Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Fakulteten för veterinärmedicin och husdjursvetenskap

Improving silage digestibility with fibrolytic enzymes

Förbättrad ensilagesmältbarhet med fibrolytiska enzymer

Thomas Eliasson

Institutionen för husdjurens utfodring och vård

Examensarbete 30 hp - 629

Uppsala 2018

Improving silage digestibility with fibrolytic enzymes

Förbättrad ensilagesmältbarhet med fibrolytiska enzymer

Thomas Eliasson

Handledare: Kamyar Mogodiniyai Kasmaei**,** SLU
Institution: Institutionen för husdjurens utfodring och vård

Bitr. handledare: Rolf Spörndly, SLU
Institution: Institutionen för husdjurens utfodring och vård

Examinator: Thomas Pauly, SLU
Institution: Institutionen för husdjurens utfodring och vård

Omfattning: 30 hp
Nivå och fördjupning: A2E
Kurstitel: Examensarbete i husdjursvetenskap
Kurskod: EX0551
Program: Agronomprogrammet - Husdjur

Utgivningsort: Uppsala
Utgivningsår: 2018
Omslagsbild:
Serienamn / delnummer: Examensarbete / Sveriges lantbruksuniveritet, Institutionen för husdjurens utfodring och vård, 629
Elektronisk publicering: http://stud.epsilon.slu.se

Nyckelord: ensilage, smältbarhet, fiber, växtfiber
Keywords: forage, roughage, dietary fiber

Sveriges Lantbruksuniversitet
Swedish University of Agricultural Sciences

Fakulteten för veterinärmedicin och husdjursvetenskap
Institutionen för husdjurens utfodring och vård

Abstract

In Sweden, the dairy cow is an important producer of both milk and meat. The production is highly dependent on forage as a source of nutrients for the cow. Ruminants are excellent converters of plant material that otherwise is highly indigestible for humans. Though, the vegetation period in Sweden is short and must supply all forage needed to feed the cow throughout the year. By harvesting later than normal, more plant biomass is produced on the expense of digestibility. This thesis comprises two parts, one literature review followed by a laboratory trial. The first part evaluates what happens inside the plant during plant maturation and if there is a way to improve digestibility in a late harvested crop. The effect of maturation was different between types of plants. The fiber content increased at a more major extent for legumes than temperate grasses (C_3). On the contrary, grasses reduced their digestibility at a more major extent than legumes. The observed nutritional changes during maturation mainly originated from cell wall growth within tissues of the plant stems; mostly in vascular tissue of legumes and spread over different tissues in grass.

From these observations, a laboratory trial was setup to test if different fibrolytic enzymes can digest the cell walls and improve digestibility of a late harvested crop. A late second cut harvest of mixed grasses and red clover was chopped, pre-wilted (~35 % DM) and mixed (1:1). The forage mix was treated with hemi-cellulases & cellulases (Hem & Cell), pectinases (Pect) and three ferulic acid esterases (FAE 1, 2 & 3). The five treatments and a control were ensiled as triplicates in 1.7 L glass jars for 60 days. Silage pH showed significant differences for the Hem & Cell (4.68) and FAE 3 (4.82) treatments in comparison to the control (4.75). The generally higher silage pH than normal was believed to be caused by a low water-soluble carbohydrate content (3.54 % of DM) in the forage. Further silage quality analysis showed an average ammonia-N of 8.17 % of total N and an average DM loss during ensiling of 3.3 % of DM. Silage was evaluated stable for 10 days of exposure to oxygen.

DM, OM and in vitro OM digestibility showed no significant differences between treatments. The fiber content (NDF) and calculated hemi-cellulose showed no significant differences between treatments. For ADF, a significantly reduced concentration (% of DM) was seen for the Hem & Cell (27.9) and Pect (27.8) treatments compared to the control (28.9). In vitro gas production kinetics was estimated with the AMPTS II system from Bioprocess Control, Lund, Sweden. Total volume per observation was 350 ml with a 1:9 rumen fluid to buffer ratio and ~4 g DM of fresh silage sample was used. Silage triplicates were considered as blocks and run as separate runs. Treatments did not affect cumulative gas production. The gas flow (% of total) at 24–36 h were significantly lower for the Hem & Cell (16.3) treatment compared to the control (19.5). The effect of block was significant for both cumulative gas production and gas flow. Finally, no significant differences were found for OM and DM disappearance estimations of residues from the in vitro gas production kinetics analysis. The conclusion from the trial was that enzyme treatments had no clear effect on digestibility in the late harvested forage.

