximum improvement in NDFD for E4 was 17% after 24 h and 5% after 48 h, and in Exp. 2, 15% and 10%, respectively; for ADFD these were 39% after 24 h and 5% after 48 h in Exp. 1 and 29% and 11%, respectively, in Exp. 2. This enzyme additive was used in a study by Holtshausen et al. (2011) in which 60 dairy cows in early lactation were fed diets containing no enzyme, low enzyme (E4; 0.5 ml of enzyme/kg of diet DM), and high enzyme (E4; 1.0 ml of enzyme/kg of diet DM). The diet contained 520 g/kg roughage, including 206 g/kg barley silage, 206 g/kg alfalfa silage and 108 g/kg alfalfa hay (DM basis). Adding enzyme to the diet linearly increased milk production efficiency (kg of 3.5% fat-corrected milk/kg of DM intake) by up to 11%. It is interesting to note that when the authors used E4 in a 24 h *in vitro* batch culture using each of the forages individually, improvements in NDFD and ADFD were only observed for alfalfa hay, and only at a higher dose rate (2 ml of enzyme/kg of forage DM). Given the improvements in NDFD and ADFD observed in our study using corn silage compared to the very minor improvements seen *in vitro* by Holtshausen et al. (2011) for other forages, it is possible that E4 would result in positive effects if used *in vivo* with a diet containing corn silage.

One of the objectives of our study was to determine the dose response of the enzymes to determine optimum dose for each product. Given that the response to enzyme dose differed for the variables measured and the incubation time, optimum dose is somewhat subjective. Thus, we considered optimum dose to be the dose at which NDFD and ADFD were increased compared with the control, with only minor further improvements with higher doses of enzyme. For E1, highest NDFD and ADFD were observed at the highest level for both incubation times in both studies, and the optimum dose was 1.0 μl/g DM. For E2, the optimum dose was 0.5 μl/g DM because it increased ADFD, with only minor further improvements at higher doses,

and responses at low dose rates in Exp. 2 were generally linear. For E3, optimum dose rate was 4 μl/g DM, because a further increase in enzyme addition failed to further increase ADFD. For E4, optimum dose rate was 2 μl/g DM. Thus, the effect of enzyme dose on improving fiber digestion differed amongst enzyme products. As each enzyme additive provides a unique array of enzymic activities, differences in the responses among additives, and in optimum dose rate, was anticipated. Similarly, Eun et al. (2007) reported that *in vitro* degradability of NDF and ADF from alfalfa hay and corn silage were increased by exogenous fibrolytic enzymes, but the response depended upon the enzyme and its dose, with some additives effective for both forages when added at 1.4 mg/g of DM, but others only moderately effective for either forage.

Increasing NDFD and ADFD of corn silage through the addition of enzyme additives would be expected to increase ruminal fiber digestibility and DM intake of dairy cows, through the reduction of physical fill in the rumen. Digestibility of NDF measured *in vitro* or *in situ* has been shown to be a good indicator of the potential of forage to enhance DM intake (Oba and Allen, 1999). Jung, Raeth-Knight, and Linn (2004) reported that an increase of one percentage unit in *in vitro* NDF digestibility of corn silage resulted in a 0.14 kg/d increase in 3.5% fat-corrected milk yield and a 0.12 kg/d increase in DM intake by dairy cows fed a diet high in corn silage proportion (400 g/kg DM). Moreover, Oba and Allen (1999) also reported a positive relationship between forage NDF digestibility (*in vitro* or *in situ*) and milk production and DM intake. The magnitude of the responses in NDFD and ADFD to increasing dose differed amongst enzyme additives in a manner that could not be explained by activity of xylanase (oat spelt) or endoglucanase. In other words, enzyme activity alone could not be used to predict improvement in NDFD or ADFD. One probable reason for this

is that enzyme activities are measured on model substrates that do not represent the complexity of plant cell wall material (Beauchemin et al., 2004). Based on TGP and DMD there was little to no differentiation in the effectiveness of the enzymes at 24 h in both studies, and at 48 h, E1, E2, and E3 were more effective than E4. Thus, degradation of NDF and ADF was more useful in differentiating the enzymes compared with DM and TGP.

Volatile fatty acids are end-products of rumen microbial fermentation and represent the main supply of energy for ruminants. The observed increases in total VFA concentration with added enzymes, and the changes in molar proportions of VFA, were somewhat inconsistent between 24 and 48 h and between Exp. 1 and Exp. 2. The increases in total VFA concentration did not correspond to increases in DMD, NDFD or ADFD. Furthermore, in Exp. 1 at 24 h, E1 had the highest, and E4 had the lowest, acetate to propionate ratio, but these differences were not maintained at 48 h. Those differences in acetate to propionate ratio were not observed at 24 h in Exp. 2, and by 48 h E4 actually had the highest ratio and E1 had the lowest ratio. In comparison, in *in vivo* study by Arriola et al. (2011), total VFA increased and acetate : propionate ratio decreased with added enzyme (E1). Chung et al. (2012) reported no effect of adding enzyme (E4) to a diet that did not contain corn silage on ruminal fluid concentrations of total VFA or molar proportions of individual VFA. Our data suggest that measuring effects of enzyme on VFA concentrations are not a particularly useful way of screening the potential effects of the enzyme additives *in vivo*. Our *in vitro* assay focused on both 24 and 48 h of incubation, as it was not clear whether both time periods would produce similar results. Most *in vitro* incubation times used to evaluate fiber degradability range between 24 and 48 h to reflect the mean retention time of forages in the rumen. In a review of the literature, Owens and Goetsch (1986) reported

that passage rate of roughage in beef and dairy cattle consuming 4.25% of body weight averaged 4.5%/h (mean retention time of 22 h), and for cattle consuming diets containing 20-50% concentrate, passage rate of roughage was 3.7%/h (mean retention time of 27 h). The National Research Council (2001) uses a 48 h *in vitro* incubation to predict ruminal NDF digestibility of dairy cows at maintenance. Eun and Beauchemin (2007) focused on 24 h *in vitro* batch culture fermentation and found exogenous enzymes improved *in vitro* degradability of alfalfa and corn silage. When considering fiber degradation at higher dose rates (Exp. 1), the results were similar at 24 and 48 h, thus only one time point would have been necessary. However, at lower dose rates (Exp. 2) differences amongst enzymes were more pronounced at 48 h. Furthermore, for ADFD the enzyme dose response depended upon the enzyme. Thus, the 48 h incubation was most useful in terms of defining the dose response and differentiating amongst enzyme products. Overall, in terms of using an *in vitro* assay to evaluate enzymes for further study *in vivo*, we recommend examining the effects on NDFD and ADFD at both 24 and 48 h, to achieve a more comprehensive understanding of the enzyme forage response.

3.7 Conclusions

The enzyme additives evaluated in the present study supplied a unique range of endoglucanase, exoglucanase and xylanase activities. All four additives evaluated increased NDFD and ADFD of corn silage *in vitro*, with E1and E2 being more effective than E3 and E4. Dose response was enzyme dependent for most variables. For E1, maximum response was observed at the highest level (8 μl/g DM), optimum dose rate was 1.0 μl/g DM, because a further increase in enzyme addition increase ADFD. For E2, a dose of 0.5 μl/g DM increased ADFD, with limited further increase

with increasing dose rate. For E3, optimum dose rate was 4 μl/g DM, because a further increase in enzyme addition failed to further increase ADFD. For E4, optimum dose rate was 2 μl/g DM. In general, NDF and ADF were more useful in differentiating the enzymes compared with DM and TGP. Based on the responses observed, further study of these additives for dairy cows fed corn silage diets is recommended.

3.8 References

Arriola, K.G., Kim, S.C., Staples, C.R., and Adesogan, A.T. (2011). Effect of fibrolytic enzyme application to low- and high-concentrate diets on the performance of lactating dairy cattle. **J. Dairy Sci.** 94: 832-841.

Beauchemin, K.A., Colombatto, D., and Morgavi, D.P. (2004). A rationale for the development of feed enzyme products for ruminants. **Can. J. Anim. Sci.** 84: 23-36.

Chung, Y.H., Zhou, M., Holtshausen,L., Alexander, T.W., McAllister, T.A., Guan, L.L., Oba, M., and Beauchemin, K.A. (2012). A fibrolytic enzyme additive for lactating Holstein cow diets: Ruminal fermentation, rumen microbial populations and enteric methane emissions. **J. Dairy Sci.** 95: 1419-1427.

Colombatto, D., and Beauchemin, K.A. (2003). A proposed methodology to standardize thedetermination of enzymic activities present in enzymeadditives used in ruminant diets. **Can. J. Anim. Sci.** 83: 559-568.

Eun, J.S., and Beauchemin, K.A. (2007). Assessment of the efficacy of varying experimental exogenous fibrolytic enzymes using *in vitro* fermentation characteristics. **Anim. Feed Sci. Technol.** 132: 298-315.

Eun, J.S., Beauchemin, K.A., and Schulze, H. (2007). Use of exogenous fibrolytic enzymes to enhance *in vitro* fermentation of alfalfa hay and corn silage. **J. Dairy Sci.** 90: 1440-1451.

Ghatora, S.K., Chadha, B.S., Badhan, A.K., Saini, S.H., and Bhat, M.K. (2006). Identifiaction and characterization of diverse xylanases from thermophilic and thermotolerant fungi. **BioResourses.** 1(1): 18-33.

Giraldo, L.A., Ranilla, M.J., Tejido, M.L., and Carro, M.D. (2007). Influence of exogenous fibrolytic enzymes and fumarate on methane production, microbial growth and fermentation in Rusitec fermenters. **Br. J. Nutr.** 98: 753-761.

Goering, H.K., and Van Soest, P.J. (1970). **Forage fiber analyses (apparatus, reagents, procedures, and some applications).** Washington DC: Agric. Handbook.

Holtshausen, L., Chung, Y.H., Gerardo-Cuervo, H., Oba, M., and Beauchemin, K.A. (2011). Improved milk production efficiency in early lactation dairy cattle with dietary addition of a developmental fibrolytic enzyme additive. **J. Dairy Sci.** 94: 899-907.

Jung, H.G., Raeth-Knight, M., and Linn, J.G. (2004). **Forage fiber digestibility: Measurement, variability, and impact.** In Proceedings of 65th Minnesota Nutrition Conference (pp. 105-125).

Knowlton, K.F., McKinney, J.M., and Cobb, C. (2002). Effect of a direct-fed fibrolytic enzyme formulation on nutrient intake, partitioning, and excretion in early and late lactation Holstein cows. **J. Dairy Sci.** 85: 3328-3335.

Kormelink, F.J.M., and Voragen, A.G.J. (1993). Degradation of different [(glucurono)arabino] xylans by a combination of purified xylan-degrading enzymes. **Appl. Microbiol. Biotechnol.** 38: 688-695.

Kung, Jr.L., Cohen, M.A., Rode, L.M., and Treacher, R.J. (2002). The effect of fibrolytic enzymes sprayed onto forages and fed in a total mixed ratio to lactating dairy cows. **J. Dairy Sci.** 85: 2396-2402.

Lewis, G.E., Sanchez, W.K., Hunt, C.W., Guy, M.A., Pritchard, G.T., Swanson, B.I., and Treacher, R.J. (1999). Effect of direct-fed fibrolytic enzymes on the lactational performance of dairy cows. **J. Dairy Sci.** 82: 611-617.

Mauricio, R.M., Mould, F.L., Dhanoa, M.S., Owen, E., Channa, K.S., and Theodorou, M.K. (1999). A semi-automated *in vitro* gas production technique for ruminant feedstuff evaluation. **Anim. Feed Sci. Technol.** 79: 321-330.

Oba, M., and Allen, M.S. (1999). Evaluation of the importance of digestibility of neutral detergent fiber from forage: Effects on dry matter intake and milk yield of dairy cows. **J. Dairy Sci.** 82: 589-596.

Owens, F.N., and Goetsch, A.L. (1986). Digesta passage and microbial protein synthesis. In L.P. Milligan, W.L. Grovum, and A. Dobson (eds.). **Control of digestion and metabolism in ruminants** (pp 196-223). New York: Prentice Hall.

Van Soest, P.J., Robertson, J.B., and Lewis, B.A. (1991). Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal production. **J. Dairy Sci.** 74: 3583-3597.

Wang, Y., and McAllister, T.A. (2002). Rumen microbes, enzymes and feed digestion: A review. **Asian-Aust. J. Anim. Sci.** 15: 1659-1676.

Yang, H.J., and Xie, C.Y. (2010). Assessment of fibrolytic activities of 18 commercial enzyme products and their abilities to degrade the cell wall fraction of corn stalks in *in vitro* enzymatic and ruminal batch cultures. **Anim. Feed Sci. Technol.** 159: 110-121.

Yang, W.Z., Beauchemin. K.A., And Rode, L.M. (2000). A comparison of methods of adding fibrolytic enzymes to lactating cow diets. **J. Dairy Sci.** 83: 2512-2520.

CHAPTER IV

EFFECTS OF EXOGENOUS FIBROLYTIC ENZYMES ON *IN VITRO* RUMINAL FERMENTATION OF FOUR CORN SILAGES

4.1 Abstract

This study was conducted to evaluate the effect of four enzyme additives on ruminal fermentation of four corn silages using a 48 h batch culture *in vitro* assay with medium and ruminal fluid. The experiment was conducted as a completely randomized design with two runs and four replicates. The enzyme additives (E1, E2, E3, and E4) were commercial products that provided a range in endoglucanase, exoglucanase, and xylanase activities. The four enzymes were added at 0, and 4 µl/g of corn silage dry matter (DM). Gas production (GP) was measured at 3, 6, 12, 18, 24, and 48 h post incubation. Degradability of DM (DMD), neutral detergent fiber (NDFD), acid detergent fiber (ADFD), and volatile fatty acid concentrations (VFA; total and individual molar proportions) were determined after 24 and 48 h. At 24 and 48 h incubation, there was difference ($P<0.05$) between enzyme additives and control in terms of their effects on DMD, NDFD, ADFD, and TGP. Except TGP at 24 h incubation, all enzyme additives were similar, but were difference ($P<0.05$) in their effects between enzyme additive and control. The E1 and E2 had higher DMD, NDFD, and ADFD than E3, E4 and control ($P<0.05$); however, E3 and E4 had higher effects than control ($P<0.05$). At both incubation times, the effects of corn silage

substrates, there were difference ($P<0.05$) between corn silage substrate in terms of their effects on DMD, NDFD, ADFD, and TGP. For all parameters, DMD, NDFD, ADFD and TGP, there were no enzyme × corn silage substrate interactions ($P\geq0.05$) at either 24 or 48 h of incubation. At 24 and 48 h incubations, there were no difference ($P>0.05$) between enzyme additives and control in terms of their effects on rumen fermentation profile. For the effects of corn silage substrates, there were differences ($P<0.05$) between corn silage substrate in terms of their effects on ruminal fermentation profile. Overall, enzyme additives show positive response in all corn silage substrates (increased DMD, NDFD, ADFD, and TGP), however, product E1 and E2 were more effective than E3 and E4.

4.2 Introduction

The use of fibrolytic enzyme additive in ruminant diets is generally attributed to improve feed utilization and animal performance. Most researches focused on cellulase and xylanase that degrade the major plant structural polysaccharides, cellulose, and hemicellulose. Supplemental fibrolytic enzyme additives have been shown to improve *in vitro* fiber digestion and enhance the nutritive value of forages (Yang and Xie, 2010; Eun et al., 2007), enhance attachment by rumen microorganisms (Nsereko et al., 2002). *In vivo* studies have also shown positive responses when fibrolytic enzymes were fed to ruminants (Arriola et al., 2011; Holtshausen et al., 2011, Phakachoed, Suksombat, Colombatto, and Beauchemin, 2013). However, the responses to fibrolytic enzyme additive vary in effectiveness depending upon factors such as enzyme activity, type and dose of enzyme, type of diet, enzyme application method, and animal physiological status (Beauchemin et al., 2003). It is not possible to predict the potential effects of feed enzymes for ruminant

based on their enzymatic activities (Colombatto, Morgavi, Furtado, and Beauchemin, 2003). The present project focused on corn silages because it is fed to cattle in many parts of the world and has relatively high nutritive value. But corn silage in many parts of the world is different in quality depending upon factors such as growth stage, planting area, plant nutrient accumulation, and plant part (i.e., leaves, stem). In addition, no study has been conducted to investigate how different corn silage quality affects the response to fibrolytic enzyme additive.

4.3 Objective

The objective of the present study was to evaluate the potential of various enzyme additives with different enzyme activities to enhance *in vitro* ruminal degradation fiber and fermentation profile of four corn silages.

4.4 Materials and methods

The experiment was conducted as a completely randomized design with 2 runs (batches) and 4 replicates per run, where the runs were conducted on separate days.

4.4.1 Substrate and enzyme preparation

The different four corn silages used as the substrates are shown in Table 4.1. All substrates were dried at 60°C for 48 h in hot air oven, and ground with mill (Wiley mill standard model 4, Arthur H. Thomas, Philadelphia, PA, USA) through 1 mm screen. Substrates were stored in plastic bags for determination of chemical components and *in vitro* fermentation. Approximately 0.9 g DM of the ground corn silage was weighed into an acetone washed and preweighed filter bag (F57, Ankom Technology, Macedon, NY). Four replications were prepared for each treatment for

each batch culture incubation time. The enzyme products were diluted with water and then added (200 µl) directly onto substrates (corn silage) in the filter bags (before sealing) at 2 doses of each enzyme : 0 (control), and 4 µl/g of substrate DM. The bag was heat-sealed, and then placed into a 125 ml bottle and incubated at room temperature for 3 h.

Table 4.1 Chemical composition of substrates incubated *in vitro*.

	Dry matter (%)	**Crude Protein (%)**	**Neutral detergent fibre (%)**	**Acid detergent Fibre (%)**	**Starch (%)**
Corn silage 1	33.47	7.58	40.84	25.07	29.66
Corn silage 2	26.12	8.28	46.85	30.35	24.39
Corn silage 3	23.42	8.75	48.90	31.91	19.78
Corn silage 4	22.17	9.08	54.12	35.34	6.11

4.4.2 *In vitro* fermentations

Rumen fluid was collected from 3 cannulated cows approximately 3 h after the morning feeding, and strained through 4 layers of cheese cloth into a flask and flushed with oxygen-free CO_2. Rumen fluid was transported in insulated flasks to the laboratory within less than 1 h of collection. Anaerobic buffer medium (60 ml; Goering and Van Soest, 1970) containing tryptone, buffer, macro and micro mineral solution, resazurin and water (see Appendix) was adjusted to pH 6.0 using 1 *M* trans-aconitic acid (Sigma Chemicals, St. Louis, MO), and then added to each bottle. In addition to buffer, rumen fluid (15 ml) were added to each bottle in a ratio of 1 : 4 (rumen fluid : anaerobic buffer medium) under continuous flushing with CO_2. The bottles were closed with rubber stoppers and aluminium seal caps immediately after

loading and the bottles were incubated at 39°C on a rotary shaker for 24 and 48 h. Negative control (rumen fluid plus anaerobic buffer medium) and blanks (filter bags plus anaerobic buffer medium and rumen fluid) were also incubated using 4 replications for correction of gas production and degradability, respectively. Head space gas production (GP) resultant of substrate fermentation was measured at 3, 6, 12, 18, 24, and 48 h post incubation. The GP was measured by inserting a 23 gauge (0.6 mm) needle attached to a pressure transducer connected to a visual display. After 24 and 48 h of incubation, 4 bottles for each treatment were removed from the incubator, gas pressure was measured, and then bottles were placed on cold water to stop the fermentation. The gas pressure was converted to gas volume using the equation reported by Mauricio et al. (1999).

$$\text{Gas volume} = 0.18 + (3.697 \times \text{gas pressure}) + (0.0824 \times \text{gas pressure}^2)$$

Total cumulative gas production at 24 and 48 h were calculated by summing the gas volumes at each previous measurement time.

4.4.3 Determination of pH and VFA at 24 and 48 h

The pH was measured immediately with a pH-meter. Concentration of VFA was measured at 24 and 48 h incubation after measuring gas and pH. A 5 ml sample of fluid was added to 1 ml of 25-% meta-phosphoric acid for measurement of VFA concentrations. The VFA concentration was analyzed by using a gas chromatograph (model 5890, Hewlett-Packard Lab, Palo Alto, CA)with a capillary column (30 m 0.32 mm i.d., 1μm phase thickness, Zebron ZB-FAAP, Phenomenex, Torrance, CA), and flame ionization detection. The oven temperature was 150°C (no hold time), which was then increased by 20°C/min to 210°C, and held at this temperature for

2 min. The injector temperature was 225°C, the detector temperature was 250°C, and the carrier gas was helium.

4.4.4 DM, NDF, and ADF degradability

After 24 h and 48 h of incubation, the filter bags were removed (4 filter bags for each time and treatment) from bottles and washed under a stream of cold water until the water ran clear. The bags were dried in an oven at 55°C for 48 h to completely dry and DM degradability was determined by the loss of DM from the bags. The contents of the bags were then assayed for NDF and ADF content. The NDF and ADF analyses were conducted sequentially using an ANKOM200 Fiber analyzer unit based on the procedure described by Van Soest et al. (1991). Sodium sulfite (10 g/l NDF solution) and heat-stable bacterial amylase (2 ml/l NDF solution) were used in the analysis of NDF. The NDF and ADF degradability were calculated NDFD and ADFD.

4.4.5 Statistical analysis

Data analyses were conducted using the MIXED model procedure of SAS (SAS Institute Inc., Cary, NC). Data were analyzed as a completely randomized design with enzyme additive, corn silage and their interaction included in the model as fixed effects. Significance was declared at $P<0.05$.

4.4.6 Experimental site

The experiment was conducted at, Agriculture and Agri-Food Canada, Lethbridge Research Center, Lethbridge, AB, Canada.

4.4.7 Duration

The duration of the present experiment was from January to December 2012.