Sammanfattning

I Sverige är mjölkkon en viktig producent av både mjölk och kött. Produktionen är mestadels baserad på grovfoder då idisslare är utmärkta omvandlare av växtmaterial. Dock är vegetationsperioden i Sverige relativt kort, vilket innebär att grovfoderproduktionen är koncentrerad till endast en del av året, men ska samtidigt vara tillräcklig för hela året. Genom att skörda senare än normalt kan mer växtmaterial produceras, men då på bekostnad av smältbarheten. Denna avhandling består av två delar, en litteraturstudie följt av ett laboratorieförsök. Den första delen utvärderar vad som händer i växten under mognadsprocessen och om det finns någon möjlighet att förbättra smältbarheten i en sent skördad gröda. Det konstaterades i litteraturen att effekten av mognad skiljer sig åt mellan olika typer av växter. Fiberinnehållet ökade i större utsträckning i baljväxter än för gräs (C3). Tvärtom reducerades gräsets smältbarhet i större utsträckning än för baljväxter. De observerade förändringarna i näringsvärde under mognad härstammar huvudsakligen från tillväxt av cellväggar i stammens olika vävnader, främst i kärlvävnad hos baljväxter och mer utspritt över olika vävnadstyper hos gräs.

Från dessa observationer upprättades ett försök för att testa om tillsats av olika fibrolytiska enzymer kan bryta ned cellväggarna och förbättra smältbarheten hos en sent skördad gröda. En sen andra skörd av gräs och rödklöver hackades, för-torkades (~35 % TS) och blandades (1:1). Blandningen behandlades med hemi-cellulaser och cellulaser (Hem & Cell), pektinaser (Pect) och tre ferulsyraesteraser (FAE 1, 2 & 3). De fem behandlingarna och en kontroll ensilerades som triplikat i 1,7 liters glasburkar under 60 dagar. pH-värdet i ensilaget visade signifikanta skillnader för Hem & Cell (4,68) och FAE 3 (4,82) behandlingarna i jämförelse med kontrollen (4,75). pH-värdet i ensilaget var något högre än förväntat, detta antogs bero på en för låg koncentration av vattenlösliga kolhydrater (3,54 % av TS) i grönmassan. Ytterligare analyser visade ett genomsnittligt ammoniak-tal på 8,17% av totalt-kväve och en genomsnittlig TS-förlust vid ensilering av 3,3% DM. Ensilaget var stabilt under 10 dagar i syrerik miljö.

Inga signifikanta skillnader mellan behandlingar sågs för TS, organisk substans och VOS. Det totala innehållet av NDF och beräknad hemi-cellulosa uppvisade inga signifikanta skillnader mellan behandlingarna. För ADF sågs en signifikant minskad koncentration (% av TS) för Hem & Cell (27,9) och Pect (27,8) behandlingarna, jämfört med kontrollen (28,9). In vitro gasproduktions kinetik analyserades med AMPTS II-systemet från Bioprocess Control, Lund, Sverige. Total volym per prov var 350 ml med ett 1:9 förhållande av våmvätska och buffert, ~4 g TS ensilage användes. Ensilagebehandlingarnas triplikat betraktades som block och analyserades därmed som separata körningar. Ingen av behandlingarna påverkade den kumulativa gasproduktionen. Gasflödet (% av total produktion) vid 24–36 h var signifikant lägre för Hem & Cell (16,3) behandlingen, jämfört med kontrollen (19,5). Blockeffekten var signifikant för både den kumulativa gasproduktionen och gasflödet. Det var inte några skillnader mellan behandlingar för TS och organisk substans i resterna från analysen av in vitro gasproduktions kinetiken. Slutsatsen från studien var att enzymbehandlingarna inte hade någon tydlig inverkan på smältbarheten i det sent skördade grovfodret.

Table of contents

1 Introduction

The ruminant possesses the ability to convert highly indigestible plant biomass into more digestible products, such as milk and meat. The key is not the ruminant itself but the microbes living inside the rumen. Bacteria, archaea, fungi and other microbes are forming a diverse community that are equipped with a versatile arsenal of enzymes. Together, these microbes digest a major part of the biomass, partly or completely, making it more available for themselves and ultimately for the ruminant (Puniya et al., 2015). This symbiosis is found in other animals as well, even in our own digestive tract, but to a smaller extent. Utilization of nutrients from microbial digestion is highly dependent on where in the digestive tract it occurs, making the ruminant with its forestomaches unique in this aspect (McDonald et al., 2011).

In Swedish agriculture, the dairy cow is an important converter of plant biomass, producing both milk and meat for human consumption. This conversion is as in most types of animal production dependent on feed production. Forages, such as grass and clover, are harvested during the vegetation period and preserved for the year to come. This is a very delicate task, as average nutritional needs of the animal population, do not change during the year. Dairy production in Sweden is not following the vegetation period, as seen in figure 1.

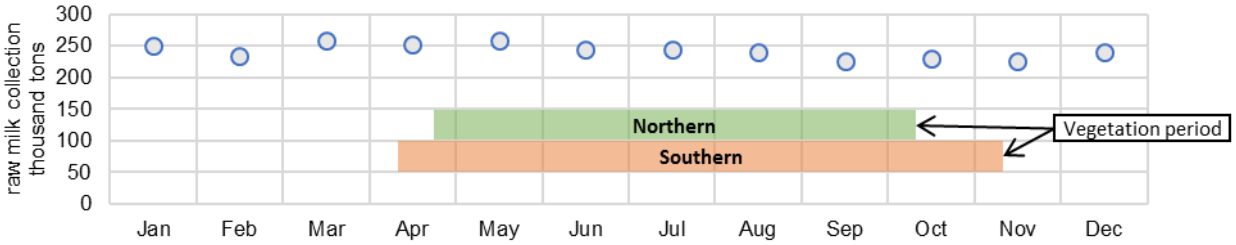

Figure 1 – Average raw milk collection (dots) during 2014 to 2016 with average vegetation period of northern and southern Sweden as colored blocks. Milk data from Eurostat (2017) and climate data from SMHI (2015).

There are many factors affecting the final quality and quantity of a forage crop. Soil preparation, fertilization and type of crops to grow can easily be affected by the farmer. Factors related to time of harvest are harder to account for. The farmer must decide when to harvest depending on when the crop is believed to have the right nutrient composition. As plants mature, both yield and digestibility are affected. Digestibility decreases, making the nutrients less available for the animal. Yield increases, though, not enough to compensate for the decreased digestibility (Buxton and O'Kiely, 2003).

The trend in Swedish dairy production has for many years been to harvest at a very early stage of plant maturity. Mainly to guarantee a high digestibility and nutrient concentration in the forage. However, not without implications, harvesting early means that the farmer harvests more often. Leading to more total inevitable harvest losses and more expenses for the farmer in form of working hours and maintenance. It is also shown that an increased harvesting frequency is negatively correlated to total biomass production (Vinther, 2006).

If the farmer could harvest at a later stage of maturity without the loss of digestibility, both harvest cost and total yield would be positively affected. Therefore, the idea for this thesis is to find ways of increasing the digestibility of a late harvested crop. The thesis will consist of two parts, a literature review for evaluation of literature on the topic and a trial for evaluating if digestibility can be improved, based on the findings in the literature review.

2 Literature review

Digestibility is a general term used to describe how much of a feed that is digested throughout the animals' digestive tract, either by microbial or animal digestive enzymes. Residues that leaves the animal through feces are considered indigestible (Van Soest, 1994). As mentioned in the introduction, digestibility decreases as plants mature. Why this happens will be clarified by evaluation of literature related to staging, development and physiology of plants. Together with a comparison between the most common forage crops in Swedish dairy production, temperate grasses (C_3) and legumes. Followed by a short review on forage preservation. And finally, an idea on how to increase digestibility based on what is found.

2.1 Plant maturation in relation to digestibility

There are many aspects that needs consideration to understand the plant maturation process. Swedish forage production mainly includes temperate grasses (C_3) and legumes, with different developmental strategies. For a comparison of nutritional values between plant types at the same level of maturity, staging systems for plant maturity has been developed. This was done by Moore et al. (1991) for perennial grasses, Ohlsson and Wedin (1989) for red clover (*Trifolium pratense)* and Kalu and Fick (1981) for alfalfa (*Medicago sativa*). These studies relate specific phases of plant development to a number, defining a more precise and standardized stage of maturity, more comparable between plant types and species.