4.5 Results

At 24 h incubation, there was significant difference ($P<0.05$) between enzyme additives and the control in terms of their effects on DMD, NDFD, and ADFD. All enzyme additives were similar to TGP, but different ($P<0.05$) effects between enzyme additives and control (Table 4.2). The E1 and E2 had higher DMD, NDFD, and ADFD than E3, E4 and control ($P<0.05$); however, E3 and E4 had higher than the control ($P<0.05$). At 48 h incubation, there was also difference ($P<0.05$) between enzyme additives and control in terms of their effects on DMD, NDFD, ADFD, and TGP. The E1 and E2 had higher DMD, NDFD, and ADFD than others ($P<0.05$), but the E3 and E4 were similar in DMD and ADFD to control. For all enzyme additives, the effects of enzyme additive on TGP were higher than the control ($P<0.05$).

At both incubation times, the effects of corn silage substrate, there was difference ($P<0.05$) between corn silage substrate in terms of their effects on DMD, NDFD, ADFD, and TGP (Table 4.2). The corn silage 1 had highest DMD, NDFD, and TGP ($P<0.05$), but lowest ADFD ($P<0.05$). In contrast, corn silage 4 had highest ADFD ($P<0.05$), but lowest DMD and TGP ($P<0.05$). For all parameters, DMD, NDFD, ADFD and TGP, there were no enzyme × corn silage substrate interactions ($P>0.05$) at either 24 or 48 h of incubation (Table 4.2).

Table 4.2 Effect of enzyme (E) and corn silage on nutrient degradability (%) and total gas production (GP) from corn silage after 24 and 48 h of incubation (N = 8).

	Enzyme[1]	24 h				48 h			
		Degradability (%)			Total GP	Degradability (%)			Total GP
		DM	NDF	ADF	(mL/ g DM)	DM	NDF	ADF	(mL/ g DM)
Corn 1	**Mean**	**49.0[a]**	**25.1[a]**	**8.5[c]**	**78.2[a]**	**58.7[a]**	**30.6[a]**	**15.0[c]**	**109.2[a]**
	Control	47.5	23.5	6.6	74.8	57.9	28.1	11.2	104.0
	E1	49.7	26.3	10.7	79.2	59.8	32.2	17.6	109.2
	E2	50.1	25.7	9.8	78.8	59.1	32.3	17.9	110.4
	E3	49.4	25.1	8.7	78.8	58.4	30.3	13.7	110.9
	E4	48.2	24.7	7.0	79.5	58.4	30.1	14.5	111.1
Corn 2	**Mean**	**42.4[b]**	**15.2[b]**	**11.5[b]**	**74.3[b]**	**51.9[b]**	**22.5[b]**	**18.5[b]**	**103.4[b]**
	Control	41.1	12.5	8.5	69.7	50.8	20.0	15.7	97.0
	E1	42.8	16.2	14.0	77.1	52.8	23.8	21.5	106.4
	E2	43.2	16.8	13.4	75.0	52.1	23.8	19.8	104.4
	E3	42.6	15.3	11.4	74.2	51.8	22.3	18.3	105.1
	E4	42.3	14.9	10.4	75.3	51.7	22.7	17.2	104.0

Table 4.2 Effect of enzyme (E) and corn silage on nutrient degradability (%) and total gas production (GP) from corn silage after 24 and 48 h of incubation (N = 8) (Continued).

	Enzyme[1]	24 h				48 h			
		Degradability (%)			Total GP	Degradability (%)			Total GP
		DM	NDF	ADF	(mL/ g DM)	DM	NDF	ADF	(mL/ g DM)
Corn 3	**Mean**	**40.4^{c}**	**13.9^{c}**	**9.1^{c}**	**66.0^{c}**	**48.2^{c}**	**19.9^{c}**	**16.1^{c}**	**91.3^{c}**
	Control	38.8	12.5	6.9	64.1	47.2	18.7	14.5	85.9
	E1	40.4	14.3	10.6	66.8	49.2	21.5	18.6	91.3
	E2	40.6	14.4	11.7	65.9	49.0	21.6	18.2	93.3
	E3	41.5	14.0	8.1	67.1	47.6	18.8	14.6	92.6
	E4	40.6	14.1	8.2	66.3	48.1	19.1	14.6	93.4
Corn 4	**Mean**	**38.5^{d}**	**14.8^{b}**	**13.4^{a}**	**60.0^{d}**	**45.2^{d}**	**22.7^{b}**	**21.2^{a}**	**79.6^{d}**
	Control	38.0	13.5	11.9	54.8	44.5	21.3	20.2	71.5
	E1	38.5	15.3	14.0	60.4	45.2	23.8	22.0	81.3
	E2	39.3	15.8	15.3	62.6	46.8	24.0	23.9	86.1
	E3	38.5	14.9	13.5	61.5	44.7	22.1	19.2	79.2
	E4	38.1	14.7	12.1	60.7	44.6	22.3	22.7	79.9
Enzyme	Control	41.3^{c}	15.5^{c}	8.3^{d}	65.8^{b}	50.1^{b}	22.0^{c}	15.4^{b}	89.6^{b}
	E1	42.8^{ab}	18.0^{a}	12.3^{a}	70.9^{a}	51.8^{a}	25.3^{a}	19.9^{a}	97.0^{a}

Table 4.2 Effect of enzyme (E) and corn silage on nutrient degradability (%) and total gas production (GP) from corn silage after 24 and 48 h of incubation (N = 8) (Continued).

	Enzyme[1]	24 h				48 h			
		Degradability (%)			Total GP	Degradability (%)			Total GP
		DM	NDF	ADF	(mL/ g DM)	DM	NDF	ADF	(mL/ g DM)
Enzyme	E2	43.3^{a}	18.2^{a}	12.5^{a}	70.6^{a}	51.7^{a}	25.4^{a}	20.0^{a}	98.6^{a}
	E3	43.0^{ab}	17.3^{b}	10.5^{b}	70.4^{a}	50.6^{b}	23.4^{b}	16.4^{b}	97.0^{a}
	E4	42.3^{b}	17.1^{b}	9.4^{c}	70.5^{a}	50.7^{b}	23.6^{b}	16.7^{b}	97.2^{a}
Corn silage SEM		0.56	0.74	0.26	2.16	0.26	0.27	0.63	2.59
Enzyme SEM		0.57	0.75	0.30	2.21	0.29	0.31	0.66	2.70
Interaction SEM		0.73	0.83	0.65	2.84	0.57	0.65	1.10	3.99
P									
Corn silage		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Enzyme		<0.001	<0.001	<0.001	0.002	<0.001	<0.001	<0.001	0.002
Enzyme*Corn silage		0.62	0.24	0.34	0.99	0.84	0.89	0.38	0.99

[1]E1 : 75 : 25 combination of Cellulase PLUS and Xylanase PLUS (Dyadic International, Florida, USA); E2 : Rovabio Excel LC2 (Adisseo USA Inc, Georgia, USA); E3 : Cinabio (Adisseo USA Inc, Georgia, USA), and E4 : Econase RDE (AB Vista, Marlborough, UK).

[a-d] Mean within the same column for the main effects of enzyme or does having different letters are different at P<0.05.

At 24 h incubation, there was no difference (P>0.05) between enzyme additives and control in terms of their effects on total VFA, molar proportions of acetate, propionate, butyrate and acetate to propionate ratio (Table 4.3). For the effects of corn silage substrate, there was difference (P<0.05) between corn silage substrate in terms of their effects on all parameters i.e. total VFA, molar proportions of acetate, propionate, butyrate, and acetate to propionate ratio (Table 4.3). Corn silage 2 had higher total VFA than other 3 corn silages (P<0.05). Corn silage 2 and corn silage 3 had higher molar proportion of acetate than corn silage 1 and corn silage 4 (P<0.05), but corn silage 1 and corn silage 4 had higher molar proportion of propionate than corn silage 2 and corn silage 3 (P<0.05). Corn silage 1 and Corn silage 2 had highest molar proportions of butyrate and acetate to propionate ratio (P<0.05), respectively. For corn silage 1, corn silage 2 and corn silage 3, molar proportion of acetate, propionate, butyrate and acetate to propionate ratio, there were no enzyme × corn silage substrate interaction (P>0.05). But for corn silage 1 and corn silage 2, the effects of enzyme on total VFA depended upon the enzyme additive (enzyme × corn silage interaction, P<0.05). For corn silage 4, molar proportion of acetate, butyrate and acetate to propionate ratio, there were no enzyme × corn silage substrate interaction (P>0.05), but the effects of enzyme on total VFA and molar proportion of propionate depended upon the enzyme additive (enzyme × corn silage interaction, P<0.05).

At 48 h incubation, there was no difference (P>0.05) between enzyme additives and control in terms of their effects on molar proportion of acetate, propionate, butyrate, and acetate to propionate ratio, but enzyme additives were different (P<0.05) in their effects on total VFA (Table 4). The E3 and E4 had higher total VFA than control (P<0.05), where the E3 had highest total VFA (P<0.05). For

the effects of corn silage substrate, there was difference ($P<0.05$) between corn silage substrate in terms of their effects on all parameters i.e. total VFA, molar proportions of acetate, propionate, butyrate, and acetate to propionate ratio (Table 4.3).

Corn silage 4 had lowest total VFA than those in other 3 corn silages ($P<0.05$). Corn silage 3 and corn silage 4 had higher molar proportion of acetate than corn silage 1 and corn silage 2 ($P<0.05$), but corn silage 1 and corn silage 2 had highest molar proportions of propionate and butyrate ($P<0.05$), respectively. Corn silage 3 and corn silage 4 had higher acetate to propionate ratio than corn silage 1 and corn silage 2 ($P<0.05$). For all corn silages, total VFA and molar proportion of butyrate, there was no enzyme × corn silage substrate interaction ($P>0.05$). But for corn silage 3 and corn silage 4, the effects of enzyme on molar proportion of acetate depended upon the enzyme additive (enzyme × corn silage interaction, $P<0.05$). All corn silage substrates, the effects of enzyme on molar proportion of propionate and acetate to propionate ratio depended upon the enzyme additive (enzyme × corn silage interaction, $P<0.05$).

Table 4.3 Effect of enzyme additive (E) and dose (D) on volatile fatty acid (VFA) concentrations from corn silage after 24 and 48 h of incubation (N = 8).

	Enzyme[1]	24 h					48 h				
		Total VFA (m*M*)	Molar proportions[2]			Ac : Pr	Total VFA (m*M*)	Molar proportions[2]			Ac : Pr
			Ac	Pr	Bu			Ac	Pr	Bu	
Corn 1	**Mean**	**108.9^{B}**	**58.9^{B}**	**18.9^{A}**	**13.3^{A}**	**3.12^{C}**	**125.4^{A}**	**56.1^{C}**	**20.3^{A}**	**13.7^{B}**	**2.79^{C}**
	Control	105.7^{b}	58.5	18.5	13.9	3.16	121.8	55.8	20.8^{a}	14.3	2.69^{b}
	E1	115.9^{a}	58.8	18.9	13.5	3.12	128.1	56.5	20.0^{b}	14.2	2.80^{ab}
	E2	109.2^{ab}	59.0	18.9	13.2	3.13	127.1	56.2	20.2^{b}	14.3	2.85^{a}
	E3	109.3^{b}	59.3	19.2	12.8	3.10	123.9	55.7	20.4^{ab}	14.5	2.74^{ab}
	E4	108.5^{ab}	59.1	19.1	13.1	3.10	126.3	56.4	20.0^{b}	14.3	2.84^{a}
Corn 2	**Mean**	**112.5^{A}**	**61.6^{A}**	**16.3^{C}**	**12.7^{B}**	**3.79^{A}**	**123.1^{A}**	**59.0^{B}**	**18.1^{B}**	**14.4^{A}**	**3.27^{B}**
	Control	114.3^{ab}	61.5	15.9	12.8	3.88	122.8	59.5	17.6^{b}	13.7	3.40^{a}
	E1	119.9^{a}	61.9	16.5	12.6	3.77	121.6	58.8	18.4^{a}	13.6	3.21^{b}
	E2	108.9^{b}	61.8	16.4	12.7	3.80	119.5	58.9	18.0^{ab}	13.8	3.31^{ab}
	E3	105.4^{b}	61.3	16.3	12.9	3.76	125.2	58.7	18.3^{a}	13.7	3.22^{b}
	E4	110.1^{b}	61.6	16.6	12.7	3.73	126.2	59.0	18.4^{a}	13.5	3.22^{b}

Table 4.3 Effect of enzyme additive (E) and dose (D) on volatile fatty acid (VFA) concentrations from corn silage after 24 and 48 h of incubation (N = 8) (Continued).

		24 h					48 h				
	Enzyme[1]	**Total VFA (m*M*)**	**Molar proportions[2]**			**Ac : Pr**	**Total VFA (m*M*)**	**Molar proportions[2]**			**Ac : Pr**
			Ac	**Pr**	**Bu**			**Ac**	**Pr**	**Bu**	
Corn 3	**Mean**	**104.7^{C}**	**61.6^{A}**	**17.4^{B}**	**12.0^{C}**	**3.54^{B}**	**123.3^{A}**	**60.0^{A}**	**17.5^{C}**	**13.1^{C}**	**3.45^{A}**
	Control	102.8	61.7	17.0	12.2	3.63	116.6	59.2^{b}	17.5^{ab}	13.6	3.41^{b}
	E1	103.3	61.1	17.4	12.2	3.52	120.7	59.8^{b}	17.9^{a}	13.0	3.36^{b}
	E2	108.1	61.6	17.6	11.9	3.52	124.9	60.0^{ab}	17.6^{ab}	13.0	3.43^{ab}
	E3	105.3	61.9	17.5	11.8	3.55	128.0	60.8^{a}	17.2^{b}	12.8	3.58^{a}
	E4	103.6	61.4	17.6	12.0	3.50	126.5	60.2^{ab}	17.5^{ab}	13.0	3.47^{ab}
Corn 4	**Mean**	**95.4^{D}**	**60.4^{B}**	**19.1^{A}**	**11.5^{D}**	**3.16^{C}**	**116.4^{B}**	**59.9^{A}**	**17.6^{C}**	**12.8^{D}**	**3.43^{A}**
	Control	90.4^{b}	59.6	19.6^{a}	11.4	3.04	110.5	59.5^{b}	18.3^{a}	12.6	3.27^{c}
	E1	91.9^{ab}	60.3	19.1^{a}	11.3	3.09	114.0	59.4^{b}	17.7^{b}	13.1	3.38^{bc}
	E2	97.9^{a}	60.5	19.2^{ab}	11.5	3.15	119.0	59.9^{ab}	17.7^{b}	12.8	3.42^{ab}
	E3	98.0^{a}	60.5	18.7^{b}	11.8	3.24	119.9	60.6^{a}	17.1^{b}	12.7	3.56^{a}
	E4	98.6^{a}	61.0	18.6^{b}	11.6	3.29	118.6	60.3^{ab}	17.3^{b}	12.7	3.51^{ab}

Table 4.3 Effect of enzyme additive (E) and dose (D) on volatile fatty acid (VFA) concentrations from corn silage after 24 and 48 h of incubation (N = 8) (Continued).

		24 h					48 h				
	Enzyme[1]	**Total VFA (m*M*)**	**Molar proportions**[2]			**Ac : Pr**	**Total VFA (m*M*)**	**Molar proportions**[2]			**Ac : Pr**
			Ac	**Pr**	**Bu**			**Ac**	**Pr**	**Bu**	
Enzyme	Control	103.3	60.3	17.8	12.6	3.43	117.9^{C}	58.5	18.6	13.5	3.19
	E1	107.8	60.5	18.1	12.4	3.38	120.1^{BC}	58.6	18.5	13.5	3.21
	E2	106.1	60.7	18.0	12.3	3.40	122.9^{ABC}	58.8	18.4	13.5	3.24
	E3	104.4	60.8	17.9	12.3	3.41	125.0^{A}	59.0	18.3	13.5	3.28
	E4	105.2	60.6	18.0	12.4	3.40	124.4^{AB}	59.0	18.3	13.4	3.26
Corn silage SEM		5.82	0.23	0.38	0.28	0.100	2.12	1.23	1.24	0.18	0.292
Enzyme SEM		5.86	0.24	0.04	0.28	0.101	2.26	1.23	1.24	0.19	0.292
Interaction SEM		6.29	0.37	0.44	0.36	0.113	3.83	1.26	1.25	0.24	0.30
P											
Corn silage		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table 4.3 Effect of enzyme additive (E) and dose (D) on volatile fatty acid (VFA) concentrations from corn silage after 24 and 48 h of incubation (N = 8) (Continued).

Enzyme[1]	24 h					48 h				
	Total VFA (m*M*)	Molar proportions[2]			Ac : Pr	Total VFA (m*M*)	Molar proportions[2]			Ac : Pr
		Ac	Pr	Bu			Ac	Pr	Bu	
Enzyme	0.17	0.20	0.46	0.49	0.77	0.03	0.11	0.23	0.89	0.11
Corn silage*Enzyme	0.01	0.34	0.03	0.36	0.11	0.96	0.03	<0.001	0.17	<0.001

[a-d]Means within the same columns within substrate having different letters are different at P<0.05.

[A,B,C, D]Mean within the same column for the main effects of enzyme or substrate having different letters are different at P<0.05.

[1]E1 : 75 : 25 combination of Cellulase PLUS and Xylanase PLUS (Dyadic International, Florida, USA); E2 : Rovabio Excel LC2 (Adisseo USA Inc, Georgia, USA); E3 : Cinabio (Adisseo USA Inc, Georgia, USA), and E4 : Econase RDE (AB Vista, Marlborough, UK).

[2]Expressed as individual VFA, mol/100 mol; Ac=acetate, Pr=propionate, and Bu=butyrate, not included isobutyrate, isovalerate, valerate, and caproate.

4.6 Discussion

The four enzyme additives used in this study were commercial product that supplied a range in xylanase and endoglucanase activities. The enzyme activities were determined at pH 6 and 39°C as suggested by Colombatto and Beauchemin (2003) to reflect the ruminal conditions typically observed in the rumen of dairy cows fed a diet containing forage and concentrate. Each enzyme additive showed a wide range of activity for both endoglucanase and xylanase, depending on the organism used to produce the enzyme (Beauchemin et al., 2004) temperature, substrate (Colombatto and Beauchemin, 2003), and pH (Kung et al., 2002). Kung et al. (2002) reported that different activity profiles when assayed at different pH values and suggested that the effect of pH on enzyme activity may be an important factor when identifying fibrolytic enzyme for using in ruminant diets.

The present study had designed to evaluate the use of two doses of four fibrolytic enzyme additives to improve the degradation of four corn silages in *in vitro* techniques. The *in vitro* techniques can be used more reliably as a bioassay to predict *in vivo* response to exogenous enzymes. This project focused on corn silages because it is fed to cattle in many parts of the world and has relatively high nutritive value. But corn silage in many parts of the world is different in quality. The differences in NDF, ADF, and starch contents between the corn silages were probably due to differences in the stage of growth and plant part. The fibrolytic enzyme additive increased DMD, DNDF, ADF, and TGP of four corn silages at both 24 and 48 h incubation; however, the degradability of corn silage had showed different response in each enzyme product dependent on type of enzyme; additives of E1 and E2 were more effective than the other two enzyme additives. Similarly, Eun et al. (2007) reported on *in vitro* study that

the degradability of NDF and ADF from alfalfa hay and corn silage were increased by fibrolytic enzyme additive but vary in response depending on type of enzyme. However, each commercial enzyme additive showed different responses; additives E1 and E2 were more effective than the other two enzyme additives. The effects of corn silage substrate, there was difference between corn silage substrate in terms of their effects on DMD, NDFD, ADFD, and TGP. This might be due to the differences in composition between corn silage used in this study. Corn silage 1 had highest DMD and TGP, because corn silage 1 had higher starch content than other 3 corn silages. For all parameters, DMD, NDFD, ADFD, and TGP, there was no enzyme × corn silage substrate interaction at either 24 or 48 h of incubation. These observations suggest that the response to fibrolytic enzyme additive is similar to all corn silages. The current study found that fibrolytic enzyme additive had no effect on total VFA and molar proportions of individual VFA. This was in agreement with Chung et al. (2012), in a study that added enzyme to the diet did not affect ruminal fluid concentrations of total VFA and molar proportions of individual VFA. However, the effects of corn silage substrate were difference between corn silage substrate in terms of their effects on total VFA and molar proportions of individual VFA, because the proportion of ruminal VFA can be changed depending on nutrition compositions in the rumen. Phakachoed et al. (2013) suggested that measuring effects of enzyme on VFA concentrations are not a particularly useful way of screening the potential effects of the enzyme additives *in vivo*.

4.7 Conclusions

All enzyme additives show positive response by increasing DMD, NDFD, ADFD and TGP in all corn silage. That mean enzyme additives can be applied to improve fiber degradation for other forages. However, E1 and E2 showed more effective than E3 and E4.

4.8 References

Arriola, K.G., Kim, S.C., Staples, C.R., and Adesogan, A.T. (2011). Effect of fibrolytic enzyme application to low- and high-concentrate diets on the performance of lactating dairy cattle. **J. Dairy Sci.** 94: 832-841.

Beauchemin, K.A., Colombatto, D., Morgavi, D.P., and Yang, W.Z. (2003). Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. **J. Anim. Sci.** 81(Suppl. 2): 37-47.

Beauchemin, K.A., Colombatto, D., and Morgavi, D.P. (2004). A rationale for the development of feed enzyme products for ruminants. **Can. J. Anim. Sci.** 84: 23-36.

Chung, Y.H., Zhou, M., Holtshausen, L., Alexander, T.W., McAllister, T.A., Guan, L.L., Oba, M., and Beauchemin, K.A. (2012). A fibrolytic enzyme additive for lactating Holstein cow diets: Ruminal fermentation, rumen microbial populations, and enteric methane emissions. **J. Dairy Sci.** 95: 1419-1427.

Colombatto, D., and Beauchemin, K.A. (2003). A proposed methodology to standardize the determination of enzymic activities present in enzyme additives used in ruminant diets. **Can. J. Anim. Sci.** 83: 559-568.

Colombatto, D., Morgavi, D.P., Furtado, A.F., and Beauchemin, K.A. (2003). Screening of exogenous enzymes for ruminant diet: Relationship between biochemical characteristics and *in vitro* ruminal degradation. **J. Anim. Sci.** 81: 2628-2638.

Eun, J.S., Beauchemin, K.A., and Schulze, H. (2007). Use of exogenous fibrolytic enzymes to enhance *in vitro* fermentation of alfalfa hay and corn silage. **J. Dairy Sci.** 90: 1440-1451.

Goering, H.K., and Van Soest, P.J. (1970). **Forage fiber analyses (apparatus, reagents, procedures, and some applications).** Washington DC: Agric. Handbook.

Holtshausen, L., Chung, Y.H., Gerardo Cuervo, H., Oba, M., and Beauchemin, K.A. (2011). Improved milk production efficiency in early lactation dairy cattle with dietary addition of a developmental fibrolytic enzyme additive. **J. Dairy Sci.** 94: 899-907.

Kung, Jr.L., Cohen, M.A., Rode, L.M., and Treacher, R.J. (2002). The effect of fibrolytic enzymes sprayed onto forages and fed in a total mixed ratio to lactating dairy cows. **J. Dairy Sci.** 85: 2396-2402.

Mauricio, R.M., Mould, F.L., Dhanoa, M.S., Owen, E., Channa, K.S., and Theodorou, M.K. (1999). A semi-automated *in vitro* gas production technique for ruminant feedstuff evaluation. **Anim. Feed Sci. Technol.** 79: 321-330.

Nsereko, V.L., Beauchemin, K.A., Morgavi, D.P., Rode, L.M., Furtado, A.F., McAllister, T.A., Iwaasa, A.D., Yang, W.Z., and Wang, Y. (2002). Effect of a fibrolytic enzyme preparation from *Trichoderma longibrachiatum* on the rumen microbial population of dairy cows. **Can. J. Microbiol.** 48: 14-20.

Phakachoed, N., Suksombat, W., Colombatto, D., and Beauchemin, K.A. (2013). Use of fibrolytic enzymes additives to enhance *in vitro* ruminal fermentation of corn silage. **Livest. Sci.** 157: 100-112.

Van Soest, P.J., Robertson, J.B., and Lewis, B.A. (1991). Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal production. **J. Dairy Sci.** 74: 3583-3597.

Yang, H.J., and Xie, C.Y. (2010). Assessment of fibrolytic activities of 18 commercial enzyme products and their abilities to degrade the cell wall fraction of corn stalks in *in vitro* enzymatic and ruminal batch cultures. **Anim. Feed Sci. Technol.** 159: 110-121.

CHAPTER V

EFFECTS OF EXOGENOUS FIBROLYTIC ENZYMES ON *IN VITRO* RUMINAL FERMENTATION OF RICE STRAW

5.1 Abstract

This study was conducted to evaluate the effect of eight enzyme additives on ruminal fermentation of rice straw using a 48 h batch culture *in vitro* assay with medium and ruminal fluid. The experiment was conducted as a completely randomized design with two runs and four replicates. The all enzyme additives were commercial products that provided a range in endoglucanase, exoglucanase, and xylanase activities. The eight enzymes were added at 0, 2 and 4 µl/g of rice straw dry matter (DM). Gas production (GP) was measured at 3, 6, 12, 18, 24, and 48 h post incubation. Degradabilities of DM (DMD), neutral detergent fiber (NDFD), and acid detergent fiber (ADFD) were determined after 24 and 48 h. After 24 h incubation, DMD, NDFD, and total gas production (TGP) were not affected by enzyme additives, however ADFD was increased by enzyme additives ($P<0.05$). After 48 h incubation, the effects of enzyme additives on DMD and TGP were also similar ($P>0.05$). But NDFD was increased ($P<0.05$) by enzyme additives. At both time points, there were differences ($P<0.05$) between enzyme additives in terms of their effects on ADFD. The supplemental exogenous fibrolytic enzymes increased *in vitro* degradation of rice

straw, but in each enzyme product shows different response dependent on enzyme dose (enzyme × dose interaction, $P<0.05$). There were differences amongst the additives for total VFA at 24 and 48 h ($P<0.05$); increasing enzyme dose increased total VFA after 24 and 48 h, such that all doses were higher than the control ($P<0.001$). Overall, enzyme additives evaluated increased NDFD and ADFD of rice straw *in vitro*, however, in each fibrolytic enzyme additive showed different response dependent on enzyme dose. Based on the responses observed, E1 and E3 should be further evaluated on *in vivo* studies using diets based on rice straw.

5.2 Introduction

Rice straw is an abundant by-product of rice production which is widely used as the main source of roughages for ruminant animals in Thailand, because the cost of good quality forages is often high and forage availability is limited. However, rice straw contains high lignocellulosic content, low crude protein content, poor palatability, and low organic matter (OM) degradation in the rumen (Jung, Buxton, Hatfield, and Ralph, 1993). Increasing the digestibility of low quality feeds using enzyme feed additives could lead to significant improvements in animal performance in many parts of the world. However, the enzyme activities of feed enzymes tested under controlled optimal conditions cannot predict their ability to enhance ruminant feed digestion (McAllister, Hristov, Beauchemin, Rode, and Cheng, 2001). Therefore, the effects of enzyme additive before use as ruminant feed enzyme additives need to be tested and screened enzyme product and optimum dose rates in a ruminal condition (Colombatto and Beauchemin, 2003). This part of the study focused on *in vitro*

screening method with rice straw as substrate, before enzyme can be used in further evaluation on *in vivo* study using diets based on rice straw.

5.3 Objectives

The objective of the present study was to evaluate the potential of various enzyme additives different in enzyme activities to enhance *in vitro* ruminal fiber degradation and fermentation profile of rice straw, and to determine the optimum dose rate of individual enzyme product.

5.4 Materials and methods

The experiment was conducted as a completely randomized design with 2 runs (batches) and 4 replicates per run with 24 treatments arranged as a factorial (8 enzyme additives × 3 doses). The runs were conducted on separate days and the rice straw was used as substrate (71.80% of NDF and 42.03% of ADF). 8 commercial enzyme feed additives were used in this study, and 3 different doses were used for each enzyme : 0, 2, and 4 µl/g DM of substrate.

5.4.1 Substrate and enzyme preparation

Rice straw was dried at 55°C until dry (48 h) and was ground with mill (Wiley mill standard model 4, Arthur H. Thomas, Philadelphia, PA, USA) through 1 mm screen. Approximately 0.9 g DM of the ground rice straw was weighed into an acetone washed and preweighed filter bag (F57, Ankom Technology, Macedon, NY). Four replications were prepared for each treatment for each batch culture incubation time. The enzyme products were diluted with water and then added (200 µl) directly onto substrates (rice straw) in the filter bags (before sealing) at 3 doses of each

enzyme : 0 (control), 2 and 4 µl/g of substrate DM. The bag was heat-sealed, and then placed into a 125 ml bottle and incubated at room temperature for 3 h.

5.4.2 *In vitro* fermentations

Rumen fluid was collected from 3 cannulated cows approximately 3 h after the morning feeding, and strained through 4 layers of cheese cloth into a flask and flushed with oxygen-free CO_2. Rumen fluid was transported in insulated flasks to the laboratory within less than 1 h of collection. Anaerobic buffer medium (60 ml; Goering and Van Soest, 1970) containing tryptone, buffer, macro and micro mineral solution, resazurin and water (see Appendix) was adjusted to pH 6.5 using 1 *M* trans-aconitic acid (Sigma Chemicals, St. Louis, MO), and then added to each bottle. In addition to buffer, rumen fluid (15 ml) were added to each bottle at a ratio of 1 : 4 (rumen fluid : anaerobic buffer medium) under continuous flushing with CO_2. The bottles were closed with rubber stoppers and aluminium seal caps immediately after loading and the bottles were incubated at 39°C on a rotary shaker for 24 and 48 h. Negative control (rumen fluid plus anaerobic buffer medium) and blanks (filter bags plus anaerobic buffer medium and rumen fluid) were also incubated using 4 replications for correction of gas production and degradability, respectively. Head space gas production (GP) resultant of substrate fermentation was measured at 3, 6, 12, 18, 24, and 48 h post incubation. The GP was measured by inserting a 23 gauge (0.6 mm) needle attached to a pressure transducer connected to a visual display. After 24 and 48 h of incubation, 4 bottles for each treatment were removed from the incubator, gas pressure was measured, and then bottles were placed on cold water to stop the fermentation. The gas pressure was converted to gas volume using the equation reported by Mauricio et al. (1999).

$$\text{Gas volume} = 0.18 + (3.697 \times \text{gas pressure}) + (0.0824 \times \text{gas pressure}^2)$$

Total cumulative gas production at 24 and 48 h were calculated by summing the gas volumes at each previous measurement time.

5.4.3 Determination of VFA at 24 and 48 h

The pH was measured immediately with a pH-meter. Concentration of VFA was measured at 24 and 48 h incubation after measuring gas. A 5 ml sample of fluid was added to 1 ml of 25-% meta-phosphoric acid for measurement of VFA concentrations. The VFA concentration was analyzed by using a gas chromatograph (model 5890, Hewlett-Packard Lab, Palo Alto, CA)with a capillary column (30 m 0.32 mm i.d., 1 μm phase thickness, Zebron ZB-FAAP, Phenomenex, Torrance, CA), and flame ionization detection. The oven temperature was 150°C (no hold time), which was then increased by 20°C/min to 210°C, and held at this temperature for 2 min. The injector temperature was 225°C, the detector temperature was 250°C, and the carrier gas was helium.

5.4.4 DM, NDF, and ADF degradability

After 24 h and 48 h of incubation, the filter bags were removed (4 filter bags for each time and treatment) from bottles and washed under a stream of cold water until the water ran clear. The bags were dried in an oven at 55°C for 48 h to completely dry and DM degradability was determined by the loss of DM from the bags. The contents of the bags were then assayed for NDF and ADF content, and NDF and ADF degradability were calculated (NDFD and ADFD). The NDF and ADF analyses were conducted sequentially using an ANKOM200 Fiber analyzer unit based on the procedure described by Van Soest et al. (1991). Sodium sulfite (10 g/l NDF

solution) and heat-stable bacterial amylase (2 ml/l NDF solution) were used in the analysis of NDF.

5.4.5 Statistical analysis

Data analyses were conducted using the MIXED model procedure of SAS (SAS Institute Inc., Cary, NC). Data were analyzed as a completely randomized design with enzyme additive, rice straw and their interaction included in the model as fixed effects. Significance was declared at $P<0.05$.

5.4.6 Experimental site

The experiment was conducted at, Agriculture and Agri-Food Canada, Lethbridge Research Center, Lethbridge, AB, Canada.

5.4.7 Duration

The duration of the present experiment was from January to December 2012.

5.5 Results

Enzyme activity

Enzymes activities were calculated based on activities measured at optimal conditions for ruminal incubation, all enzyme additives supplied xylanase, endo-glucanase and, exo-glucanase activity and amylase, except only E1 and E7 not present amylase activity (Table 5.1). For xylanase activity, the enzyme products (per ml) were ranked E6>E8>E5>E2>E7>E1>E3>E4, the xylan substrate used was oat spelt. For endo-glucanaseand activity, the enzyme products (per ml) were ranked E2>E8>E1>E5>E6>E3>E7>E4, the cellulose substrate used was medium-viscosity carboxymethylcellulose. Thus, E6 was the highest concentrated source of xylanase

and E2 was highest concentrated source of endo-glucanase, while E4 was the lowest concentrated product.

***In vitro* fermentation**

After 24 h incubation, DMD, NDFD, and TGP were not affected by enzyme additives, however enzyme additives increased ADFD ($P<0.05$) (Table 5.2). After 48 h incubation, the effects of enzyme additives on DMD and TGP were also similar ($P>0.05$). But NDFD was increased ($P<0.05$) by enzyme additives. At both time points, there were differences ($P<0.05$) between enzyme additives in terms of their effects on ADFD.

Table 5.1 Enzyme activity of the 8 enzyme additives (pH 6.5).

Enzyme product	Enzymatic activity[1]			
	Xylanase	Endoglucanase	Exoglucanase	Amylase
Dyadic (E1)	1457±20	250±26	11±0.8	-
Econase (E2)	2820±124	265±18	7±0.4	0.6
Rovabio (E3)	880±42	106±6	6±0.9	0.5
Cinabio (E4)	156±16	28±0.5	2±0.4	0.1
EL2012 059L (E5)	2903±71	230±25	7±0.6	1.4
EL2012 060L (E6)	3347±34	209±12	10±1	0.5
EL2012 062L (E7)	2741±81	53±6	4±0.3	-
EL2012 063L (E8)	3246±59	263±1	8±0.9	2.0

[1]Endoglucanase, exoglucanase and amylase activitywere expressed as µmoles of glucose released per minuteper millilitre enzyme.

Xylanase activity was expressed as µmoles of xylose released per minute per millilitre enzyme.

At 48 h after incubation, E3 showed highest NDFD; in contrast NDFD was lowest in E4. At both time points, E3 also showed highest ADFD, but ADFD was

lowest in E5. For all parameters, DMD, NDFD, ADFD, and TGP, were increased ($P<0.05$) compared with control at 24 and 48 h after incubations. In contrast, the effect of enzyme dose on DMD was similar ($P>0.05$) between control and enzyme dose at 24 h after incubation. At both time points, enzyme × dose interaction of DMD and TGP were not observed ($P>0.05$), and enzyme × dose interaction of NDFD was not observed at 24 h after incubation.

At 48 h after incubation, the effect of enzyme dose on NDFD depended upon the enzyme additive (enzyme × dose interaction, $P<0.05$). At both time points, the effect of enzyme dose on ADFD depended upon the enzyme additive (enzyme × dose interaction, $P<0.05$). At 24 h after incubation, E2, E3, E6 and E7, all enzyme doses increased ADFD compared with the control ($P<0.05$), but between 2 and 4 µl/g DM, they were similar. For E1, all enzyme doses increased ADFD compared with the control ($P<0.05$), and ADFD at 4 µl/g DM dose was higher than the 2 µl/g DM dose. For E4, ADFD was similar ($P>0.05$) for all enzyme doses compared with the control. For E5 and E8, the ADFD at 2 µl/g DM was higher than the control, but the highest dose (4 µl/g DM) was similar with control ($P>0.05$).

At 48 h after incubation, E1, E2, E3, E4, E7 and E8, all enzyme doses increased NDFD compared with the control ($P<0.05$), but between 2 and 4 µl/g DM, they were similar. For E6, all enzyme dose increased NDFD compared with the control ($P<0.05$), but the NDFD at 4 µl/g DM was higher than at 2 µl/g DM.

For E5, the response to enzyme dose for NDFD, the 2 µl/g DM dose was higher ($P<0.05$) than the control, but the highest dose (4 µl/g DM) was similar to the control ($P>0.05$). For E1, E2, E3, E6, E7 and E8, all enzyme doses increased NDFD compared with the control ($P<0.05$), but between 2 and 4 µl/g DM, they were similar.

For E4 and E5, ADFD was similar (P>0.05) for all enzyme doses compared with the control.

For 24 h incubation, total VFA, molar proportion of acetate and acetate to propionate ratio were different between enzymes and doses (P<0.05). Total VFA, molar proportion of acetate and acetate to propionate ratio were higher for E8 than for the other enzyme additives (Table 5.3). At 2 and 4 µl/g DM supplementation of enzymes increased total VFA and molar proportion of acetate compared with the control (P<0.05), but between 2 and 4 µl/g DM, they were similar. The proportion of propionate was not affected by enzyme additives and doses (P>0.05). The proportion of butyrate were unaffected by enzyme additives (P>0.05). At 2 and 4 µl/g DM supplementation of enzymes increased proportion of butyrate compared with the control (P<0.05), but between 2 and 4 µl/g DM, they were similar. For all parameters, enzyme × dose interaction of total VFA, molar proportion of acetate, propionate, butyrate and acetate to propionate ratio were not observed (P>0.05).

For 48 h incubation, total VFA, molar proportion of acetate and acetate to propionate ratio were different between enzymes (P<0.05) (Table 5.3). Total VFA was higher for E2 than for other enzyme additives. The proportion of acetate was higher for E2, E3, and E4 than for the other enzyme additives. The proportion of butyrate was higher for E6 and E8 than for other enzyme additives. The acetate to propionate ratio was higher for E1, E2, E4, and E7 than for other enzyme additives. Total VFA, molar proportion of acetate, propionate and butyrate were not affected by doses (P>0.05). The dose of 4 µl/g DM decreased acetate to propionate ratio (P<0.05), but the doses of 0 µl/g DM and 2 µl/g DM were similar. For all parameters, enzyme × dose interaction of total VFA, molar proportion of acetate, propionate, butyrate and acetate to propionate ratio were also not observed (P>0.05)

Table 5.2 Effect of enzyme (E) and dose (D) on the degradability (%) of dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF), and total gas production (GP) from rice straw after 24 and 48 h of incubation with ruminal fluid.

Enzyme[1]	Level µL/g DM	24 h				48 h			
		Degradability (%)			Total GP	Degradability (%)			Total GP
		DM	NDF	ADF	mL/ g DM	DM	NDF	ADF	mL/ g DM
E1	**Mean**	**25.0**	**13.3**	**12.3^{A}**	**51.4**	**32.1**	**22.9^{B}**	**22.8^{BC}**	**81.2**
	0	24.8	11.4	10.3^{c}	49.1	31.6	20.5^{c}	20.9^{b}	75.6
	2	25.1	13.6	12.5^{b}	52.4	32.4	24.0^{a}	23.4^{a}	84.4
	4	25.2	14.9	14.2^{a}	52.6	32.3	24.2^{a}	24.0^{a}	83.8
E2	**Mean**	**24.8**	**13.0**	**12.0^{AB}**	**51.3**	**32.4**	**22.9^{B}**	**22.8^{BC}**	**80.5**
	0	24.8	11.4	10.3^{b}	49.1	31.6	20.5^{b}	20.9^{b}	75.6
	2	24.3	13.3	12.2^{a}	50.3	32.0	24.3^{a}	23.2^{a}	79.7
	4	25.5	14.4	13.5^{a}	54.6	33.5	23.8^{a}	24.4^{a}	86.4
E3	**Mean**	**25.0**	**12.7**	**12.4^{A}**	**53.3**	**33.4**	**24.3^{A}**	**24.8^{A}**	**84.1**
	0	24.8	11.4	10.3^{b}	49.1	31.6	20.5^{b}	20.9^{b}	75.6
	2	25.2	12.9	13.6^{a}	56.4	34.7	25.9^{a}	26.5^{a}	91.3
	4	25.1	13.9	13.3^{a}	54.3	33.9	26.4^{a}	27.1^{a}	85.3
E4	**Mean**	**24.8**	**12.2**	**10.7^{C}**	**51.1**	**31.9**	**21.9^{C}**	**21.8^{CD}**	**80.6**
	0	24.8	11.4	10.3	49.1	31.6	20.5^{b}	20.9	75.6

Table 5.2 Effect of enzyme (E) and dose (D) on the degradability (%) of dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF), and total gas production (GP) from rice straw after 24 and 48 h of incubation with ruminal fluid (continued).

Enzyme[1]	Level µL/g DM	24 h				48 h			
		Degradability (%)			Total GP	Degradability (%)			Total GP
		DM	NDF	ADF	mL/ g DM	DM	NDF	ADF	mL/ g DM
E4	2	25.0	12.1	10.9	52.4	31.6	22.3^{a}	21.9	83.0
	4	24.6	12.9	10.9	51.9	32.5	22.8^{a}	22.5	83.1
E5	**Mean**	**25.1**	**12.9**	**11.6^{AB}**	**51.5**	**31.6**	**21.6^{C}**	**21.6^{D}**	**77.9**
	0	24.8	11.4	10.3^{b}	49.1	31.6	20.5^{b}	20.9	75.6
	2	25.4	14.4	13.1^{a}	53.2	31.6	22.3^{a}	21.4	79.4
	4	25.1	12.8	11.3^{b}	52.2	31.5	22.1^{ab}	22.4	78.6
E6	**Mean**	**24.9**	**12.4**	**11.6^{AB}**	**51.9**	**32.6**	**23.4^{AB}**	**23.7^{AB}**	**81.4**
	0	24.8	11.4	10.3^{b}	49.1	31.6	20.5^{c}	20.9^{b}	75.6
	2	25.2	12.9	12.5^{a}	53.1	32.2	23.5^{b}	24.3^{a}	82.5
	4	24.7	12.8	12.0^{a}	53.7	34.0	26.2^{a}	26.0^{a}	86.2
E7	**Mean**	**24.8**	**12.8**	**11.7^{AB}**	**52.7**	**32.3**	**22.9^{B}**	**23.2^{B}**	**80.7**
	0	24.8	11.4	10.3^{b}	49.1	31.6	20.5^{b}	20.9^{b}	75.6

Table 5.2 Effect of enzyme (E) and dose (D) on the degradability (%) of dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF), and total gas production (GP) from rice straw after 24 and 48 h of incubation with ruminal fluid (continued).

Enzyme[1]	Level µL/g DM	24 h				48 h			
		Degradability (%)			Total GP mL/ g DM	Degradability (%)			Total GP mL/ g DM
		DM	NDF	ADF		DM	NDF	ADF	
E7	2	25.1	13.7	12.7^{a}	53.3	32.2	24.5^{a}	24.5^{a}	81.5
	4	24.6	13.2	12.2^{a}	55.7	33.1	23.7^{a}	24.1^{a}	85.1
E8	**Mean**	**24.5**	**12.7**	**11.3^{BC}**	**51.3**	**32.0**	**22.6^{BC}**	**22.6^{BCD}**	**79.7**
	0	24.8	11.4	10.3^{b}	49.1	31.6	20.5^{b}	20.9^{b}	75.6
	2	24.7	13.4	11.9^{a}	52.9	32.8	23.9^{a}	24.0^{a}	82.8
	4	24.0	13.4	11.7^{ab}	51.9	31.6	23.4^{a}	22.9^{a}	80.9
		0.61	1.14	1.03	2.14	1.45	1.47	0.91	4.30
Dose	0	24.8	11.4^{B}	10.3^{B}	49.1^{B}	31.6^{B}	20.5^{B}	20.2^{B}	75.6^{B}
	2	25.0	13.3^{A}	12.4^{A}	53.0^{A}	32.4^{A}	23.8^{A}	23.6^{A}	83.1^{A}
	4	24.8	13.5^{A}	12.4^{A}	53.4^{A}	32.8^{A}	24.1^{A}	24.0^{A}	83.7^{A}

Table 5.2 Effect of enzyme (E) and dose (D) on the degradability (%) of dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF) and total gas production (GP) from rice straw after 24 and 48 h of incubation with ruminal fluid (continued).