However, crop samples will always contain plants at different stages of maturity. This can be accounted for by sorting the crop samples into fractions, depending on the stage of maturity for each plant. Each fraction is then counted or weighed and a mean stage count (MSC) or mean stage weight (MSW) is calculated. The MSC and MSW values can then be used to relate crops harvested at different stages of maturity to nutritional values. This was done in a study by Hoffman et al. (1993), a part of the data is summarized in Figure 2.

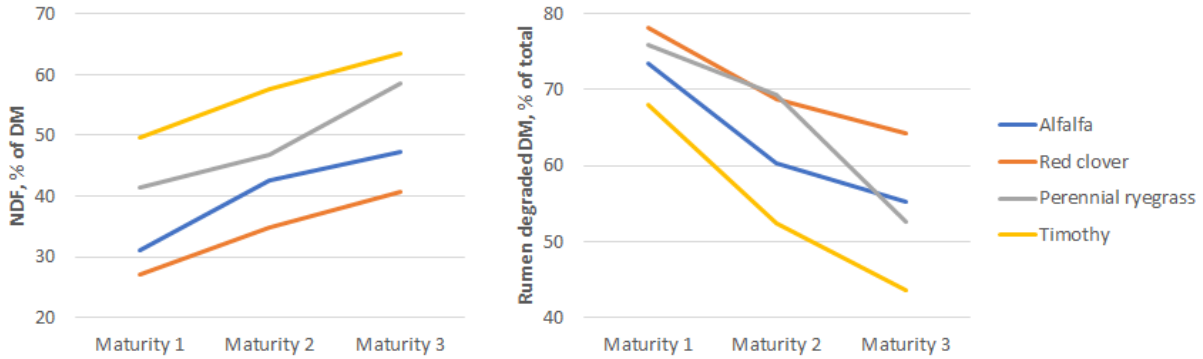

Figure 2 – NDF content and rumen degradable DM in relation to plant maturity in alfalfa (*Medicago sativa*), red clover (*Trifolium pratense*), perennial ryegrass (*Lolium perenne*) and Timothy (*Phleum pratense*). Adapted from Hoffman et al. (1993).

Maturation has a major impact on increasing the fiber fraction (NDF) of both grasses and legumes, and by that, decreasing dry matter degradability at a major extent. Figure 2 also shows that maturation affects NDF at a higher extent in grasses than legumes. According to Buxton et al. (1995), an explanation for this is that grasses generally contains more cell walls in relation to cell contents, compared to legumes.

The NDF fraction is basically describing the proportion of cell walls in a plant sample. The analytical procedure for NDF is a standardized treatment with neutral detergents, dissolving most of what is not bound to the cell wall (Van Soest et al., 1991). However, one issue with the

NDF method is loss of digestible cell wall constituents, such as pectin. Legumes contain major amounts of pectin, compared to grasses. As neutral detergents dissolves most of the pectin, NDF represents less of the cell wall fraction in legumes than for grasses (Jung et al., 1993).

Though, this does not explain the difference in NDF content and dry matter degradability between grasses and legumes. A closer look on the plant morphology in relation to nutritional value of grasses and legumes shows that there are major differences between plant parts. Rinne and Nykänen (2000) harvested timothy (*Phleum pretense*) and red clover (*Trifolium pretense*) at three different dates, and divided the harvest into stems and leaves of each plant species. Parts of the data is shown in figure 3.