Enzyme[1]	Level	24 h				48 h			
		Degradability (%)			Total GP	Degradability (%)			Total GP
	µL/g DM	DM	NDF	ADF	mL/g DM	DM	NDF	ADF	mL/g DM
P-values									
Enzyme		0.87	0.21	0.003	0.26	0.25	<0.001	<0.001	0.33
Dose		0.60	<0.001	<0.001	<0.001	0.02	<0.001	<0.001	<0.001
Enzyme*Dose		0.93	0.28	0.04	0.35	0.71	0.008	0.01	0.69

[1]Enzyme E1, E2, E3, E4, E5, E6, E7, and E8 are identified in Table 5.1.

[a, b, c]Means within the same column within enzyme having different letters are different at P<0.05.

[A, B, C, D]Mean within the same column for enzyme and does having different letters are different at P<0.05.

SEM : standard error of the mean.

For 24 h incubation, total VFA, molar proportion of acetate and acetate to propionate ratio were different between enzymes and doses ($P<0.05$). Total VFA, molar proportion of acetate and acetate to propionate ratio were higher for E8 than for the other enzyme additives (Table 5.3). At 2 and 4 µl/g DM supplementation of enzymes increased total VFA and molar proportion of acetate compared with the control ($P<0.05$), but between 2 and 4 µl/g DM, they were similar. The proportion of propionate was not affected by enzyme additives and doses ($P>0.05$). The proportion of butyrate were unaffected by enzyme additives ($P>0.05$). At 2 and 4 µl/g DM supplementation of enzymes increased proportion of butyrate compared with the control ($P<0.05$), but between 2 and 4 µl/g DM, they were similar. For all parameters, enzyme × dose interaction of total VFA, molar proportion of acetate, propionate, butyrate and acetate to propionate ratio were not observed ($P>0.05$).

For 48 h incubation, total VFA, molar proportion of acetate and acetate to propionate ratio were different between enzymes ($P<0.05$) (Table 5.3). Total VFA was higher for E2 than for other enzyme additives. The proportion of acetate was higher for E2, E3, and E4 than for the other enzyme additives. The proportion of butyrate was higher for E6 and E8 than for other enzyme additives. The acetate to propionate ratio was higher for E1, E2, E4, and E7 than for other enzyme additives. Total VFA, molar proportion of acetate, propionate and butyrate were not affected by doses ($P>0.05$). The dose of 4 µl/g DM decreased acetate to propionate ratio ($P<0.05$), but the doses of 0 µl/g DM and 2 µl/g DM were similar. For all parameters, enzyme × dose interaction of total VFA, molar proportion of acetate, propionate, butyrate and acetate to propionate ratio were also not observed ($P>0.05$).

Table 5.3 Effect of enzyme additive (E) and dose (D) on volatile fatty acid (VFA) concentrations from rice straw after 24 and 48 h of incubation (N = 8).

Enzyme[1]	Dose (µl/g DM)	24					48				
		Total VFA (mM)	Molar proportions[2]			Ac : Pr	Total VFA (mM)	Molar proportions[2]			Ac : Pr
			Ac	Pr	Bu			Ac	Pr	Bu	
E1	**Mean**	**53.26^{B}**	**60.47^{C}**	**16.35**	**11.56^{A}**	**3.70^{C}**	**66.02^{AB}**	**62.04^{AB}**	**17.51**	**10.57^{B}**	**3.55^{A}**
	0	51.01	60.20	16.41	11.65	3.67	62.16	61.94	17.44	10.62	3.55
	2	57.71	60.94	16.19	11.49	3.76	68.33	62.10	17.53	10.52	3.55
	4	51.03	60.31	16.45	11.54	3.67	68.07	62.07	17.57	10.58	3.54
E2	**Mean**	**51.26^{B}**	**61.41^{BC}**	**16.44**	**11.15^{AB}**	**3.74^{BC}**	**69.31^{A}**	**62.45^{A}**	**17.60**	**10.41^{B}**	**3.55^{A}**
	0	51.01	60.20	16.41	11.65	3.67	62.16	61.94	17.44	10.62	3.55
	2	48.04	62.57	16.25	10.76	3.85	73.81	63.19	17.44	10.22	3.62
	4	54.73	61.48	16.65	11.04	3.70	71.97	62.22	17.91	10.40	3.48
E3	**Mean**	**53.43^{B}**	**61.54^{ABC}**	**16.45**	**11.17^{AB}**	**3.74^{BC}**	**66.22^{AB}**	**62.34^{A}**	**17.64**	**10.38^{B}**	**3.54^{AB}**
	0	51.01	60.20	16.41	11.65	3.67	62.16	61.93	17.44	10.62	3.55
	2	52.51	62.10	16.57	10.92	3.75	76.59	63.07	17.99	10.02	3.51
	4	56.79	62.33	16.38	10.93	3.70	59.40	62.02	17.49	10.50	3.55

Table 5.3 Effect of enzyme additive (E) and dose (D) on volatile fatty acid (VFA) concentrations from rice straw after 24 and 48 h of incubation (N = 8) (Continued).

Enzyme[1]	Dose (µl/g DM)	24					48				
		Total VFA (mM)	Molar proportions[2]			Ac : Pr	Total VFA (mM)	Molar proportions[2]			Ac : Pr
			Ac	Pr	Bu			Ac	Pr	Bu	
E4	**Mean**	**55.99^{AB}**	**61.58^{ABC}**	**16.30**	**11.22^{AB}**	**3.78^{ABC}**	**63.62^{ABC}**	**62.27^{A}**	**17.39**	**10.40^{B}**	**3.60^{A}**
	0	51.01	60.20	16.41	11.65	3.67	62.16	61.94	17.44	10.62	3.55
	2	56.70	62.32	16.21	10.98	3.85	66.03	62.40	17.39	10.28	3.61
	4	60.26	62.42	16.28	11.02	3.83	61.42	62.48	17.33	10.30	3.63
E5	**Mean**	**53.82^{B}**	**61.53^{ABC}**	**16.36**	**11.23^{AB}**	**3.76^{ABC}**	**55.58^{C}**	**61.46^{ABC}**	**17.45**	**10.80^{AB}**	**3.52^{AB}**
	0	51.01	60.20	16.41	11.65	3.67	62.16	61.94	17.44	10.62	3.55
	2	55.71	61.98	16.32	11.11	3.80	53.47	61.00	17.54	10.94	3.48
	4	54.73	62.42	16.35	10.95	3.82	51.93	61.45	17.38	10.84	3.54
E6	**Mean**	**56.97^{AB}**	**62.29^{AB}**	**16.31**	**10.95^{B}**	**3.82^{AB}**	**55.77^{C}**	**61.24^{BC}**	**17.54**	**11.08^{A}**	**3.46^{BC}**
	0	51.01	60.20	16.41	11.65	3.67	62.16	61.94	17.44	10.62	3.55
	2	62.95	63.59	16.25	10.54	3.91	51.23	6120	17.43	11.42	3.43
	4	56.96	63.07	16.26	10.68	3.88	54.14	60.57	17.75	11.21	3.39

Table 5.3 Effect of enzyme additive (E) and dose (D) on volatile fatty acid (VFA) concentrations from rice straw after 24 and 48 h of incubation (N = 8) (Continued).

Enzyme[1]	Dose (µl/g DM)	24					48				
		Total VFA (m*M*)	Molar proportions[2]			Ac : Pr	Total VFA (m*M*)	Molar proportions[2]			Ac : Pr
			Ac	Pr	Bu			Ac	Pr	Bu	
E7	**Mean**	**57.49^{AB}**	**62.45^{AB}**	**16.36**	**10.87^{B}**	**3.81^{ABC}**	**59.28^{BC}**	**62.09^{AB}**	**17.52**	**10.46^{B}**	**3.54^{A}**
	0	51.01	60.20	16.41	11.65	3.67	62.16	61.94	17.44	10.62	3.55
	2	63.32	64.17	16.36	10.28	3.92	56.76	61.95	17.42	10.47	3.56
	4	58.86	63.00	16.31	10.69	3.88	59.27	62.38	17.71	10.28	3.53
E8	**Mean**	**61.51^{A}**	**62.87^{A}**	**16.26**	**10.76^{B}**	**3.87^{A}**	**56.87^{C}**	**60.91^{C}**	**17.53**	**11.13^{A}**	**3.42^{C}**
	0	51.01	60.20	16.41	11.65	3.67	62.16	61.94	17.44	10.62	3.55
	2	63.10	63.91	16.22	10.40	3.94	51.91	60.25	17.60	11.18	3.40
	4	70.61	64.52	16.14	10.27	3.99	57.20	60.51	17.56	11.64	3.30
Dose	0	51.01^{B}	60.20^{B}	16.41	11.65^{A}	3.67^{B}	62.16	61.94	17.44	10.62	3.55^{A}
	0	51.01^{B}	60.20^{B}	16.41	11.65^{A}	3.67^{B}	62.16	61.94	17.44	10.62	3.55^{A}
	2	57.32^{A}	62.70^{A}	16.35	10.81^{B}	3.85^{A}	62.26	61.91	17.54	10.63	3.52^{AB}
	4	57.99^{A}	62.42^{A}	16.30	10.89^{B}	3.82^{A}	59.99	61.73	17.58	10.70	3.49^{B}
SEM		3.522	0.786	0.135	0.321	0.032	4.813	0.552	0.163	0.233	0.047

Table 5.3 Effect of enzyme additive (E) and dose (D) on volatile fatty acid (VFA) concentrations from rice straw after 24 and 48 h of incubation (N = 8) (Continued).

Enzyme[1]	Dose (µl/g DM)	24					48				
		Total VFA (m*M*)	Molar proportions[2]			Ac : Pr	Total VFA (m*M*)	Molar proportions[2]			Ac : Pr
			Ac	Pr	Bu			Ac	Pr	Bu	
P											
Enzyme		0.021	0.011	0.645	0.092	0.039	0.002	0.004	0.695	0.001	0.001
Dose		0.001	0.001	0.237	0.001	0.001	0.584	0.723	0.175	0.715	0.044
Enzyme*Dose		0.199	0.604	0.785	0.906	0.585	0.214	0.341	0.388	0.066	0.074

[A,B,C]Mean within the same column for the main effects of enzyme or substrate having different letters are different at P<0.05.

SEM : standard error of the mean.

[1]Enzyme E1, E2, E3, E4, E5, E6, E7, and E8 are identified in Table 5.1.

[2]Expressed as individual VFA, mol/100 mol; Ac=acetate, Pr=propionate, and Bu=butyrate, not included isobutyrate, isovalerate, valerate, and caproate.

5.6 Discussion

The present study evaluated several commercially produced fibrolytic enzyme additives for their potential to be used as ruminant feed additives for rice straw based diets. The effects of enzyme product on the animal response cannot be predicted from the enzyme activity (Beauchemin et al., 2004). Therefore, the *in vitro* method was used to screen the effects of enzyme product and optimum dose rate before enzyme products can be used in commercial ruminant farm, because *in vitro* method can screen large number of samples or treatments. The *in vitro* assay was used in the study to recommend enzymes for future use in dairy cow feeding studies, the pH of the buffer used was adjusted to pH 6.5 to reflect the typical pH in the rumen of dairy cows that offered rice straw.

At the both time points, all enzyme products had no effect on TGP when compared to the control. Similarly, Wang, Spratling, Wiedmeier, and McAllister (2004) reported no effect on TGP during 30 h of incubation when exogenous enzymes with endoglucanase, xylanase, and amylase activities were added to untreated wheat straw. Liu and Ørskov (2000) reported that treatment of untreated rice straw with varying dose levels of cellulase (0, 4, 8 to 16 unit per gram straw) had no effect on GP at 24 h of incubation. However, Yang et al. (2011) reported that the enzyme additives increased TGP from alfalfa hay after 12 h of incubation with ruminal fluid, but the enzymes did not affect TGP from rice straw.

Only some enzyme treatments improved TGP from rice straw at 48 h of incubation. Yang et al. (2011) suggesting that the recalcitrant fiber of rice straw took longer before the effects of the enzyme additives occurred. Therefore, these results

indicate that enzymes may have a greater potential to be used to improve the quality of higher quality forages than poorer quality forages.

At both time points, 24 and 48 h after incubation, DMD was not significantly different ($P>0.05$) for all enzyme treatments, the results reflected the results from TGP. Rice straw contains large amounts of polysaccharides that are potential sources of energy for the rumen microbe, however, it contains low crude protein (CP) and high lignocellulose content. Waghorn and McNabb (2003) reported that esterified bonds between cellulose, hemicellulose, and lignin restrict the digestion of recalcitrant cereal straws by ruminal microorganisms. Eun, Beauchemin, Hong, and Bauer (2006) reported that DMD from untreated rice straw at 24 h incubation was not affected by some enzymes (cellulases or xylanases) added. In contrast, Yang et al. (2011) reported that all enzyme treatments increased DMD at 12 and 48 h after incubations.

The ADFD was significantly different ($P<0.05$) by all enzyme dose treatments at both time points, 24 and 48 h of incubation, except for E4 and E5 which all enzyme doses were similar to control. However, NDFD was only increased at 48 h of incubation. Beauchemin et al. (1999) suggested that the application of enzyme directly to feed results in the slow release of enzyme into ruminal fluid as the feed is digested. Similarly, Yang et al. (2011) reported that NDFD was increased by all enzyme treatments at both incubation times (12 and 48 h), but ADFD was only enhanced at 48 h. In contrast, Eun et al. (2006) reported that enzyme products did not significantly affect NDFD and ADFD from untreated rice straw at 24 h of incubation, however, degradability of the fibrous fractions NDF and ADF were increased with enzyme that contained mainly protease activity. The different results of TGP, DMD, NDFD and ADFD could be due to the different rice straw substrate or specificity of enzyme products used. Beauchemin et al. (2003) have suggested that enzyme

additives vary in effectiveness depending upon factors such as enzyme activity, type and dose of enzyme, type of diet, enzyme application method, and animal physiological status.

Based on improvements in NDFD and ADFD of rice straw after 24 and 48 h of incubations in *in vitro* study (Figure 1). Maximum improvements in NDFD for E1 were increased respectively by up to 31% and 18% at 24 and 48 h, ADFD were respectively increased by up to 38% and 15% at 24 and 48 h. Similarly, E2 increased NDFD respectively by up to 26% and 19% at 24 and 48 h, ADFD were respectively increased by up to 21% and 17% at 24 and 48 h. Maximum improvements in NDFD for E3 were respectively 22% and 29%, for ADFD, these were respectively increased by 32% and 30% after 24 and 48 h. Maximum improvements in NDFD for E4 were 13% after 24 h and 11% after 48 h, respectively; for ADFD these were 6% after 24 h and 8% after 48 h respectively. For enzyme E1, E2, E3 and E4 that had been used previously in *in vitro* studies with corn silage (Chapter 3) where positive results had been reported. The results also reported that two additives, E1 (Dyadic) and E3 (Rovabio) were more effective than E2 (Econase) and E4 (Cinabio) after both 24 and 48 h of incubations. In addition, two additives E1 and E2 that had been used previously in feeding studies with dairy cows where positive results had been reported (Arriola et al., 2011; Holtshausen et al., 2011). However, there was no any report study on other four additives with rice straw in both *in vitro* and *in vivo*. It is recommended that E1 and E3 at 2.0 µl/g DM dose should be further evaluated in *in vivo* studies using diets based on rice straw.

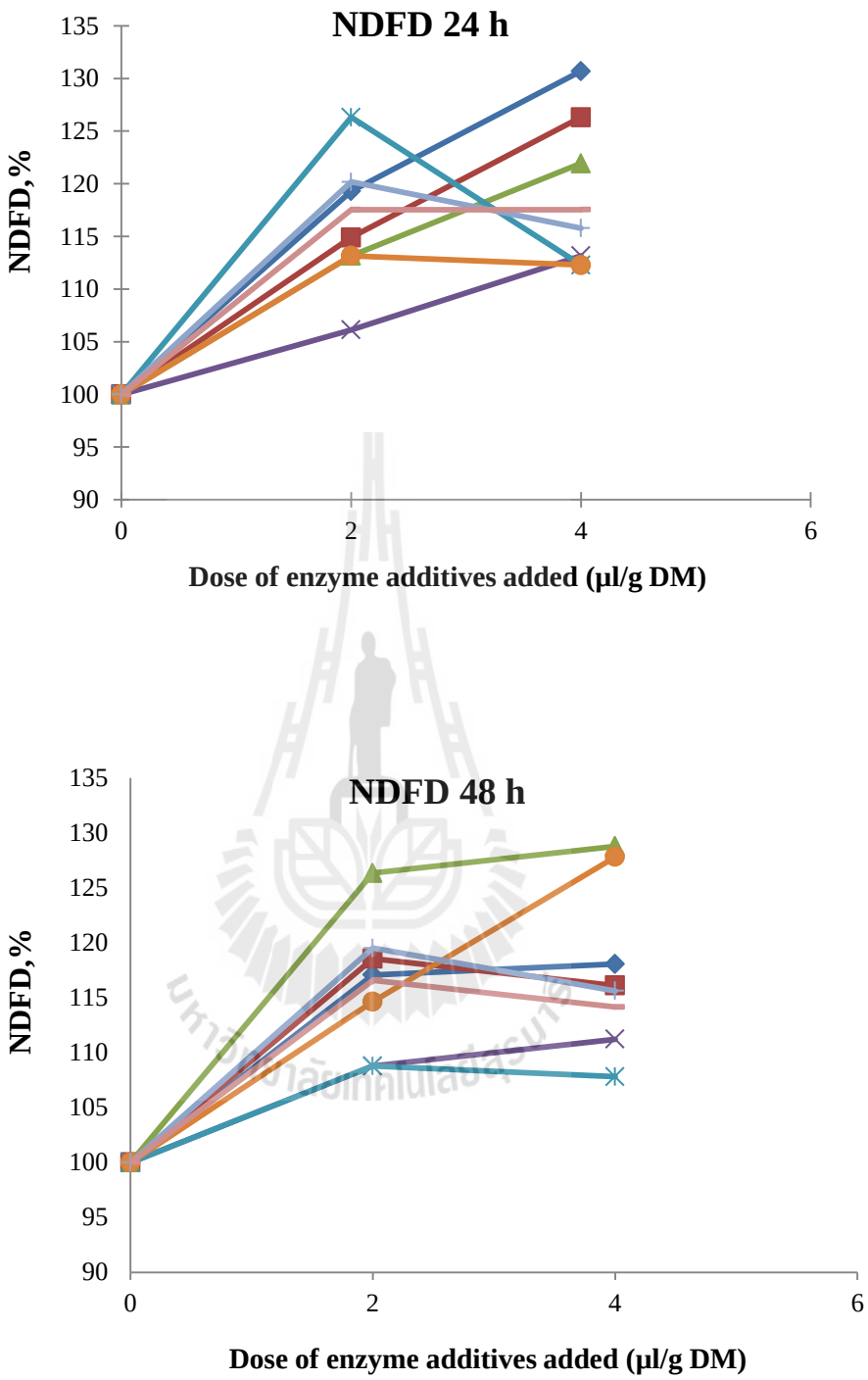

Figure 5.1 Percentage increase in neutral detergent fiber disappearance (NDFD) and acid detergent fiber disappearance (ADFD) from rice straw after 24 and 48 h of incubation. Enzyme E1, E2, E3, E4, E5, E6, E7, and E8 are identified in Table 5.1.

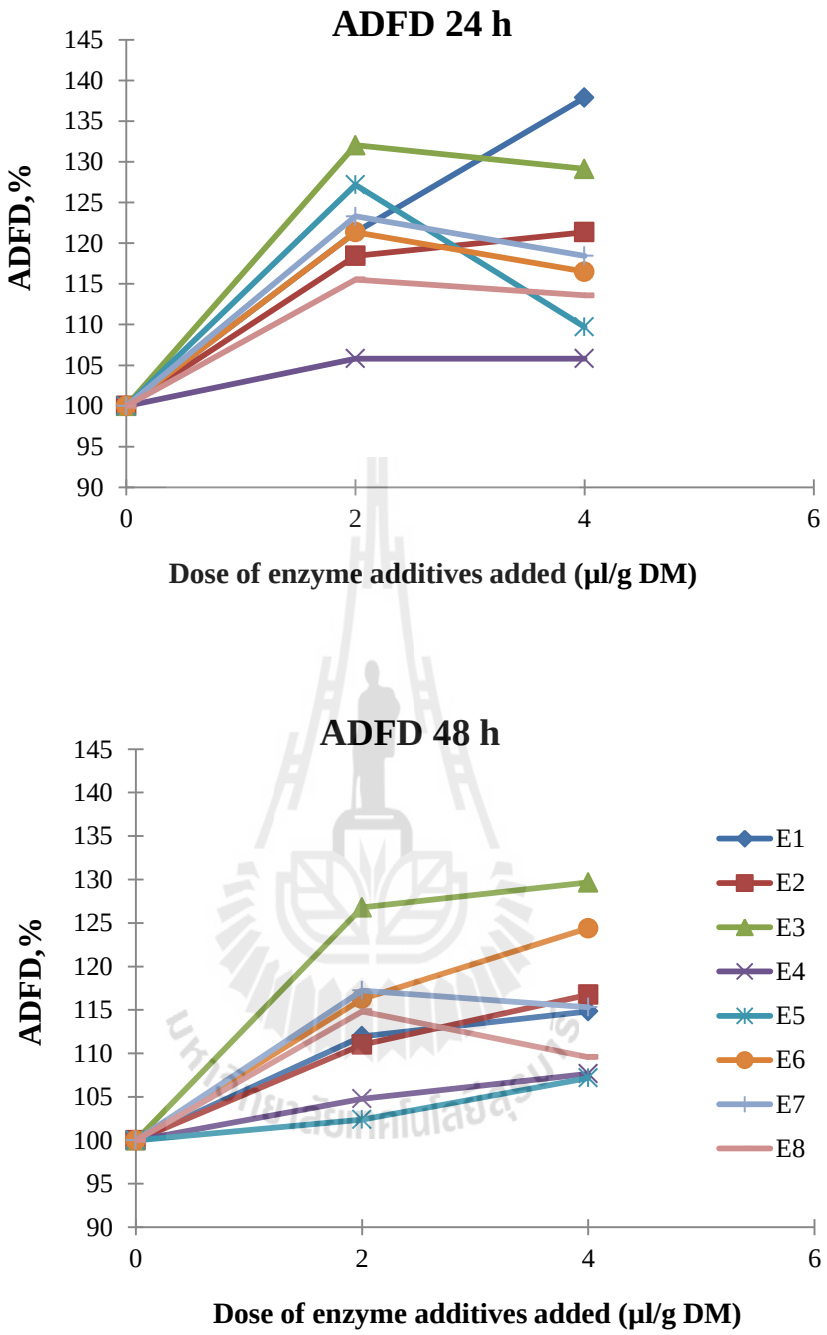

Figure 5.1 Percentage increase in neutral detergent fiber disappearance (NDFD) and acid detergent fiber disappearance (ADFD) from rice straw after 24 and 48 h of incubation. Enzyme E1, E2, E3, E4, E5, E6, E7, and E8 are identified in Table 5.1 (Continued).

Maximum improvements in NDFD for E5 were increased respectively by up to 26% and 9% at 24 and 48 h, ADFD were respectively increased by up to 27% and 7% at 24 and 48 h (Figure 1). Maximum improvements for E6 increased NDFD respectively by up to 13% and 27% at 24 and 48 h, ADFD were respectively increased by up to 21% and 24% at 24 and 48 h. Maximum improvements in NDFD for E7 were respectively 20% and 20%, for ADFD, these were respectively increased by 23% and 17% after 24 and 48 h. Maximum improvements in NDFD for E8 were 17% after 24 h and 17% after 48 h, respectively; for ADFD these were 16% after 24 h and 15% after 48 h, respectively. For enzymes E5, E6, E7, and E8 were developmental fibrolytic enzyme additives in liquid form.

Volatile fatty acids are end-products of rumen microbial fermentation and represent the main supply of energy for ruminants. The current study found that fibrolytic enzyme additives increased total VFA concentration, molar proportions of acetate and butyrate, which correspond to increases in DMD, NDFD or ADFD. Eun et al. (2006) reported that *in vitro* study on total VFA production was higher for ammoniated rice straw (ARS) compared to untreated rice straw (URS) and addition of enzyme to URS or ARS did not influence total VFA production. Some rumen microbes are capable of switching fermentation end-products depending on their growth rate (Russell and Wallace, 1997). However, Yang et al. (2011) reported that total VFA was not affected for all enzyme treatments after 12 h of incubation of rice straw with ruminal fluid, but the proportion of acetate was increased by enzyme additives. However, total VFA concentrations tended to be greater than the control after 48 h of incubation. Yang et al. (2011) suggested a slight increase in carbohydrate fermentation resulting from the addition of enzyme additives.

5.7 Conclusions

The all enzyme additives evaluated in the present study supplied a unique range of endoglucanase, exoglucanase and xylanase activities. All additives evaluated increased NDFD and ADFD of rice straw *in vitro*. However, in each fibrolytic enzyme additive shows different response dependent on enzyme dose. Based on the responses observed, E1 and E3 should be further evaluated on *in vivo* studies using diets based on rice straw.

5.8 References

Arriola, K.G., Kim, S.C., Staples, C.R., and Adesogan, A.T. (2011). Effect of fibrolytic enzyme application to low- and high-concentrate diets on the performance of lactating dairy cattle. **J. Dairy Sci.** 94: 832-841.

Beauchemin, K.A., Colombatto, D., and Morgavi, D.P. (2004). A rationale for the development of feed enzyme products for ruminants. **Can. J. Anim. Sci.** 84: 23-36.

Beauchemin, K.A., Colombatto, D., Morgavi, D.P., and Yang, W.Z. (2003). Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. **J. Anim. Sci.** 81(Suppl. 2): 37-47.

Beauchemin, K.A., Yang, W.Z., and Rode, L.M. (1999). Effects of grain source and enzyme additive on site and extent of nutrient digestion in dairy cows. **J. Dairy Sci.** 82: 378-390.

Colombatto, D., and Beauchemin, K.A. (2003). A proposed methodology to standardize the determination of enzymic activities present in enzyme additives used in ruminant diets. **Can. J. Anim. Sci.** 83: 559-568.

Eun, J.S., Beauchemin, K.A., Hong, S.H., and Bauer, M.W. (2006). Exogenous enzymes added to untreated or ammoniated rice straw: Effects on *in vitro* fermentation characteristics and degradability. **Anim. Feed Sci. Technol.** 131: 86-101.

Goering, H.K., and Van Soest, P.J. (1970). **Forage fiber analyses (apparatus, reagents, procedures, and some applications).** Washington DC: Agric. Handbook.

Holtshausen, L., Chung, Y.H., Gerardo-Cuervo, H., Oba, M., and Beauchemin, K.A. (2011). Improved milk production efficiency in early lactation dairy cattle with dietary addition of a developmental fibrolytic enzyme additive. **J. Dairy Sci.** 94: 899-907.

Jung, H.G., Buxton, D.R., Hatfield, R.D., and Ralph, J. (1993). **Forages cell wall structure and digestibility.** USA: American Society for Agronnomics.

Liu, J.X., and Ørskov, E.R. (2000). Cellulase treatment of untreated and steam pre-treated rice straw effect on *in vitro* fermentation characteristics. **Anim. Feed Sci. Technol.** 88: 189-200.

McAllister, T.A., Hristov, A.N., Beauchemin, K.A., Rode, L.M., and Cheng, K.J. (2001). Enzymes in ruminant diets. In M.R. Bedford and G.G. Partridge (eds.). **Recent advances in enzymes in farm animal nutrition** (pp. 273-298). Wallingford , CABI Publishing.

Mauricio, R.M., Mould, F.L., Dhanoa, M.S., Owen, E., Channa, K.S., and Theodorou, M.K. (1999) A semi-automated *in vitro* gas production technique for ruminant feedstuff evaluation. **Anim. Feed Sci. Technol.** 79: 321-330.

Russell, J.B., and Wallace, R.J. (1997). Energy-yielding and energy-consuming reactions. In P.N. Hobson and C.S. Stewart (eds.), **The rumen microbial ecosystem** (2nd ed., pp 246-882). New York: Blackie academic & professional.

Van Soest, P.J., Robertson, J.B., and Lewis, B.A. (1991). Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal production. **J. Dairy Sci.** 74: 3583-3597.

Waghorn, G.C., and McNabb, W.C. (2003). Consequence of plant phenolic compounds for productivity and health of ruminants. **Proc. Nutr. Soc.** 62: 383-392.

Wang, Y., Spratling, B.M., Wiedmeier, D.R., and McAllister, T.A. (2004). Effect of alkali pretreatment of wheat straw on the efficacy of exogenous fibrolytic enzymes. **J. Anim. Sci.** 82: 198-208.

Yang, H.E., Son, Y.S., and Beauchemin, K.A. (2011). Effects of exogenous enzymes on ruminal fermentation and degradability of alfalfa hay and rice straw. **Asian-Aust. J. Anim. Sci.** 24: 56-64.

CHAPTER VI

EFFECTS OF XYLANASE SUPPLEMENTATION ON RUMINAL DIGESTIBILITY IN FISTULATED NON-LACTATION DAIRY COWS FED RICE STRAW

6.1 Abstract

The effect of xylanase product on ruminal disappearance and rumen fermentation of rice straw based diet was evaluated in fistulated non-lactating dairy cows. Three fistulated non-lactating dairy cows were used in 3 × 3 Latin squares design; the trial consisted of 3 periods of 21 d in each period. Treatments were : 1) control, 2) 10 g xylanase/cow/d, and 3) 20 g xylanase/cow/d. The commercial xylanase enzyme (endo-1, 4-beta-xylanase, EC 3.2.1.8) which was a fibrolytic enzyme powder (Porzyme® 93010; Danisco Animal Nutrition) was used in this study. Diets offered as 3 kg/d of concentrate containing 17% CP together with *ad libitum* rice straw and clean water. Enzyme did not change dry matter intake, ruminal pH and NH_3-N concentrations. DM and ADF potential disappearance fraction, DM and ADF total disappearances were unaffected by the enzyme supplementation. However, NDF potential disappearance fraction, and NDF total disappearance were increased when the enzyme was added at a high dose. Hemicellulose degradability at 0, 3, 6, 12, 24, 48, 72, and 96 hour were unaffected by supplementation of xylanase. Total volatile

fatty acid concentrations, molar proportion of acetate, propionate and butyrate; and ratio of acetate : propionate at each hour were unaffected by xylanase.

6.2 Introduction

The degradation of plant cell walls by ruminants is of major economic importance in the developing countries. Increasing the efficiency with which the ruminal micro-organisms degrades fiber has been the subject of extensive research for many years. Recently, research has demonstrated that supplementing dairy cow and feedlot cattle diets with fiber degrading enzymes has significant potential to improve feed utilization and animal performance. Ruminant feed enzyme additives, primarily xylanases and cellulases, are concentrated extracts resulting from bacterial or fungal fermentations that have specific enzymatic activities. Improvements in animal performance due to the use of enzyme additives can be attributed mainly to improvements in ruminal fiber digestion resulting in increased digestible energy intake. However, most research has been carried out in developed countries where roughage contains less fiber than in the tropics. In addition, the enzyme products are mixed enzymes of unknown quantities of each enzyme. Several studies reported that supplementation fibrolytic enzyme mixture in high xylanase activity have shown positive response in feedlot cattle (Beauchemin et al., 1999), dairy cattle (Arriola et al., 2011). Xylanase is common enzymes that appear in rumen produced by some of microbial fermentation, which is the main enzyme involved in degrading the xylan polymer of plant cell wall into soluble sugars. The present research aims to evaluate the use of a single enzyme xylanase in non-lactating dairy cows fed rice straw. Rice straw is an abundant by-product of rice production and the main source of

roughages in Thailand. However, its nutritive value is low, containing high lignocellulosic content, low crude protein (CP) content, and poor palatability resulting in low intake and incomplete fiber digestion affecting poor feed efficiency and poor animal production. Recently, several studies showed that adding exogenous fibrolytic enzymes to ruminant diets increased digestion of DM and fiber (Bowman et al., 2002; Rode et al., 1999; Yang et al., 2000). Furthermore, Yang et al. (1999) reported increased digestion of OM and NDF in the rumen and in the total tract in dairy cows supplemented with an enzyme mixture, and total tract digestion of DM and OM increased when a small amount of enzyme was used (Beauchemin et al., 2000). However, not all studies report improved digestion due to the use of exogenous fibrolytic enzymes (Knowlton et al., 2002; Lewis et al., 1999) and viewed across a variety of enzyme products and experimental conditions the response to feed enzymes by ruminants has been variable.

6.3 Objectives

The objective of this study was to evaluate the effect of two levels of xylanase (endo-1, 4-beta-xylanase, EC 3.2.1.8) on ruminal disappearance and rumen fermentation of rice straw based diets in fistulated non-lactating dairy cow.

6.4 Materials and methods

6.4.1 Feed and animal management

6.4.1.1 Animal and feeding managements

Three fistulated Crossbred Holstein Friesian non-lactating dairy cows housed in individual pens were assigned to one of three treatments in 3 × 3 Latin

squares design. The trial consisted of 3 periods, with 21 d in each period, 14 d for adaptation to diets and 7 d for ruminal sample collection and *in vivo* disappearance trial. Treatments were : 1) control, 2) 10 g xylanase/cow/d, and 3) 20 g xylanase/cow/d. The commercial xylanase enzyme (endo-1, 4-beta-xylanase, EC 3.2.1.8) which was a fibrolytic enzyme powder (Porzyme® 93010; Danisco Animal Nutrition) derived from dried *Trichoderma longibrachiatum* fermentation (xylanase activity 40000 U/g) was used in this study.

6.4.2 Measurements and chemical analysis

6.4.2.1 Feed intake

Diets offered as 3 kg/d of concentrate containing 17% CP, divided into 2 equal meals at 0700 and 1500 h together with *ad libitum* rice straw and clean water. Feed offered and feed refused were measured and recorded daily during the experimental periods. Dry matter content (48 h at 65°C) of the corn silage for individual cows was determined daily to calculate DMI.

The samples were ground through a 1 mm screen for chemical analysis. Dry matter (DM) of rice straw and concentrate were determined by oven drying at 105°C to a constant weight. The samples were analyzed for crude protein (CP), ether extract (EE), ash (AOAC, 1995), neutral detergent fiber (NDF), detergent fiber acid (ADF), and acid detergent lignin (ADL) (Van Soest et al., 1991). Rice straw was ground through a 2 mm screen for *in vivo* ruminal disappearance determination. Approximately 5 g of 2 mm ground rice straw samples were placed into 8 × 11 cm nylon bags with 47 μm pore size. Samples of rice straw were suspended in the rumen of each fistulated non-lactating dairy cow for 0, 3, 6, 12, 24, 48, and 72 h, and all bags were retrieved and placed in ice water to stop the fermentation. The bags were washed

under a gentle stream of water until the water ran clear and the bags were oven-dried at 65°C for 48 h. After weighing each bag individually, the residues were analyzed for DM, NDF, and ADF content. The disappearance values were determined and expressed as a proportion of DM, NDF, and ADF incubated, respectively.

6.4.2.2 Collection of rumen fluid

To evaluate ruminal fermentation, on the last day of each experimental period (d 21), ruminal fluid samples were collected from each fistulated non-lactating dairy cow at 0, 2, 4, and 6 h after the morning feeding. The pH of rumen fluid were immediately determined at the time of sampling by pH meter. For VFAs and ammonia N determination, 36 ml of rumen fluid was put into 50 ml centrifuge tube containing 4 ml of 1 M H_2SO_4, then centrifuged at 1895 rpm for 15 min. Supernatants was collected and put into 25 ml test tube, then capped and stored at -20°C until analysis. Analysis of acetic, propionic and butyric acids used GC (Hewlett Packard GC system HP6890, USA, 19091N-113 INNOWAX, Length (meters) 30, I.D. (mm) 0.32 WIDEBORE, Film (um) 0.25). Ammonia N concentrations was determined by Kjeldahl analysis (AOAC, 1995)

6.4.3 Statistical analysis

Measurements of intake, pH, ammonia N, VFAs and ruminal disappearance coefficients of each period were analyzed by ANOVA using the Statistical Analysis System (SAS, 1996). Differences between treatment means were statistically compared using Least Significant Differences.

6.4.4 Experimental site

The experiment was conducted at Suranaree University of Technology's farm, and the center for Scientific and Technological Equipment building 1 and 3, Suranaree University of Technology.

6.4.5 Duration

The duration of the present experiment was from May to August 2011.

6.5 Results

6.5.1 Feed compositions

Chemical composition of concentrate and rice straw used in the present experiment is given in Table 6.1. Mean values for DM, CP, EE, NDF, ADF and ADL of concentrate and rice straw were 91.33 and 92.45, 17.23 and 3.43, 2.65 and 0.51, 31.29 and 67.27, 24.06 and 47.19, 5.51 and 7.68%, respectively.

Table 6.1 Chemical composition of feeds.

Item	Concentrate	Rice straw
Dry matter	91.33	92.45
	……..……..% of DM……	
Ash	10.10	15.36
Crude protein	17.23	3.43
Ether extract	2.65	0.51
Neutral detergent fiber	31.29	67.27
Acid detergent fiber	24.06	47.19
Acid detergent lignin	5.51	7.86

6.5.2 Intake and ruminal fermentation

Feed intake, ruminal pH and ammonia N concentration were showed in Table 6.2. Concentrate DM intake of the three groups were similar at 2.74 kg/d (data not showed), total DM feed intake were 10.27, 10.55, and 10.24 kg/d, respectively which were unaffected by supplementation of xylanase ($P>0.05$). No effect of xylanase was detected on ruminal pH and ammonia N concentration of each hour ($P>0.05$).

Table 6.2 Effect of xylanase supplementation on feed intake, ruminal pH and NH_3-N of fistulated non-lactating dairy cows.

Item	Control	10 g xylanase/cow/d	20 g xylanase/cow/d	SEM	P-value
Feed	10.27	10.55	10.24	2.840	0.98
Intake(kg/day)					
pH					
Hour 0	6.62	6.67	6.71	0.002	0.37
Hour 2	6.61	6.60	6.65	0.003	0.67
Hour 4	6.42	6.48	6.39	0.001	0.24
Hour 6	6.42	6.50	6.37	0.003	0.32
NH_3-N (mg/l)					
Hour 0	25.51	25.45	23.60	2.68	0.57
Hour 2	43.19	43.41	48.41	6.24	0.32
Hour 4	28.14	26.54	33.94	6.92	0.21
Hour 6	17.90	19.02	20.35	2.08	0.44

In addition, Table 6.3 showed volatile fatty acid concentrations in the rumen. Total volatile fatty acid concentration, molar proportion of acetate, propionate and butyrate and ratio of acetate : propionate of each hour were unaffected by supplementation of xylanase (P>0.05).

6.5.3 Ruminal disappearance of rice straw

In vivo DM, NDF, and ADF disappearance of rice straw are shown in Table 6.4. DM soluble fraction (a), DM potential disappearance fraction (b), DM disappearance rate (c), and DM total disappearance (a + b) of rice straw were unaffected by supplementation of xylanase (P>0.05). NDF soluble fraction (a) and NDF disappearance rate (c) of rice straw were unaffected by supplementation of xylanase (P>0.05), however NDF potential disappearance fraction (b) and NDF total

disappearance (a + b) of rice straw were higher in 20 g/cow/d xylanase than in control and 10 g/cow/d xylannase (P<0.05). ADF soluble fraction (a), ADF potential disappearance fraction (b), ADF disappearance rate (c), and ADF total disappearance (a + b) of rice straw were unaffected by supplementation of xylanase (P>0.05).

Hemicellulose degradability is shown in Table 6.5. Hemicellulose degradability at 0, 3, 6, 12, 24, and 48 hour were unaffected by supplementation of xylanase (P>0.05), however, hemicellulose degradability tended to increase particularly at 20 g/cow/d xylanase dose at 72 and 96 h incubations (P≤0.10).

Table 6.3 Effect of xylanase supplementation on volatile fatty acid (VFAs) of fistulated non-lactating dairy cows.

Item	Control	xylanase/cow/d		SEM	P-value
		10 g	20 g		
Acetate (C2)		**mol/100mol**			
Hour 0	75.52	75.07	74.63	1.59	0.82
Hour 2	72.68	72.07	72.54	2.68	0.94
Hour 4	73.05	73.46	73.26	2.71	0.97
Hour 6	73.66	73.31	73.26	2.62	0.97
Propionate (C3)		**mol/100mol**			
Hour 0	14.47	15.09	14.32	1.70	0.85
Hour 2	15.69	15.69	15.92	1.33	0.98
Hour 4	15.04	15.25	15.26	1.17	0.98
Hour 6	14.52	14.81	14.71	2.11	0.98
Butytate (C4)		**mol/100mol**			
Hour 0	10.00	10.27	10.60	0.92	0.85
Hour 2	11.63	11.86	11.55	1.51	0.97
Hour 4	11.90	11.26	11.48	1.62	0.90
Hour 6	11.83	11.85	11.88	1.31	1.00

Table 6.3 Effect of xylanase supplementation on volatile fatty acid (VFAs) of fistulated non-lactating dairy cows (Continued).

Item	Control	xylanase/cow/d		SEM	P-value
		10 g	20 g		
TotalsVFA		**Mmol**			
Hour 0	51.32	51.39	49.46	3.07	0.60
Hour 2	63.78	58.21	65.98	5.67	0.17
Hour 4	65.40	59.88	67.86	5.77	0.18
Hour 6	62.31	58.17	64.98	4.09	0.17
C2:C3					
Hour 0	5.23	4.98	5.25	0.26	0.87
Hour 2	4.65	4.65	4.57	0.16	0.98
Hour 4	4.88	4.82	4.81	0.17	0.98
Hour 6	5.10	4.98	4.93	0.29	0.95

Table 6.4 Effect of xylanase supplementation on ruminal disappearance coefficients of rice straw.

Item	Control	xylanase/cow/d		SEM	P-value
		10 g	20 g		
Dry matter					
Soluble fraction (a)	9.07	13.10	10.10	1.79	0.19
Potential disappearance (b)	48.80	48.07	53.10	4.63	0.27
Disappearance rate (c)	0.021	0.018	0.018	< 0.01	0.20
a+b	57.87	61.20	63.17	2.88	0.19
Neutral detergent fiber					
Soluble fraction (a)	1.00	1.47	0.97	0.064	0.32
Potential disappearance (b)	57.13^{b}	55.63^{b}	61.50^{a}	0.62	0.04
Disappearance rate (c)	0.023	0.021	0.020	< 0.01	0.16
a+b	57.93^{b}	57.10^{b}	62.47^{a}	0.76	0.04

Table 6.4 Effect of xylanase supplementation on ruminal disappearance coefficients of rice straw (Continued).

Item	Control	xylanase/cow/d		SEM	P-value
		10 g	20 g		
Acid detergent fiber					
Soluble fraction (a)	0.37	0.97	0.50	0.047	0.21
Potential disappearance (b)	58.23	56.67	59.13	5.32	0.66
Disappearance rate (c)	0.0176	0.0183	0.0176	< 0.01	0.20
a+b	58.57	57.63	59.57	4.56	0.74

[a,b]Means within the same row having different letters are different (P<0.05).

Table 6.5 Effect of xylanase supplementation on hemicellulose degradability (%).