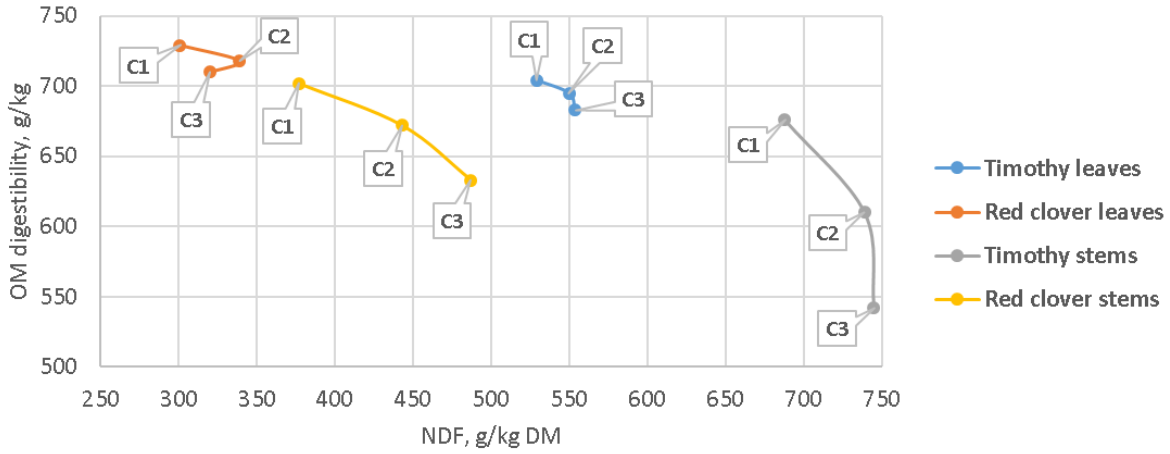

Figure 3 – NDF in relation to organic matter digestibility of stems and leaves from timothy (*Phleum pretense*) and red clover (*Trifolium pretense*), cut at three different dates (C1, C2 and C3). Data from Rinne and Nykänen (2000).

Figure 3 shows that stems generally contains more NDF than leaves, the difference is especially obvious when it comes to the grass. As the plants are harvested later, from early cut (C1) to late cut (C3), an interesting development is seen. NDF increases dramatically in legume stems, the same is not seen for the grass stems. On the contrary, OM digestibility just shows a minor decrease for the legume stems in comparison to grass stems. Leaves from both plants do not show any obvious changes in nutritional value as maturity increases. It is also important to mention that as maturation proceeds, stem to leaf ratio also increases, increasing NDF and decreasing OM digestibility even further (Kuoppala et al., 2009; Rinne and Nykänen, 2000). These findings lead to two conclusions; 1) stems contributes most to an increased cell wall concentration and decreased digestibility as maturation proceeds, 2) cell walls are constructed differently in grasses and legumes.

2.2 Plant stem tissues and their development

The stem is built up by different combinations of tissues depending on the plant type, both grass and legume stems contain major amounts of parenchyma. This tissue is mainly deposited in the center of the stem and is easily digested due to its loose arrangement and thin cell walls. The second most abundant tissues in grasses and legumes are vascular tissues, sclerenchyma and mesophyll. The vascular tissues build up transport vessels for water and nutrients throughout the plant, comprising mostly elements of xylem and phloem. This tissue is mainly situated around the parenchyma, and is much more abundant in legumes (Wilson, 1993). Cells in the phloem are easily digested due to thin cell walls, while most cells building up the xylem have thick cell walls, making them highly indigestible (Akin, 1989). The xylem cells play an especially important role in the maturation process of legumes, they continue to grow

throughout plant maturation and contributes highly to the reduced digestibility. This is not seen in grass stems; vascular tissue is mainly formed during plant elongation. Instead, sclerenchyma cells are of more importance for changes in digestibility during grass stem maturation. This tissue is mainly found as a ring layer outside the vascular tissue layer. Sclerenchyma tissue do not continue to grow throughout plant maturation, in comparison to legumes where the stem continues to increase in diameter due to xylem tissue growth. Instead, the sclerenchyma cell wall increases in size on expense of the cell lumen and by this, contributing to an increased stem strength and reduced digestibility. As maturation proceeds, growth of cell walls also increases in the parenchyma cells close to the sclerenchyma ring. Mesophyll is mostly found in legumes as a layer of easily digestible, photosynthetic cells, close to the outermost tissue layer. The last tissue to mention is epidermis, deposited as the outermost layer of both grasses and legumes, protecting the plant with a waxy cuticle layer. Both cell walls and the cuticle layer increases in size as stems mature, highly affecting digestibility; especially in grass stems (Wilson, 1993; Wilson and Hatfield, 1997).

The above described differences between plant types partly explains the differences throughout maturity, as shown in Figure 3. Grass stems show a more widespread distribution of cell wall growth between many tissues, and mostly as a modification of already existing tissue; decreasing digestibility of the whole stem, while NDF is not increasing much. Legume stems concentrate cell wall growth to their xylem tissue. Also growing the tissue itself, most tissues are still highly digestible, while NDF increases at a major extent due the growth of the xylem tissue itself (Wilson and Kennedy, 1996). Though, growth is a simplified term of what is happening in the cell walls, compositional changes also play a key role.