Item	Control	xylanase/cow/d		SEM	P-value
		10 g	20 g		
Hour 0	1.94	1.34	2.18	0.37	0.53
Hour 3	3.28	4.53	2.88	0.83	0.39
Hour 6	8.93	8.91	7.79	2.36	0.76
Hour 12	23.86	23.88	23.77	1.53	0.99
Hour 24	30.30	30.85	31.12	1.96	0.86
Hour 48	39.82	42.37	42.91	4.68	0.50
Hour 72	51.08	52.83	55.36	3.85	0.10
Hour 96	52.55	56.98	62.32	4.97	0.06

6.6 Discussion

Xylanase supplementation had no effect on dry matter intake in the present study, although xylanase increased hemicellulose degradability. Similar results (Schingoethe, Stegeman, and Treacher, 1999; Kung et al., 2000; Sutton, Phipps, Beever, Humphries, Hartnell, Vicini, and Hard, 2003; Rode et al., 1999) reported that dry matter intake was not increased by supplementation of enzymes. Yang et al. (1999) reported that dry matter intake was unaffected by enzyme supplements even though fiber digestion in total tract increased but unaffected in particulate passage rate. However, enzyme have improved dry matter intake (Lewis et al., 1999), who reported that dry matter intake of dairy cows were increased when enzyme was applied to forages. Beauchemin et al. (2004) proposed that pretreatment of dry feeds with enzymes applied in a liquid form was importance of adsorption and binding of enzyme to substrate before feeding to allow proper attachment and protection against degradation from proteolytic processes in the rumen. Contrarily, Yang et al. (2000) reported that dry matter intake was unaffected by supplementation of enzyme when applied the enzyme to TMR immediately before feeding. The effect of enzyme addition on intake appear to differ among enzyme products, but the method of applying enzymes to diets is apparently not a major factor influencing feed intake (Yang et al., 2000).

Ruminal pH was unaffected by supplementation of xylanase. In the current study, ruminal pH closed to neutrality because animals received high proportion of rice straw (data not shown). Exogenous fibrolytic enzymes seemed to work better at close to neutrality (Colombatto, Mould, Bhat, and Owen, 2007). Ammonia N concentrations at all sampling hours were unaffected by supplementation of xylanase. Similar result (Bowman et al., 2002; Sutton et al., 2003; Alvarez et al., 2009; Yang

et al., 1999) because Ammonia N in the rumen occur from degradation of protein or urea by enzyme from ruminal microorganisms, But the main effect of supplementation xylanase is increases digestion of fiber (hemicellulose) which no effects on Ammonia N concentration in rumen. VFA concentration of each hour were unaffected by supplementation of xylanase. Similar result (Yang et al., 1999; Kung et al., 2000). In contrast, Sutton et al. (2003) reported decreased acetate, increased propionate and no effects on butyrate when compared to control. Yang et al. (2000) reported increased total VFA but decreased butyrate.

DM and ADF digestion were unaffected by supplementation of xylanase because this study used xylanase alone, which effects to hemicellulose degradability. Xylanase supplementation increased NDF potential disappearance fraction (b) and NDF total disappearance (a + b) of rice straw, were higher with xylanase 20 g/cow/d than control and xylannase 10 g/cow/d. Because hemicellulose digestion tended to increase particularly at 20 g xylanase dose at 72 and 96 h incubations. Bowman et al. (2002) reported that increase DM, OM, NDF, and ADF digestibility when enzyme was applied to concentrate, because who used mixture enzymes in experiment. Similar result with Rode et al. (1999) reported increased digestibility of nutrients in the total tract of dairy cows, when enzyme was added to the concentrate portion of a TMR. Beauchemin et al. (1999) reported that applying enzymes to the TMR before feeding increased digestibility in the total tract, It was suggested that applying the enzyme to the TMR immediately before feeding caused the enzymes to be solubilized in ruminal fluid resulting in rapid passage of the enzyme from the rumen such that most of the enzyme action occurred in the hindgut (Yang et al., 2000).

6.7 Conclusions

The enzyme used in present study contained xylanase alone (endo-1, 4-beta-xylanase) activity 40000 U/g. When supplements in fistulated non-lactating dairy cows, it had no effect on feed intake, Ruminal pH, NH_3-N concentrations and rumen VFA proportions. Xylanase supplementation was unaffected on DM, ADF potential disappearance fraction, DM and ADF total disappearance. But increased NDF potential disappearance fraction, and NDF total disappearance when added in high doses. Hemicellulose degradability tended to increase particularly at 20 g xylanase dose at 72 and 96 h incubations.

6.8 References

Alvarez, G., Pinos-Rodriguez, J.M., Herrera, J.G., Garcia, J.C., Gonzalez, S.S., and Barcena, R. (2009). Effects of exogenous fibrolytic enzymes on ruminal digestibility in steers fed high fiber rations. **Livest. Sci.** 121: 150-154.

Arriola, K.G., Kim, S.C., Staples, C.R., and Adesogan, A.T. (2011). Effect of fibrolytic enzyme application to low- and high-concentrate diets on the performance of lactating dairy cattle. **J. Dairy Sci.** 94: 832-841.

Association of Official Analytical Chemists (AOAC). (1995). **Official methods of analysis** (16th ed.). USA: Arlington.

Beauchemin, K.A., Colombatto, D., Morgavi, D.P., Yang, W.Z., and Rode, L.M. (2004). Mode of action of exogenous cell wall degrading enzymes for ruminants. **Can. J. Anim. Sci.** 84: 13-22.

Beauchemin, K.A., Rode, L.M., Maekawa, M., Morgavi, D.P., and Kampen, R. (2000). Evaluation of a nonstarchpolysaccharidase feed enzyme in dairy cow

diets. **J. Dairy Sci.** 83: 543-553.

Beauchemin, K.A., Yang, W.Z., and Rode, L.M. (1999). Effect of grain source and enzyme additive on site and extent of nutrient digestion in dairy cows. **J. Dairy Sci.** 82: 378-390.

Bowman, G.R., Beauchemin, K.A., and Shelford, J.A. (2002). The proportion of the diet to which firbolytic enzyme are added affects nutrient digestion by lactating dairy cows. **J. Dairy Sci.** 85: 3420-3429.

Colombatto, D., Mould, F.L., Bhat, M.K., and Owen, E. (2007). Influence of exogenous fibrolytic enzyme level and incubation pH on the *in vitro* ruminal fermentation of alfalfa stems. **Anim. Feed Sci. Technol.** 137: 150-162.

Knowlton, K.F., McKinney, J.M., and Cobb, C. (2002). Effect of a direct-fed fibrolytic enzyme formulation on nutrient intake, partitioning, and excretion in early and late lactation holstein cows. **J. Dairy Sci.** 85: 3328-3335.

Kung, L.Jr., Treacher, R.J., Nauman, G.A., Smagala, AM., Endres, K.M., and Cohen, M.A. (2000). The effect of treating forages with fibrolytic enzymes on its nutritive value and lactation performance of dairy cows. **J. Dairy Sci.** 83: 115-122.

Lewis, G.E., Sanchez, W.K., Hunt, C.W., Guy, M.A., Pritchard, G.T., Swanson, B.I., and Treacher, R. (1999). Effect of direct-fed fibrolytic enzymes on the lactational performance of dairy cows. **J. Dairy Sci.** 82: 611-617.

Rode, L.M., Yang, W.Z., and Beauchemin, K.A. (1999). Fibrolytic enzyme supplements for dairy cows in early lactation. **J. Dairy Sci.** 82: 2121-2126.

Schingoethe, D.J., Stegeman, G.A., and Treacher, R.J. (1999). Response of lactating dairy cows to a cellulase and xylanase enzyme mixture applied to forages at the time of feeding. **J. Dairy Sci.** 82: 996-1003.

Statistical Analysis System. (1996). **SAS user' guide: Statistics.** NC: SAS Institute.

Sutton, J.D., Phipps, R.H., Beever, D.E., Humphries, D.J., Hartnell, G.F., Vicini, J.L., and Hard, D.L. (2003). Effect of method of application of a fibrolytic enzyme product on digestive processes and milk production in Holstein-friesian cows. **J. Dairy Sci.** 86: 546-556.

Van Soest, P.J., Robertson, J.B., and Lewis, B.A. (1991). Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal production. **J. Dairy Sci.** 74: 3583-3597.

Yang, W.Z., Beauchemin, K.A., and Rode, L. (1999). Effects of an enzyme feed additive on extent of digestion and milk production of lactating dairy cows. **J. Dairy Sci.** 82: 391-403.

Yang, W.Z., Beauchemin, K.A., and Rode, L.M. (2000). A Comparison of methods of adding fibrolytic enzymes to lactating cow diets. **J. Dairy Sci.** 83: 2512-2520.

CHAPTER VII

EFFECTS OF EXOGENOUS FIBROLYTIC ENZYME ON RUMINAL DIGESTIBILITY IN FISTULATED NON-LACTATING DAIRY COWS FED CORN SILAGE

7.1 Abstract

This study was conducted to evaluate the effect of two fibrolytic enzyme additives on the digestibility of corn silage and ruminal fermentation, pH and blood glucose concentration of fistulated non-lactating dairy cows. Five fistulated Crossbred Holstein Friesian non-lactating dairy cows housed in individual pens were assigned to one of five treatments in 5 × 5 Latin squares design. The trial consisted of 5 periods, with 21 d in each period, 14 d for adaptation to diets and 7 d for ruminal sample collection and *in vivo* disappearance trial. Dietary treatments were : 0 (control), 0.5 ml of E1/kg of corn silage dry matter (DM), 1.0 ml of E1/kg of corn silage DM, 1.0 ml of E2/kg of corn silage DM and 1.5 ml of E2/kg of corn silage DM. Diets offered as 3 kg/d of concentrate containing 21% crude protein (CP), divided into 2 equal meals at 0800 and 1600 h together with *ad libitum* corn silage and clean water. Addition of enzyme additives to the diet had no effect on dry matter intake (DMI), ruminal fluid concentrations of total volatile fatty acid (VFA), NH_3, molar proportions of individual VFA, ruminal pH and blood glucose concentration. The degradability of dry matter (DMD), neutral detergent fiber (NDFD), and acid detergent fiber (ADFD) were not

affected by enzyme additives at 3, 6, and 12 h of ruminal incubations (P>0.05). However, enzyme additives evaluated increased DMD, NDFD, and ADFD of corn silage after 24, 48, and 72 h of ruminal incubations (P<0.05).

7.2 Introduction

Exogenous fibrolytic enzymes that contain mainly of cellulase and xylanase activities showed positive responded to increased fiber digestion of corn silage in *in vitro* (Eun and Beauchemin, 2007; Eun et al., 2007). The *in vitro* study was conducted to evaluate the effect of fibrolytic enzyme under rumen condition at pH 6.0 to 6.5 and 39°C (Colombatto and Beauchemin, 2003). Holtshausen et al. (2011) reported that in the *in vitro* study, the developmental enzyme additive increase or tendency for an increase in NDF digestibility of the alfalfa hay, alfalfa silage and barley silage, and in the *in vivo* study, adding the same enzyme additive (1.0 ml/kg TMR DM) also showed positive effect to increased milk production efficiency and FCM production efficiency. Therefore, the effects of enzyme additive need to be confirm in a ruminal condition (*in vivo*). Rovabio Excel LC2 (E1) and 75 : 25 combination of Cellulase PLUS and Xylanase PLUS (E2) enzyme products were selected based on results obtained in Chapter 3. In this part of study focused on *in vivo* study using diets based on corn silage to confirm the effect of 2 enzyme additives (Chapter 3).

7.3 Objectives

The objective of this study was to evaluate the effect of two exogenous fibrolytic enzymes on blood glucose, ruminal disappearance and rumen fermentation of fistulated non-lactating dairy cows fed corn silage.

7.4 Materials and methods

7.4.1 Feed and animal management

Five fistulated Crossbred Holstein Friesian non-lactating dairy cows housed in individual pens were assigned to one of five treatments in 5 × 5 Latin squares design. The trial consisted of 5 periods, with 21 d in each period, 14 d for adaptation to diets and 7 d for ruminal sample collection and *in vivo* disappearance trial. Dietary treatments were : 0 (control), 0.5 ml of E1/kg of corn silage DM, 1.0 ml of E1/kg of corn silage DM, 1.0 ml of E2/kg of corn silage DM and 1.5 ml of E2/kg of corn silage DM. The enzyme additives were diluted in water. The dilution (50 ml of the dilution per 1 kg of corn silage DM) was sprayed on to the corn silage at the time before feeding. An equal amount of water (50 ml/1 kg of corn silage DM) was added to the control diet.

7.4.2 Measurements and chemical analysis

Diets offered as 3 kg/d of concentrate containing 21% CP, divided into 2 equal meals at 0800 and 1600 h together with *ad libitum* corn silage and clean water. Feed offered and feed refused were measured and recorded daily during the experimental periods. The DM content (48 h at 60°C) of the corn silage for individual cows was determined daily to calculate DMI.

The samples were ground through a 1 mm screen for chemical analysis. The DM of corn silage and concentrate were determined by oven drying at 105°C to a constant weight. The samples were analyzed for CP, ether extract (EE), ash (AOAC, 1995), NDF, ADF, and ADL (Van Soest et al., 1991). Corn silage was ground through a 2 mm screen for *in vivo* ruminal disappearance determination. Approximately 5 g of 2 mm ground corn silage samples were placed into 8 × 11 cm nylon bags with 47 μm pore size. Samples of corn silage were suspended in the rumen of each fistulated non-lactating dairy cow for 0, 3, 6, 12, 24, 48, and 72 h, and all bags were retrieved and placed in ice water to stop the fermentation. The bags were washed under a gentle stream of water until the water ran clear and the bags were oven-dried at 65°C for 48 h. After weighing each bag individually, the residues were analyzed for DM, NDF, and ADF content. The disappearance values were determined and expressed as a proportion of DM, NDF, and ADF incubated, respectively.

7.4.3 Ruminal fermentation

To evaluate ruminal fermentation, on the last day of each experimental period (d 21), ruminal fluid samples were collected from each fistulated non-lactating dairy cow at 0, 2, 4, and 6 h after the morning feeding. The pH of rumen fluid were immediately determined at the time of sampling by pH meter. For VFAs and ammonia N determination, 36 ml of rumen fluid was put into 50 ml centrifuge tube containing 4 ml of 1 M H_2SO_4, then centrifuged at 1895 rpm for 15 min. Supernatants were collected and put into 25 ml test tube, then capped and stored at -20°C until analysis. Analysis of acetic, propionic and butyric acids used GC (Hewlett Packard GC system HP6890, USA, 19091N-113 INNOWAX, Length (meters) 30, I.D. (mm) 0.32 WIDEBORE, Film (um) 0.25). Ammonia N concentration was determined by Kjeldahl analysis (AOAC, 1995)

7.4.4 Statistical analysis

Measurements of intake, pH, ammonia N, VFAs, blood glucose and ruminal disappearance coefficients of each period were analyzed by ANOVA using the Statistical Analysis System (SAS, 1996). Differences between treatment means were statistically compared using Least Significant Differences.

7.4.5 Experimental site

The experiment was conducted at Suranaree University of Technology's Farm, and the Center for Scientific and Technological Equipment, Building 10, Suranaree University of Technology.

7.4.6 Duration

The duration of the present experiment was from May to August 2013.

7.5 Results

7.5.1 Feed compositions

Chemical composition of concentrate and rice straw used in the present experiment is given in Table 7.1.

Table 7.1 Chemical composition of feeds.

Item	Concentrate	Corn silage
Dry matter	92.99	25.36
	……..……..% of DM…….	
Ash	10.21	12.61
Crude protein	21.38	7.31
Ether extract	2.85	0.91
Neutral detergent fiber	42.53	62.61
Acid detergent fiber	19.96	36.04
Acid detergent lignin	7.23	10.37

7.5.2 Intake, blood glucose and ruminal fermentation

Feed intake, blood glucose, ruminal pH and ammonia N concentration were showed in Table 7.2. All of animal groups consumed similar amount of concentrate at 2.79 kg DM/d (data not showed), total DM feed intakes were 12.43, 12.80, 12.91, 12.75, and 12.91 kg DM/d, respectively. Adding fibrolytic enzyme to the diet did not affect ruminal pH, ammonia N concentration and blood glucose concentration (of each hour) ($P>0.05$).

Ruminal volatile fatty acid concentrations were showed in Table 7.3. There were also no effect of fibrolytic enzyme addition on total volatile fatty acid concentration, molar proportion of acetate, propionate and butyrate and ratio of acetate : propionate (of each hour) ($P>0.05$).

7.5.3 Ruminal degradability of corn silage

In vivo DMD, NDFD, and ADFD of corn silage are shown in Table 7.4. At 3, 6, and 12 h of ruminal incubation, the DMD was not affected by enzyme additives. However, adding fibrolytic enzyme increased DMD after 24, 48, and 72 h ruminal incubations ($P<0.05$). At 24 and 72 ruminal incubations, all enzyme treatments increased DMD compared with the control, but DMD between enzyme groups were similar. At 48 h ruminal incubation, adding 1.0 ml of E1, 1.0 ml of E2 and 1.5 ml of E2/kg of corn silage DM increased DMD, and at 1.5 ml of E2/kg of corn silage DM also showed highest DMD.

Table 7.2 Effect of exogenous fibrolytic enzyme supplementation on feed intake, ruminal pH, NH_3-N and blood glucose of fistulated non-lactating dairy cows fed corn silage.

Item	Control	E1		E2		SEM	P-value
		0.5 ml/kg	1.0 ml/kg	1.0 ml/kg	1.5 ml/kg		
Feed Intake (kg/d)	12.43	12.80	12.91	12.75	12.91	0.230	0.59
pH							
Hour 0	6.96	6.96	6.94	6.98	7.03	0.051	0.77
Hour 2	6.38	6.42	6.36	6.34	6.44	0.044	0.54
Hour 4	6.38	6.39	6.33	6.36	6.46	0.048	0.46
Hour 6	6.48	6.55	6.57	6.53	6.59	0.188	0.71
NH_3-N (mg/l)							
Hour 0	18.66	18.08	19.01	17.73	18.60	0.521	0.47
Hour 2	39.77	40.24	39.47	41.74	40.38	1.624	0.88
Hour 4	21.47	20.44	21.90	22.15	21.72	1.171	0.86
Hour 6	15.91	14.41	14.76	14.65	14.34	0.683	0.51
Glucose (mg/dl)							
Hour 0	63.6	63.2	64.8	63.2	63.4	1.043	0.80
Hour 2	64.6	60.8	63.6	62.8	62.2	1.314	0.36
Hour 4	59.4	60.4	61.0	61.4	60.0	1.189	0.77
Hour 6	64.0	64.4	64.6	65.8	65.6	0.623	0.25

E1 : Rovabio Excel LC2 (Adisseo USA Inc, Georgia, USA); E2 : 75 : 25 combination of Cellulase PLUS and Xylanase PLUS (Dyadic International, Florida, USA. SEM : standard error of the mean.

Table 7.3 Effect of exogenous fibrolytic enzyme supplementation on volatile fatty acid (VFAs) of fistulated non-lactating dairy cows fed corn silage.

Item	Control	E1		E2		SEM	P-value
		0.5 ml/kg	1.0 ml/kg	1.0 ml/kg	1.5 ml/kg		
Acetate (C2)				**mol/100mol**			
Hour 0	75.66	75.67	75.64	75.75	76.58	0.376	0.38
Hour 2	67.59	66.84	67.40	67.27	67.41	0.752	0.96
Hour 4	69.35	69.50	69.34	69.15	69.40	0.517	0.99
Hour 6	71.05	71.15	71.83	71.15	71.95	0.294	0.14
Propionate (C3)				**mol/100mol**			
Hour 0	15.91	16.25	16.22	16.26	15.75	0.249	0.67
Hour 2	22.51	22.41	22.60	21.80	22.34	0.539	0.74
Hour 4	19.86	19.74	19.78	20.11	19.84	0.474	0.98
Hour 6	18.32	18.12	18.07	18.30	17.89	0.355	0.91
Butytate (C4)				**mol/100mol**			
Hour 0	8.43	8.07	8.14	7.99	7.67	0.363	0.69
Hour 2	9.89	10.74	10.00	10.92	10.19	0.423	0.37
Hour 4	10.79	10.76	10.93	10.74	10.76	0.335	0.99
Hour 6	10.63	10.73	10.09	10.55	10.16	0.271	0.39
Totals VFA				**Mmol**			
Hour 0	69.08	71.04	67.81	69.36	68.92	1.387	0.60
Hour 2	88.69	92.47	91.36	89.75	88.36	2.423	0.72
Hour 4	92.46	95.17	96.47	95.82	94.71	1.991	0.67
Hour 6	88.18	94.43	90.76	91.48	92.29	2.355	0.47

Table 7.3 Effect of exogenous fibrolytic enzyme supplementation on volatile fatty acid (VFAs) of fistulated non-lactating dairy cows fed corn silage (Continued).

Item	Control	E1		E2		SEM	P-value
		0.5 ml/kg	1.0 ml/kg	1.0 ml/kg	1.5 ml/kg		
C2:C3							
Hour 0	4.76	4.68	4.67	4.66	4.88	0.088	0.41
Hour 2	3.04	3.01	2.98	3.10	3.04	0.106	0.94
Hour 4	3.52	3.54	3.51	3.27	3.33	0.157	0.64
Hour 6	3.89	3.94	3.99	3.89	4.06	0.096	0.69

E1 : Rovabio Excel LC2 (Adisseo USA Inc, Georgia, USA); E2 : 75 : 25 combination of Cellulase PLUS and Xylanase PLUS (Dyadic International, Florida, USA. SEM : standard error of the mean.

The NDFD was not affected by enzyme additives at 3, 6, and 12 h of ruminal incubations (P>0.05). However, addition of fibrolytic enzyme increased NDFD after 24, 48, and 72 h of ruminal incubations (P<0.05). At 24 h of ruminal incubation, adding 1.0 and 1.5 ml of E2/kg of corn silage DM increased NDFD compared with the control, but NDFD were similar at 0.5 and 1.0 ml of E1 compared with the control. The NDFD showed higher for 1.5 ml of E2/kg of corn silage DM than for the other treatment groups at 48 h of ruminal incubation. At 72 h of ruminal incubations, all enzyme treatments increased NDFD compared with the control, but NDFD between enzyme groups were similar.

ADFD at 3 and 6 h of ruminal incubations were unaffected by supplementation of fibrolytic enzymes (P>0.05). However, ADFD were increased by enzyme additives at 12, 24, 48, and 72 h of ruminal incubations (P<0.05). The ADFD

showed higher for 1.5 ml of E2/kg of corn silage DM than for the other treatment groups at 24 and 48 h of ruminal incubation.

7.6 Discussion

In the present study, DMI was not affected by enzyme additives even though DMD, NDFD and ADFD were increased. Similar results (Rode et al., 1999; Sutton et al., 2003 and Chung et al., 2012) reported that DMI was not affected by supplementation of enzymes. In addition, Yang et al. (1999) reported that DMI was also unaffected by enzyme even though fiber digestion in total tract was increased.

Table 7.4 Effect of exogenous fibrolytic enzyme supplementation on the degradability (%) of dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF) of fistulated non-lactating dairy cows fed corn silage.

Item	Control	E1		E2		SEM	P-value
		0.5 ml/kg	1.0 ml/kg	1.0 ml/kg	1.5 ml/kg		
DMD							
Hour 3	22.01	22.38	22.70	22.77	22.67	0.257	0.26
Hour 6	24.16	26.23	26.14	25.28	26.88	0.691	0.12
Hour 12	27.35	29.97	30.86	30.33	30.77	0.946	0.11
Hour 24	34.59^{b}	37.67^{a}	37.56^{a}	38.09^{a}	38.43^{a}	0.690	0.01
Hour 48	44.53^{c}	47.12^{bc}	48.09^{ab}	49.31^{ab}	50.30^{a}	0.882	0.01
Hour 72	50.71^{b}	53.76^{a}	53.44^{a}	54.46^{a}	54.29^{a}	0.545	0.01

Table 7.4 Effect of exogenous fibrolytic enzyme supplementation on the degradability (%) of dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF) of fistulated non-lactating dairy cows fed corn silage (Continued).

Item	Control	E1		E2		SEM	P-value
		0.5 ml/kg	1.0 ml/kg	1.0 ml/kg	1.5 ml/kg		
NDFD							
Hour 3	4.38	3.32	3.68	3.35	3.61	0.381	0.34
Hour 6	6.71	7.24	7.33	7.02	7.57	0.392	0.62
Hour 12	10.71	12.67	12.97	12.67	13.01	0.765	0.24
Hour 24	19.99^{c}	22.09^{bc}	22.24^{bc}	23.83^{ab}	24.68^{a}	0.759	0.01
Hour 48	34.70^{c}	36.66^{b}	37.69^{b}	37.87^{b}	39.76^{a}	0.570	0.01
Hour 72	41.39^{b}	44.10^{a}	45.04^{a}	44.63^{a}	44.86^{a}	0.740	0.03
ADFD							
Hour 3	4.36	3.88	3.97	3.43	3.92	0.362	0.27
Hour 6	5.95	5.84	5.97	6.23	6.30	0.282	0.74
Hour 12	9.11^{c}	10.69^{b}	11.23^{ab}	12.04^{a}	11.67^{ab}	0.408	0.01
Hour 24	16.94^{c}	18.64^{b}	18.88^{b}	19.47^{ab}	20.16^{a}	0.378	0.01
Hour 48	29.36^{d}	31.50^{c}	32.36^{bc}	33.61^{ab}	34.34^{a}	0.519	0.01
Hour 72	37.65^{c}	38.81^{bc}	39.61^{ab}	40.78^{a}	40.22^{ab}	0.521	0.01

E1 : Rovabio Excel LC2 (Adisseo USA Inc, Georgia, USA); E2 : 75 : 25 combination of Cellulase PLUS and Xylanase PLUS (Dyadic International, Florida, USA. SEM : standard error of the mean.

$^{a, b, c}$Means within the same row having different letters are different at $P<0.05$.

However, some studies have reported that supplementation of enzyme increased DMI when enzyme was applied to forage (Lewis et al., 1999). In contrast,

Holtshausen et al., (2011) reported that supplementation of enzyme to the diet decreased DMI, with the decline in intake proportional to the dose of the enzyme.

Addition of enzyme additives to the diet had no effect on ruminal fluid concentrations of total VFA, NH_3, molar proportions of individual VFA and ruminal pH, even though DMD, NDFD and ADFD were increased. Similar with Chung et al. (2012) who reported that supplementation of enzyme additives to the diet did not affect ruminal fluid concentrations of total VFA, NH_3, molar proportions of individual VFA and ruminal pH. Sutton et al. (2003) reported decreased acetate, increased propionate and no effects on butyrate when compared to control. Moreover, Beauchemin et al. (2000) reported an increase in the proportion of acetate in ruminal fluid by fibrolytic enzymes.

Blood glucose concentration was similar between cow consumed the control diet and cow consumed the control diet with enzyme additives. Holtshausen et al., (2011) reported that supplementation of enzyme additives to the diet did not affect blood glucose concentration; however blood insulin concentration was increased for the cows receiving the control diet with 1.0 ml/kg of TMR DM compared with cows receiving the control diet without enzyme additive. Their study indicated that the enzymes increased the energy availability even the feed intake was decreased for cows fed enzyme diets.

Additions of fibrolytic enzyme additives to the corn silage of fistulated Crossbred Holstein Friesian non-lactating dairy cows increased DMD, NDFD and ADFD in the rumen compared to the control. Based on the results in both studies *in vitro* and *in vivo*, for *in vitro* study, at 0.5 and 1.0 ml of E1/kg DM corn silage increased NDFD by up to 9% and 9%, respectively, and increased ADFD by up to 18% and 21%, respectively. At 1.0 ml of E2/kg DM corn silage increased NDFD by

up to 8%, and increased ADFD by up to 22%. For *in vivo* study, at 0.5 and 1.0 ml of E1/kg DM corn silage increased NDFD by up to 6% and 9%, respectively, and increased ADFD by up to 7% and 10%, respectively. At 1.0 and 1.5 ml of E2/kg DM corn silage increased NDFD by up to 9% and 15%, respectively, and increased ADFD by up to 14% and 17%, respectively. Those similar results were observed in both studies *in vitro* (Chapter 3) and *in vivo*; it might be due to increase the utilization of nutrients of corn silage when treated with enzyme additive. Holtshausen et al. (2011) reported that in the *in vitro* study, addition of the developmental enzyme additive (Econase RDE) increase or tendency for an increase in NDF digestibility of the alfalfa hay, alfalfa silage and barley silage, and in the *in vivo* study, adding the same enzyme additive (1.0 ml/kg TMR DM) increased milk production efficiency (kg of milk/kg of DMI) by 10.7% and FCM production efficiency (kg of FCM/kg of DMI) by 11.3%. Based on the results from meta analysis 17 observations from 7 studies, an increase of one percentage unit for *in vitro* NDFD of corn silage resulted in a 0.14 kg/d increase in 3.5% FCM yield and a 0.12 kg/d increase in DMI by dairy cow fed a diet high in corn silage proportion (>40% of the dietary DM) (Jung et al., 2004). Moreover, Oba and Allen (1999) also reported a positive relationship between forage NDFD (*in vitro* or *in situ*) and milk production and DMI, one percentage unit increase in NDFD was associated with a 0.25 kg/d increase in 4% FCM and 0.17 kg/d increase in DMI. The optimal dose rate for the fibrolytic enzyme additive was determined in the current study. For E1, optimal dose rate was 0.5 ml/kg of corn silage DM; for E2, optimal dose rate was 1.0 ml/kg of corn silage DM. Because its lower dose rate to increased fiber degradability compared with the control.

7.7 Conclusions

Addition of an exogenous fibrolytic enzyme additive to the diet of fistulated Crossbred Holstein Friesian non-lactating dairy cows did not alter DMI, ruminal fermentation, rumen pH and blood glucose concentration. However, supplementation of exogenous fibrolytic enzyme additive increased fiber degradability of corn silage. That might be due to increase the utilization of nutrients of corn silage when treated with enzyme additive. Based on the responses observed, further work is needed to determine these enzyme additives to measure animal performance of dairy cows fed corn silage diets.

7.8 References

Association of Official Analytical Chemists (AOAC). (1995). **Official methods of analysis** (16th ed.). USA: Arlington.

Beauchemin, K.A., Rode, L.M., Maekawa, M., Morgavi, D.P., and Kampen, R. (2000). Evaluation of a nonstarchpolysaccharidase feed enzyme in dairy cow diets. **J. Dairy Sci.** 83: 543-553.

Colombatto, D., and Beauchemin, K.A. (2003). A proposed methodology to standardize the determination of enzymic activities present in enzyme additives used in ruminant diets. **Can. J. Anim. Sci.** 83: 559-568.

Chung, Y.H., Zhou, M., Holtshausen, L., Alexander, T.W., McAllister, T.A., Guan, L.L., Oba, M., and Beauchemin, K.A. (2012). A fibrolytic enzyme additive for lactating Holstein cow diets: ruminal fermentation, rumen microbial populations and enteric methane emissions. **J. Dairy Sci.** 95: 1419-1427.

Eun, J.S., and Beauchemin, K.A. (2007). Enhancing in vitro degradation of alfalfa hay and corn silage using feed enzymes. **J. Dairy Sci.** 90: 2839-2851.

Eun, J.S., Beauchemin, K.A., and Schulze, H. (2007). Use of exogenous fibrolytic enzymes to enhance in vitro fermentation of alfalfa hay and corn silage. **J. Dairy Sci.** 90: 1440-1451.

Holtshausen, L., Chung, Y.H., Gerardo-Cuervo, H., Oba, M., and Beauchemin, K.A. (2011). Improved milk production efficiency in early lactation dairy cattle with dietary addition of a developmental fibrolytic enzyme additive. **J. Dairy Sci.** 94: 899-907.

Jung, H.G., Raeth-Knight, M., and Linn, J.G. (2004). **Forage fiber digestibility: Measurement, variability, and impact.** In Proceedings of 65th Minnesota Nutrition Conference (pp. 105-125).

Lewis, G.E., Sanchez, W.K., Hunt, C.W., Guy, M.A., Pritchard, G.T., Swanson, B.I., and Treacher, R.J. (1999). Effect of direct-fed fibrolytic enzymes on the lactational performance of dairy cows. **J. Dairy Sci.** 82: 611-617.

Oba, M., and Allen, M.S. (1999). Evaluation of the importance of digestibility of neutral detergent fiber from forage: Effects on dry matter intake and milk yield of dairy cows. **J. Dairy Sci.** 82: 589-596.

Rode, L.M., Yang, W.Z., and Beauchemin, K.A. (1999). Fibrolytic enzyme supplements for dairy cows in early lactation. **J. Dairy Sci.** 82: 2121-2126.

Statistical Analysis System. (1996). **SAS User' Guide: Statistics.** NC: SAS Institute.

Sutton, J.D., Phipps, R.H., Beever, D.E., Humphries, D.J., Hartnell, G.F., Vicini, J.L., and Hard, D.L. (2003). Effect of method of application of a fibrolytic enzyme product on digestive processes and milk production in Holstein-friesian cows. **J. Dairy Sci.** 86: 546-556.

Van Soest, P.J., Robertson, J.B., and Lewis, B.A. (1991). Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal production. **J. Dairy Sci.** 74: 3583-3597.

Yang, W.Z., Beauchemin, K.A., and Rode, L. (1999). Effects of an enzyme feed additive on extent of digestion and milk production of lactating dairy cows. **J. Dairy Sci.** 82: 391-403.

CHAPTER VIII

EFFECTS OF EXOGENOUS FIBROLYTIC ENZYME ON RUMINAL DIGESTIBILITY IN FISTULATED NON-LACTATING DAIRY COW FED RICE STRAW

8.1 Abstract

This study was conducted to evaluate the effect of two fibrolytic enzyme additives on the digestibility of rice straw and ruminal fermentation, pH and blood glucose concentration of fistulated non-lactating dairy cows. Three fistulated Crossbred Holstein Friesian non-lactating dairy cows housed in individual pens were assigned to one of three treatments in 3 × 3 Latin squares design. The trial consisted of 3 periods, with 21 d in each period, 14 d for adaptation to diets and 7 d for ruminal sample collection and *in vivo* disappearance trial. Dietary treatments were : 0 (control), 2.0 ml of E1/kg of rice straw dry matter (DM), and 2.0 ml of E2/kg of rice straw DM. Diets offered as 3 kg/d of concentrate containing 21% crude protein (CP), divided into 2 equal meals at 0800 and 1600 h together with *ad libitum* rice straw and clean water. Addition of enzyme additives to the diet had no effect on dry matter intake (DMI), ruminal fluid concentrations of total volatile fatty acid (VFA), NH_3-N, molar proportions of individual VFA, ruminal pH, and blood glucose concentration. The degradability of dry matter (DMD) was not affected by enzyme additives. At 3, 6, and 12 h of ruminal incubations, degradability of neutral detergent fiber (NDFD), and

acid detergent fiber (ADFD) were not affected by enzyme additives (P>0.05). However, enzyme additives evaluated increased NDFD and ADFD of rice straw at 24, 48, and 72 h of ruminal incubations (P<0.05).

8.2 Introduction

The large amount by-product of the rice production is mainly used as a roughage source for ruminant animals in Thailand. Rice straw is rich in polysaccharides and contains high lignin and silica contents, low crude protein content, poor palatability, low voluntary intake and low degradability by ruminal microorganisms. Many methods to improve the utilization of rice straw by ruminants have been investigated such as NaOH, NH_3, and urea (Wanapat et al., 2009). In addition, the supplementation of enzyme feed additive has been investigated to improve the utilization of rice straw (Liu and Ørskov, 2000; Eun et al., 2006; Yang et al., 2011). Yang et al. (2011) reported that the degradability of NDF was increased by all the enzyme treatments at 12 and 48 h of incubations, while ADF degradability was only increased at 48 h of incubation. Therefore, the effects of enzyme additive need to be confirmed in a ruminal condition (*in vivo*). The Rovabio Excel LC2 (E1) and 75 : 25 combination of Cellulase PLUS and Xylanase PLUS (E2) enzyme products were selected based on results obtained in Chapter 6. This part of study focused on *in vivo* study using diets based on rice straw to confirm the effect of 2 enzyme additives (Chapter 6).

8.3 Objectives

The objective of this study was to evaluate the effect of two exogenous fibrolytic enzymes on blood glucose, ruminal disappearance and rumen fermentation of fistulated non-lactating dairy cows fed rice straw.

8.4 Materials and methods

8.4.1 Feed and animal management

Three fistulated Crossbred Holstein Friesian non-lactating dairy cows housed in individual pens were assigned to one of three treatments in 3 × 3 Latin squares design. The trial consisted of 3 periods, with 21 d in each period, 14 d for adaptation to diets and 7 d for ruminal sample collection and *in vivo* disappearance trial. Dietary treatments were : 0 (control), 2.0 ml of E1/kg of rice straw DM, and 2.0 ml of E2/kg of rice straw DM. The enzyme additives were diluted in water, 50 ml of the dilution per 1 kg of rice straw DM. The dilution was sprayed on to the rice straw at the time before feeding. An equal amount of water (50 ml/1 kg of rice straw DM) was added to the control diet.

8.4.2 Measurements and chemical analysis

Diets offered as 3 kg/d of concentrate containing 21% CP, divided into 2 equal meals at 0800 and 1600 h together with *ad libitum* rice straw and clean water. Feed offered and feed refused were measured and recorded daily during the experimental periods. Dry matter content (48 h at 60°C) of the rice straw for individual cows was determined daily to calculate dry matter intake (DMI).

The samples were ground through a 1 mm screen for chemical analysis. Dry matter (DM) of rice straw and concentrate were determined by oven drying at 105°C

to a constant weight. The samples were analyzed for crude protein (CP), ether extract (EE), ash (AOAC, 1995), NDF, and ADF (Van Soest et al., 1991). Rice straw was ground through a 2 mm screen for *in vivo* ruminal disappearance determination. Approximately 5 g of 2 mm ground rice straw samples were placed into 8 × 11 cm nylon bags with 47 μm pore size. Samples of rice straw were suspended in the rumen of each fistulated non-lactating dairy cow for 0, 3, 6, 12, 24, 48, and 72 h, and all bags were retrieved and placed in ice water to stop the fermentation. The bags were washed under a gentle stream of water until the water ran clear and the bags were oven-dried at 65°C for 48 h. After weighing each bag individually, the residues were analyzed for DM, NDF, and ADF contents. The disappearance values were determined and expressed as a proportion of DM, NDF, and ADF incubated, respectively.

8.4.3 Ruminal fermentation

To evaluate ruminal fermentation, on the last day of each experimental period (d 21), ruminal fluid samples were collected from each fistulated non-lactating dairy cow at 0, 2, 4, and 6 h after the morning feeding. The pH of rumen fluid were immediately determined at the time of sampling by pH meter. For VFAs and ammonia N determination, 36 ml of rumen fluid was put into 50 ml centrifuge tube containing 4 ml of 1M H_2SO_4, then centrifuged at 1895 rpm for 15 min. Supernatant was collected and put into 25 ml test tube, then capped and stored at -20°C until analysis. Analysis of acetic, propionic and butyric acids used GC (Hewlett Packard GC system HP6890, USA, 19091N-113 INNOWAX, Length (meters) 30, I.D. (mm) 0.32 WIDEBORE, Film (um) 0.25). Ammonia N concentrations was determined by Kjeldahl analysis (AOAC, 1995).

8.4.4 Statistical analysis

Measurements of intake, pH, ammonia N, VFAs, blood glucose and ruminal disappearance coefficients of each period were analyzed by ANOVA using the Statistical Analysis System (SAS, 1996). Differences between treatment means were statistically compared using Least Significant Differences.

8.4.5 Experimental site

The experiment was conducted at Suranaree University of Technology's farm, and the center for Scientific and Technological Equipment, Building 10, Suranaree University of Technology.

8.4.6 Duration

The duration of the present experiment was from October to December 2013.

8.5 Results

8.5.1 Feed compositions

Chemical composition of concentrate and rice straw used in the present experiment is given in Table 8.1.

Table 8.1 Chemical composition of feeds.

Item	Concentrate	Rice straw
Dry matter	92.99	93.22
	……..……..% of DM…….	
Ash	10.21	15.12
Crude protein	21.38	3.62
Ether extract	2.85	0.62
Neutral detergent fiber	42.53	71.23
Acid detergent fiber	19.96	46.04
Acid detergent lignin	7.23	10.33

8.5.2 Intake blood glucose and ruminal fermentation

Feed intake, blood glucose, ruminal pH and ammonia N concentration are showed in Table 8.2. All of animal groups were received similar amount of 2.79 kg DM/d (data not showed), total feed intake were unaffected by supplementation of fibrolytic enzyme ($P>0.05$). Feed intakes were 10.20, 10.75, and 10.73 kg DM/d, respectively. Ruminal pH, ammonia N concentration and blood glucose concentration of each hour were not affected by adding fibrolytic enzyme ($P>0.05$).

Ruminal volatile fatty acid concentrations are showed in Table 8.3. Total volatile fatty acid concentration, molar proportion of acetate, propionate and butyrate and ratio of acetate : propionate of each hour were also unaffected by fibrolytic enzyme addition ($P>0.05$).

8.5.3 Ruminal degradability of rice straw

In vivo DMD, NDFD, and ADFD of rice straw are shown in Table 8.4. The DMD was not affected by enzyme additives. At 3, 6, and 12 h of ruminal incubations, the NDFD was not affected by enzyme additives ($P>0.05$). However, addition of fibrolytic enzyme increased NDFD at 24, 48, and 72 h of ruminal incubations ($P<0.05$). At 24, 48, and 72 h of ruminal incubations, all enzyme treatments increased NDFD compared with the control, but NDFD between enzyme groups were similar.

Table 8.2 Effect of exogenous fibrolytic enzyme supplementation on feed intake, ruminal pH, NH_3-N and blood glucose of fistulated non-lactating dairy cows fed rice straw.

Item	Control	2.0 ml/kg of E1	2.0 ml/kg of E2	SEM	P-value
Feed Intake (kg DM/d)	10.20	10.57	10.73	0.089	0.10
pH					
Hour 0	6.79	6.66	6.95	0.240	0.72
Hour 2	6.86	6.72	6.77	0.143	0.79
Hour 4	6.46	6.53	6.57	0.129	0.83
Hour 6	6.50	6.57	6.56	0.151	0.95
NH_3-N (mg/l)					
Hour 0	15.79	15.88	17.09	0.989	0.65
Hour 2	28.57	29.71	32.99	1.789	0.38
Hour 4	21.55	20.74	18.61	0.526	0.11
Hour 6	13.87	14.10	16.83	2.079	0.62
Glucose (mg/dl)					
Hour 0	61.00	53.33	57.33	2.874	0.36
Hour 2	60.33	58.00	57.33	2.674	0.74
Hour 4	59.33	57.33	56.00	2.009	0.59
Hour 6	60.00	59.00	59.00	2.186	0.93

SEM : standard error of the mean.

The ADFD was unaffected by supplementation of fibrolytic enzymes at 3 and 6 h of ruminal incubations ($P>0.05$). However, enzyme additives increased ADFD at 24, 48, and 72 h of ruminal incubations ($P<0.05$). The enzyme treatments increased ADFD compared with the control, but ADFD between enzyme groups were similar.

8.6 Discussion

Enzyme additives did not change DMI even though NDFD and ADFD were increased. Similar result, Alvarez et al. (2009) reported that six ruminal cannula steers were used in a 3 × 3 Latin square replicated. The treatments consisted of : 1) control, 2) Fibrozyme (2 g/kg dry matter), and 3) Promote (3 ml/kg dry matter). DMI was not affected by supplementation of two enzyme additives even though NDF disappearance rate, ADF potential disappearance and ADF disappearance rate of oat straw were increased.

Table 8.3 Effect of exogenous fibrolytic enzyme supplementation on volatile fatty acid (VFAs) of fistulated non-lactating dairy cows fed rice straw.