2.3 Forage preservation

The most common way of preserving plant biomass in Sweden today is as silage. Grass, legumes or whole-grain crops are harvested and packed into air-tight silos. The anaerobic environment promotes growth of microbes, mainly lactic acid bacteria (LAB). The LAB convert easily fermented carbohydrates into acids, mainly lactic acid, which contributes to a reduction in pH. This process slightly alters the carbohydrate composition of the plant biomass (Pahlow et al., 2003).

The ensiling process can be described by dividing the process into separate phases. Rooke and Hatfield (2003) divide the process from a biochemical point of view. The first three steps of the process are summarized in Figure 4.

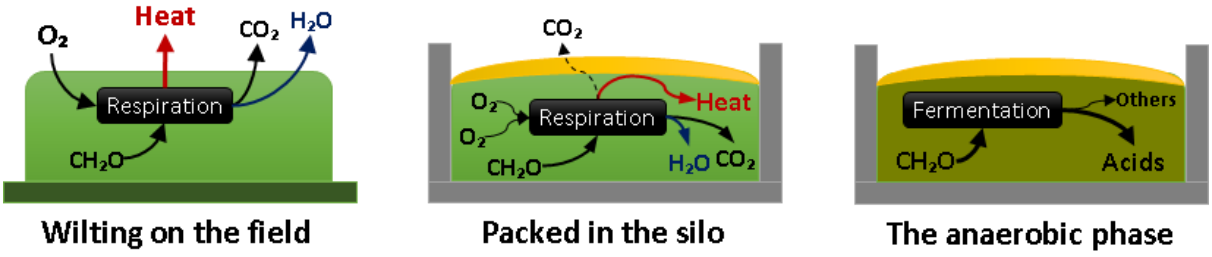

Figure 4 – Ensiling from a biochemical point of view, interpretation of Rooke and Hatfield (2003).

The crop is cut and left out for wilting, if not collected directly. Respiration of stored carbohydrates by plant enzymes is always occurring inside the plant to produce energy for growth and other processes. Though, as plants are cut, this process continues without the plants ability to replenish the storage by photosynthesis. Therefore, prolonged wilting might lead to a

reduced quantity of available substrate for LAB fermentation later in the ensiling process. Proteolysis also occurs by plant enzymes, breaking down proteins into amino acids. These can easily be further broken down into peptides and finally ammonia by spoilage bacteria, such as enterobacteria and clostridia. Finally, wilting can also influence the microbial population, both restrict LAB and promote spoilage microorganisms. As the crop is collected, chopped and packed into the silo and ultimately covered and sealed; respiration continues until all oxygen is depleted (Muck et al., 2003).

It is important to reduce the time for plant respiration as this highly affect the amount of easily fermented carbohydrates for fermentation. The efficiency of the fermentation step is dependent on a quick promotion of an anaerobic environment together with enough substrate available for the LAB to produce acids. A quick drop in pH inhibits growth of spoilage bacteria, partly also inhibiting growth of fungi. The spoilage bacteria will otherwise degrade amino acids at a major extent into ammonia, which both increases the pH and decreases the nutritional quality of the silage. Fungi spoilage is generally stimulated by a prolonged presence of oxygen and a too slow reduction in pH during the initial ensiling process. This is generally the case when compaction and sealing of the silo is insufficient. Yeast utilize easily digestible carbohydrates, and further also, acids produced by the LAB if oxygen is present, preventing the pH reduction. Mold growth is generally stimulated when oxygen leaks in during the ensiling process, resulting in accumulation of mycotoxins (McDonald et al., 1991).

2.3.1 The stable phase and feed-out

It is well defined that, as silage reaches a sufficiently low pH in which growth of spoilage microorganisms, and to some extent LAB is inhibited, silage is considered stable. This pH varies with type of silage and DM content. Kung and Shaver (2001) gives a general recommendation of a pH between 4.3–4.7 for both grass and legume silages at a DM content of ~35 % (McDonald et al., 1991).

As the silage is ready for feed-out, covering material is removed partly or completely, depending on type of storage method. In Sweden, bunker silos and round bales are the most used methods, according to Pettersson et al. (2009). The risk for aerobic deterioration is often not high in round bales, as the silage is utilized shortly after opening. However, higher during storage, as bales easily gets punctured during handling. For bunker silos, aerobic stability is an important factor. The term aerobic stability generally refers to how well the silage resists deterioration as oxygen is re-introduced to the plant material. Oxygen penetrates the surface of the silage as soon as covering material is removed. If pH was reduced to an sufficient extent and growth of spoilage microbes inhibited, as described in the previous paragraphs; silage is stable if enough plant material is removed over time and the amount of surface exposed to oxygen is limited (Muck et al., 2003; Pahlow et al., 2003)

2.4 Concluding remarks and study aim

In the bioenergy industry, specific microbes or enzymes produced thereof, are used to digest plant biomass into different products. Residues from the forest industry, specific energy crops or other plant materials are used as substrates (Himmel, 2008). This industry is, as the dairy industry, looking for new ways to feed their production more efficiently. The understanding of the maturity process of plants suggests that an improvement of digestibility can be achieved by interfering with the cell wall structure. The process of ensiling is the most common way of preserving crops in Sweden, and a relatively long period of time, suitable for addition of

compounds interfering with the cell wall structure. Therefore, this project aims to improve digestibility of plant biomass during the ensiling process by addition of fibrolytic enzymes.

3 Laboratory trial

The general idea for the trial was to add different types of fibrolytic enzymes and evaluate their effect on silage digestibility. Treatments were evaluated for their effect on general silage quality, silage chemical analysis and gas production kinetics by rumen fluid incubation. Ferulic acid esterase was chosen for the ability to break up the cell wall complex, making the fiber fraction more available for degradation in the rumen. Cellulases and hemi-cellulases were chosen for their general effect on degrading cellulose and hemi-cellulose, which are comprising a major part of the cell wall structure. Finally, pectinase was chosen for the ability to break down pectin, which mainly builds up the middle lamella that glues cell walls of neighboring cells together.

3.1 Silage preparation

Samples were obtained from two neighboring fields in Uppsala, Sweden using a scythe, one grass mixture sample and one red clover sample. Both forages were a late second-cut harvest. The forages were further chopped with a stationary chopper and wilted overnight (24 h) in a well-ventilated room at room temperature, followed by mixing (1:1) on fresh basis. DM content of the resulting forage mixture was estimated to 35 % by drying in a microwave oven. Further analytical values of the forage can be seen in section 4.2. The forage mixture was then weighed into plastic bags, 3 kg forage per bag and 6 bags in total. An additional bag was filled with the forage mixture and frozen at -20°C for chemical analyses. Enzymes, supplied by Novozymes, Denmark, were previously delivered frozen to SLU, Uppsala and thawed on the day of ensiling. Table 1 gives a short description of the enzymes and shows the trial setup.

Table 1 – Ensiling schedule with treatment names and description of enzymes.

				Optimum activity	
Treatment	**Enzyme**	**Donor**	**Spec. activity**	**pH**	**Temp.**
Control	None				
Hem & Cell	Hemi-cellulases and cellulase	Unknown*	Unknown*	Unknown*	Unknown*
Pect	Pectinase	Unknown*	Unknown*	Unknown*	Unknown*
FAE 1	Ferulic acid esterase 1	*Aspergillus*	Unknown*	5.0	~60°C
FAE 2	Ferulic acid esterase 2	*Aspergillus*	Unknown*	5.0	~60°C
FAE 3	Ferulic acid esterase 3	*Magnaporthe*	Unknown*	5.0	~40°C

*Information was not revealed by Novozymes.

The enzyme solutions were diluted with de-ionized water to reach an application level of 20 mg enzyme per kg forage DM and a total of 20 g dilution per kg fresh forage. Enzyme dilutions were sprayed into the bags with spray bottles, followed by a thorough mixing for ~2 minutes. From each bag, 827 ± 13 g forage was filled into autoclaved 1.7 L glass jars, in triplicate, by using a homemade compressing device. Each jar was then sealed with an ethanol sterilized water-lock lid, silos weighed before and after. Silos were stored at 20°C at 50 % relative humidity for 60 days.

3.2 Forage and silage analysis

Silos were opened after 60 days of ensiling, weighed before and after unsealing the jars. Samples were taken directly for silage juice extraction and aerobic stability testing. The rest of

the silage was emptied into plastic bags and frozen at -20°C in for further analysis. The weight registrations from day 0 and day 60 were used to estimate DM losses during ensiling. It was assumed that for each mole CO_2 produced, 1 mole of water was produced. Meaning that for each gram of CO_2 leaving the silo, 0.41 g of the DM was transferred into water. Therefore, DM loss was calculated from silo weight loss, multiplied by 1.41, expressed as g/kg DM.