Item	Control	2.0 ml of E1/kg	2.0 ml of E2/kg	SEM	P-value
Acetate (C2)		**mol/100mol**			
Hour 0	75.87	75.71	76.73	0.538	0.49
Hour 2	74.34	74.40	75.44	0.266	0.15
Hour 4	73.03	74.26	74.90	1.210	0.62
Hour 6	73.77	74.63	75.93	0.960	0.44
Propionate (C3)		**mol/100mol**			
Hour 0	15.31	15.16	15.06	0.287	0.83
Hour 2	16.41	17.70	16.71	0.599	0.44
Hour 4	17.39	16.71	16.51	0.718	0.91
Hour 6	16.80	15.56	15.35	0.806	0.68
Butytate (C4)		**mol/100mol**			
Hour 0	8.83	9.14	8.21	0.248	0.21
Hour 2	9.25	8.90	8.51	0.314	0.42
Hour 4	9.58	9.22	8.39	0.485	0.39
Hour 6	9.43	9.81	8.71	0.291	0.22

Table 8.3 Effect of exogenous fibrolytic enzyme supplementation on volatile fatty acid (VFAs) of fistulated non-lactating dairy cows fed rice straw (Continued).

Item	Control	2.0 ml of E1/kg	2.0 ml of E2/kg	SEM	P-value
Totals VFA		**mmol**			
Hour 0	82.49	83.55	82.48	2.769	0.95
Hour 2	99.53	98.19	96.75	1.079	0.36
Hour 4	103.88	105.28	103.27	2.255	0.83
Hour 6	102.74	102.37	103.55	1.434	0.85
C2:C3					
Hour 0	5.09	5.06	5.07	0.167	0.99
Hour 2	4.57	4.29	4.59	0.132	0.39
Hour 4	4.22	4.55	4.53	0.451	0.85
Hour 6	4.41	4.85	4.96	0.392	0.64

SEM : standard error of the mean.

Chung et al. (2012) also reported that 9 ruminally cannulated lactating Holstein cows were used in a replicated 3 × 3 Latin square design. Diets containing no enzyme, low enzyme (Econase RDE; 0.5 ml of enzyme/kg of diet DM), and high enzyme (Econase RDE; 1.0 ml of enzyme/kg of diet DM). The diet contained 700 g/kg corn silage and 300 g/kg concentrate (DM basis). DMI was not affected by enzyme, averaging 25.6, 24.4, and 25.5 kg/d for the control, low, and high enzyme diet, respectively. However, Lewis et al. (1999) reported that supplementation of fibrolytic enzyme increased DMI when enzyme was applied to forage. In contrast, Holtshausen et al. (2011) reported that 60 dairy cows in early lactation were fed diets containing no enzyme, low enzyme (Econase RDE; 0.5 ml of enzyme/kg of diet DM), and high enzyme (Econase RDE; 1.0 ml of enzyme/kg of diet DM). The diet

contained 520 g/kg roughage, including 206 g/kg barley silage, 206 g/kg alfalfa silage and 108 g/kg alfalfa hay (DM basis). DMI was decreased by supplementation of enzyme to the diet, with the decline in intake proportional to the dose of the enzyme.

Addition of enzyme additives to the diet had no effect on ruminal fluid concentrations of total VFA, NH_3, molar proportions of individual VFA and ruminal pH. Similarly, Chung et al. (2012) reported that ruminal fluid concentrations of total VFA, NH_3, molar proportions of individual VFA and ruminal pH were not affected by supplementation of enzyme additives to the diet. However, Gado et al. (2009) reported that rumen ammonia N, concentrations of total VFA, and individual VFA proportions were altered with an increase in acetate (61.0-64.8 mol/100 mol; P=0.05) before feeding, and acetate and propionate increased 3 h post-feeding (60.0-64.0 and 18.3-20.8 mol/100 mol, respectively.

Table 8.4 Effect of exogenous fibrolytic enzyme supplementation on the degradability (%) of dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF) of fistulated non-lactating dairy cows fed rice straw.

Item	Control	2.0 ml/kg of E1	2.0 ml/kg of E2	SEM	P-value
DMD					
Hour 3	16.75	17.36	17.74	0.405	0.40
Hour 6	17.81	17.77	19.22	0.821	0.50
Hour 12	22.34	23.43	23.36	0.806	0.64
Hour 24	28.25	31.69	30.76	1.301	0.35
Hour 48	41.44	46.37	46.46	0.919	0.93
Hour 72	46.88	51.50	52.82	1.663	0.22

Table 8.4 Effect of exogenous fibrolytic enzyme supplementation on the degradability (%) of dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF) of fistulated non-lactating dairy cows fed rice straw (Continued).

Item	Control	2.0 ml/kg of E1	2.0 ml/kg of E2	SEM	P-value
NDFD					
Hour 3	4.97	5.15	5.01	0.580	0.98
Hour 6	6.26	7.39	8.73	0.836	0.31
Hour 12	13.45	14.28	13.23	1.314	0.85
Hour 24	20.29[b]	23.66[a]	23.24[a]	0.186	0.01
Hour 48	37.61[b]	39.45[a]	40.42[a]	0.210	0.02
Hour 72	43.37[b]	47.73[a]	47.49[a]	0.483	0.04
ADFD					
Hour 3	2.42	2.68	3.12	0.340	0.48
Hour 6	3.60	3.83	3.97	0.559	0.37
Hour 12	10.22	11.90	11.85	0.692	0.34
Hour 24	18.86[b]	20.85[a]	20.28[a]	0.232	0.04
Hour 48	34.85[b]	37.36[a]	37.82[a]	0.311	0.04
Hour 72	41.91[b]	44.40[a]	44.22[a]	0.294	0.04

[a, b]Means within the same row having different letters are different at $P<0.05$.

SEM : standard error of the mean.

Additions of fibrolytic enzyme additives to the rice straw of fistulated Crossbred Holstein Friesian non-lactating dairy cows increased NDFD and ADFD after 24 h of ruminal incubation compared to the control. That might be due to exogenous fibrolytic enzymes need enough time to interact with the substrate. Colombatto et al. (2003) reported that addition of enzyme increased the rate of fermentation and the release of reducing from both xylan and a mixture of cellulose

and xylan when the substrates were treated with commercial mixtures of xylanases and cellulases and allowed to interact for 20 h before inoculation with ruminal fluid.

Based on the results in both studies *in vitro* and *in vivo* (48 h of incubation), for *in vitro* study, at 2 ml of E1/kg DM rice straw increased NDFD and ADFD by up to 26.34% and 26.79%, respectively. At 2 ml of E2/kg DM rice straw increased NDFD and ADFD by up to 17.07% and 11.96%, respectively. For *in vivo* study, 2 ml of E1/kg DM rice straw increased NDFD and ADFD by up to 4.89% and 7.20%, respectively. 2 ml of E2/kg DM rice straw increased NDFD and ADFD by up to 7.47% and 8.52%, respectively. Those similar results were observed in both studies *in vitro* (Chapter 5) and *in vivo*; it might be due to increase the utilization of nutrients of rice straw when treated with enzyme additive. Gado et al. (2009) reported that digestibility of DM, OM, NDF, and ADF in the total tract of (ZADO®) enzyme supplemented cows were higher than the control by up to 8-16%. Oba and Allen (1999) reported that positive relationship between forage NDFD (*in vitro* or *in situ*) and milk production and DMI, one percentage unit increase in NDFD was associated with a 0.25 kg/d increase in 4% FCM and 0.17 kg/d increase in DMI. The optimal dose rate for the fibrolytic enzyme additive was determined in the current study. For both E1 and E2, the optimal dose rate were 2.0 ml/kg of rice straw DM. Because at 2.0 ml/kg of rice straw DM increased fiber degradability compared with the control.

8.7 Conclusions

Addition of an exogenous fibrolytic enzyme additive to the diet of fistulated Crossbred Holstein Friesian non-lactating dairy cows did not alter DMI, ruminal fermentation, rumen pH and blood glucose concentration. However, supplementation

of exogenous fibrolytic enzyme additive increased fiber degradability of rice straw after 24, 48, and 72 h incubations. That might be due to improving the utilization of nutrients of rice straw when treated with enzyme additive. Based on the responses observed, further work is needed to determine these enzyme additives to measure animal performance of dairy cows fed rice straw diet.

8.8 References

Alvarez, G., Pinos-Rodriguez, J.M., Herrera, J.G., Garcia, J.C., Gonzalez, S.S., and Barcena, R. (2009). Effects of exogenous fibrolytic enzymes on ruminal digestibility in steers fed high fiber rations. **Livest. Sci.** 121: 150-154.

Association of Official Analytical Chemists (AOAC). (1995). **Official methods of analysis** (16th ed.). USA: Arlington.

Chung, Y.H., Zhou, M., Holtshausen, L., Alexander, T.W., McAllister, T.A., Guan, L.L., Oba, M., and Beauchemin, K.A. (2012). A fibrolytic enzyme additive for lactating Holstein cow diets: ruminal fermentation, rumen microbial populations and enteric methane emissions. **J. Dairy Sci.** 95: 1419-1427.

Colombatto, D., Mould, F.L., Bhat, M.K., Morgavi, D.P., Beauchemin, K.A., and Owen, E. (2003). Influence of fibrolytic enzymes on the hydrolysis and fermentation of pure cellulose and xylan by mixed ruminal microorganisms *in vitro*. **J. Anim. Sci.** 81: 1040-1050.

Eun, J.S., Beauchemin, K.A., Hong, S.H., and Bauer, M.W. (2006). Exogenous enzymes added to untreated or ammoniated rice straw: Effects on *in vitro* fermentation characteristics and degradability. **Anim. Feed Sci. Technol.** 131: 86-101.

Gado, H.M., Salem, A.Z.M., Robinson, P.H., and Hassan, M. (2009). Influence of exogenous enzymes on nutrient digestibility, extent of ruminal fermentation as well as milk production and composition in dairy cows. **Anim. Feed Sci. Technol.** 154: 36-46.

Holtshausen, L., Chung, Y.H., Gerardo-Cuervo, H., Oba, M., and Beauchemin, K.A. (2011). Improved milk production efficiency in early lactation dairy cattle with dietary addition of a developmental fibrolytic enzyme additive. **J. Dairy Sci.** 94: 899-907.

Lewis, G.E., Sanchez, W.K., Hunt, C.W., Guy, M.A., Pritchard, G.T., Swanson, B.I., and Treacher, R. (1999). Effect of direct-fed fibrolytic enzymes on the lactational performance of dairy cows. **J. Dairy Sci.** 82: 611-617.

Liu, J.X., and Ørskov, E.R. (2000). Cellulase treatment of untreated and steam pre-treated rice straw effect on *in vitro* fermentation characteristics. **Anim. Feed Sci. Technol.** 88: 189-200.

Oba, M., and Allen, M.S. (1999). Evaluation of the importance of digestibility of neutral detergent fiber from forage: Effects on dry matter intake and milk yield of dairy cows. **J. Dairy Sci.** 82: 589-596.

Statistical Analysis System. (1996). **SAS User' Guide: Statistics.** NC: SAS Institute.

Van Soest, P.J., Robertson, J.B., and Lewis, B.A. (1991). Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal production. **J. Dairy Sci.** 74: 3583-3597.

Wanapat, M., Polyorach, S., Boonnop, K., Mapato C., and Cherdthong, A. (2009). Effects of treating rice straw with urea or urea and calcium hydroxide upon intake, digestibility, rumen fermentation and milk yield of dairy cows. **Livest. Sci.** 125: 238-243.

Yang, H.E., Son Y.S., and Beauchemin, K.A. (2011). Effects of exogenous Enzymes on ruminal fermentation and degradability of alfalfa hay and rice straw. **Asian-Aust. J. Anim. Sci.** 24: 56-64.

CHAPTER IX

OVERALL CONCLUSION AND IMPLICATION

The present study was conducted to evaluate the effect of exogenous fibrolytic enzymes supplementation on ruminal fermentation and degradability of corn silage and rice straw. The study was divided into 2 parts including *in vitro* and *in vivo* studies. The *in vitro* study was conducted at the Agriculture and Agri-Food Canada's Research Centre in Lethbridge, Alberta, Canada, during January-December 2012. The *in vivo* study was conducted at the Suranaree University Dairy Farm, Suranaree University of Technology, Thailand, during June 2013-March 2014. Results from these studies can be summarized as follows :

The *in vitro* study was conducted to evaluate the effect of exogenous fibrolytic enzymes supplementation on ruminal fermentation and degradability of corn silage and rice straw using a 48 h batch culture assay with buffer and ruminal fluid. The part of *in vitro* study was divided into 3 experiments including, the first experiment was conducted to evaluate the potential of four enzyme additives to enhance *in vitro* ruminal fiber degradation and fermentation profile of corn silage. The results showed that DMD and TGP were unaffected by enzyme additives, but all enzyme additives increased NDFD and ADFD of corn silage. However, E1 and E2 had higher NDFD and ADFD at 24 and 48 h of incubation compared with E3 and E4. Responses to increasing dose of enzyme were increased NDFD and ADFD for all enzymes, with the optimum dose rate dependant on the enzyme additive. Optimum dose rates were 1.0,

0.5, 4.0, and 2.0 µl/g DM for E1, E2, E3, and E4 respectively.

The second experiment was conducted to evaluate the potential of four enzyme additives with different enzyme activities to enhance *in vitro* ruminal degradation fiber and fermentation profile of four corn silages. The study found that adding enzyme additives increased DMD, NDFD, ADFD, and TGP compared to the control at 24 and 48 h incubations. The E1 and E2 had higher NDFD and ADFD compared with E3 and E4. The effects on DMD, NDFD, ADFD, and TGP were difference between corn silage substrate. For all parameters, DMD, NDFD, ADFD, and TGP, there were no enzyme × corn silage substrate interactions at either 24 or 48 h of incubation. Adding enzyme additives had no effect on concentrations of total volatile fatty acid (VFA), NH_3, molar proportions of individual VFA at 24 and 48 h incubations. Enzyme additives show positive response in all corn silage substrates (increased DMD, NDFD, ADFD, and TGP).

The third experiment was to evaluate the potential of eight enzyme additives different in enzyme activities to enhance *in vitro* ruminal fiber degradation and fermentation profile of rice straw. The study found that DMD, NDFD, and TGP were unaffected by enzyme additives at 24 h incubation, however ADFD was increased by enzyme additives. Adding enzyme additives had also no effect on DMD and TGP at 48 h incubation, however NDFD was increased by enzyme supplementation. At both time points, ADFD were increased by enzyme additives, however, in each fibrolytic enzyme additive showed different response dependent on dose rate. For fermentation profile parameters, enzyme × dose interaction of total VFA, molar proportion of acetate, propionate, butyrate and acetate to propionate ratio were not observed.

The *in vivo* study was conducted to evaluate the effect of two exogenous fibrolytic enzymes on blood glucose, ruminal degradability and rumen fermentation of

fistulated non-lactating dairy cows. The part of *in vivo* study was also divided in to 3 experiments including, the first experiment was to evaluate the effect of two levels of xylanase (endo-1, 4-beta-xylanase, EC 3.2.1.8) on ruminal degradation and rumen fermentation of rice straw based diets in fistulated non-lactating dairy cow. The study found that DM and ADF potential disappearance fraction, DM and ADF total disappearances were unaffected by the enzyme supplementation. However, NDF potential disappearance fraction, and NDF total disappearance were increased when the enzyme was added at a high dose. Hemicellulose degradability at 3, 6, 12, 24, 48, 72, and 96 hour were unaffected by supplementation of xylanase. Furthermore, adding xylanase enzyme did not change dry matter intake, ruminal pH, NH_3-N concentrations, total volatile fatty acid concentrations, molar proportion of acetate, propionate and butyrate; and ratio of acetate: propionate at each hour.

The second experiment was conducted to evaluate the effect of two exogenous fibrolytic enzymes on blood glucose concentration, ruminal degradation and rumen fermentation of corn silage based diets in fistulated non-lactating dairy cows. The study found that addition of enzyme additives to the diet had no effect on DMI, ruminal fluid concentrations of total VFA, NH_3, molar proportions of individual VFA, ruminal pH and blood glucose concentration. Addition of enzyme additive had also no effect on DMD, NDFD, and ADFD of corn silage at 3, 6, and 12 h of ruminal incubations. However, enzyme additives increased DMD, NDFD, and ADFD of corn silage after 24, 48, and 72 h of ruminal incubations.

The third experiment was conducted to evaluate the effect of two exogenous fibrolytic enzymes on blood glucose concentration, ruminal degradation and rumen fermentation of rice straw based diets in fistulated non-lactating dairy cows. The study found that adding enzyme additive had no effect on DMD of rice straw at all time

ruminal incubations. Fibrolytic enzyme supplementation showed similar response in NDFD and ADFD at 3, 6, and 12 h of ruminal incubations. However, addition of enzyme additive increased NDFD and ADFD at 24, 48, and 72 h of ruminal incubations. Furthermore, adding enzyme additive did not change DMI, ruminal fluid concentrations of total VFA, NH_3, molar proportions of individual VFA, ruminal pH, and blood glucose concentration.

From the present study, the enzyme additives evaluated were commercial products, each with a unique range in endoglucanase, exoglucanase and xylanase activities. Enzyme additives used in this study showed different of fibrolytic activities depending on type of enzyme produces. The use of exogenous fibrolytic enzyme increased fiber degradation of corn silage and rice straw. Those similar results were observed in both studies *in vitro* and *in vivo*; it might be due to improve the utilization of nutrients of corn silage and rice straw when treated with enzyme additive. However, each enzyme additive showed different response depending on type of enzyme additive. Therefore, the effects of enzyme additive before use as ruminant feed enzyme additives need to be tested and screened enzyme product and optimum dose rates in a ruminal condition. Because the enzyme activities of feed enzymes tested under controlled optimal conditions cannot predict their ability to enhance ruminant feed digestion. Based on the results observed in chapter 4, enzyme additives improved DM, NDF, and ADF degradability in all corn silages, therefore the enzyme additives can be applied to improve fiber degradation for other forages containing NDF and ADF with in the same range as in corn silages. The present study can be identifying the optimum dose rate of each enzyme for improving fiber degradation of corn silage and rice straw. For corn silage, the optimal dose rate of E1 (Rovabio Excel LC2; Adisseo USA Inc, Georgia, USA) was 0.5 ml/kg of corn silage DM, and the

optimal dose rate of E2 (combination of Cellulase PLUS and Xylanase PLUS 75 : 25; Dyadic International, Florida, USA) was 1.0 ml/kg of corn silage DM. For rice straw, the optimal dose rate of E1 (Rovabio Excel LC2; Adisseo USA Inc, Georgia, USA) and E2 (combination of Cellulase PLUS and Xylanase PLUS 75 : 25; Dyadic International, Florida, USA) were 2.0 ml/kg of rice straw DM. From these effects to be observed, the enzyme specificity should be diverse enough and should closely match the targeted substrate.

Based on the responses observed, further work is needed to determine these enzyme additives to measure animal performance of dairy cows fed corn silage and rice straw diets.

APPENDIX

Reagents preparation

1. Buffer solution

- Ammonium bicarbonate (NH_4HCO_3) 4 g
- Sodium bicarbonate ($NaHCO_3$) 35 g
- Dissolved in water and brought up to 1 L in volumetric flask.

2. Macromineral solution

- Sodium hydrogen phosphate, dibasic (Na_2HPO_4) 5.7 g
- Potassium phosphate, monobasic (KH_2PO_4) 6.0 g
- Magnesium sulfate, heptahydrate ($MgSO_4.7H_2O$) 0.6 g
- Dissolved in water and brought up to 1 L in volumetric flask.

NOTE : Buffer and Macromineral solution could be stored refrigerated for up to 3 months and at room temperature for up to 1 month.

3. Micromineral solution

- Calcium chloride, dihydrate ($CaCl_2.2H_2O$) 13.2 g
- Manganese chloride, tetrahydrate ($MnCl_2.4H_2O$) 10.0 g
- Cobalt chloride, hexahydrate ($CoCl_2.6H_2O$) 1.0 g
- Ferric chloride, hexahydrate ($FeCl_2.6H_2O$) 8.0 g
- Dissolved in water and brought up to 100 mL in volumetric flask.

NOTE : Micromineral solution could be stored refrigerated for up to 12 months.

4. 0.1% (wt/vol) Resazurin

- Dissolved 0.1 g of resazurin 100 mL water.
- Stored in dark (amber coloured) bottle at 4°C (in fridge).

5. Medium preparation (on the day the *in vitro* was started)

**This recipe was for 1 L, increased volume as required

- Weighed out 2.5 g tryptone and dissolved completely in 500 mL water
- Added 0.125 mL micromineral solution
- Added 250 mL buffer solution and 250 mL macromineral solution
- Added 1.25 mL 0.1% resazurin solution
- Placed container with medium in water bath (39°C) and bubbled CO_2 through solution for 45 minutes
- Weighed out 0.313 g L-cysteine hydrochloride and 0.313 g sodium sulphide and added directly to medium
- Bubbled CO_2 through solution for another 15 minutes or until solution turned grey to clear.
    - A purple/pink colour indicated the presence of oxygen.
    - A grey/clear colour indicated the solution was reduced.
- Kept medium in water bath and headspace saturated with CO_2 until medium + inoculums was going to be transferred to incubation vials (at this point rumen fluid could be collected).

CURRICULUM VITAE

Mr. Noppharat Phakachoed was born on the 27th of July 1986 in Roi-Et, Thailand. He graduated Bachelor of Science from school of Animal Production Technology, Institute of Agricultural Technology, Suranaree University of Technology in 2008. After graduation, he obtained the scholarship from the Royal Golden Jubilee PhD Program, to presence a Doctor degree at the same university. He conducted the research in the topic of Effects of exogenous fibrolytic enzyme supplementation on *in vitro* and *in vivo* fermentation of corn silage and rice straw. The result of this project has been presented as 2 publications in Livestock Science Journal. He also had opportunity to do experiment at Agriculture and Agri-Food Canada's Research Centre in Lethbridge, Alberta, Canada, during January-December 2012.