3.2.1 Silage juice extraction

A mixture of 60 g silage and 60 g de-ionized water was weighed into plastic bags, one for each silo. After soaking in a fridge at 4°C for ~6 hours, the bags were stored in a freezer at -20°C overnight (24 h). This step was made to disrupt plant cells and by that ease the extraction. Each bag was thawed and pinched, and the silage juice was collected by compressing the sample with a hydraulic press. Each jar was then shaken and poured into two bottles, one for pH and one for ammonia-N (AmN) analysis. The bottles were frozen at -20°C until further analysis.

3.2.2 Aerobic stability

PVC-pipes with a diameter of 10 cm and height of 16.5 cm were covered with a glass-fiber filter on one end. The pipes were then sprayed with ethanol and left to dry overnight (12 h). Each pipe was filled with ~328 g of silage, according to DLG (2006), and mounted into an insulating holder. Individual temperature probes were inserted into each pipe and a paper cover was put on the top. Pipes were then covered with 10 mm insulating boards on top and bottom. Both boards had 10 mm drilled holes for each pipe so that air could circulate through the silage. After connecting the probes to a recorder, individual temperatures were recorded every second hour. After 10 days of recording, data was extracted, the PVC-pipes were emptied, and the silage was assessed for deterioration. Temperature readings from the probes were corrected according to the formula shown below.

$$corrected\ temperature = a_p * reading + b_p$$

The parameters *a* and *b* were previously determined for each probe (*p*) by linear regressions with water-filled jars, holding standardized temperatures of 20°C and 40°C.

3.2.3 Chemical analyses

The frozen forage (~250 g) and silage (~100 g/silo) were weighed into aluminum trays, and dried overnight (18 h) in a forced draught oven at 60°C. The trays were left to equilibrate with the environment and weighed. Samples were then milled with a knife mill through a 1 mm sieve and bottled for use in further chemical analyses and determination of in vitro organic matter digestibility (IVOMD).

DM of forage and silage was estimated by weighing 2.0 g of milled sample into pre-weighed porcelain crucibles, followed by drying at 103°C in an oven overnight (12 h). Crucibles were transferred to a desiccator to equilibrate with the room temperature and weighed. DM was calculated according to the formula below. Correction for volatile losses during drying of silage samples was made by adding 1.4 to the final DM percentage (not done for the forage). The correction is based on a study with more than 300 samples, in which DM determination methods were compared with toluene distillation followed by a Karl Fischer titration of the distillate (Lingvall & Ericson 1981, unpublished).

$$DM(\%) = \left(\frac{dried\ (g)_{60°C}}{sample\ (g)}\right) * \left(\frac{dried\ (g)_{103°C}}{dried\ (g)_{60°C}}\right) * 100 + 1.4$$

Thereafter, samples were incinerated in a muffled furnace at 550°C for 3 hours. Crucibles were left to cool and equilibrate with the room temperature in a desiccator and ash content was registered. The organic matter (OM) content was determined by subtracting the amount of ash from DM.

Water soluble carbohydrates (WSC) were analyzed in the forage sample, according to Udén (2006). The procedure comprised extraction with an acetate buffer and hydrolysis with sulfuric acid, releasing soluble glucose and fructose. Followed by a two-step enzymatic procedure that converted the glucose and fructose into 6-phoshoglucolactones, reducing $NADP^+$ to NADPH in the process. Finally, absorbance was measured at 340 nm before and after the conversion of sugars into 6-phoshoglucolactones and WSC was calculated from the increase in NADPH. Total N was estimated by the Kjelldahl method (EC No 152/2009) in the forage, crude protein (CP) was then calculated by assuming 160 g N/kg protein. NDF and ADF was run in sequence, as described by Van Soest and Robertson (1979), on both forage and silage samples. Hemi-cellulose was calculated by withdrawing ADF from NDF.

Frozen silage juice was thawed and equilibrated with room temperature. pH was measured by a Metrohm 654 pH-meter (Metrohm, Herisau, Switzerland). For the AmN analysis, a FIA star 5010 flow injection analyzer (Tecator AB, Höganäs, Sweden) was used. By injecting solutions with known concentrations of NH_4Cl, a standard curve was created. From this curve, AmN concentration in the silage juice was estimated. Finally, AmN in relation to total N was calculated by the following formula.

$$AmN\ (\%\ of\ total\ N)_{silage} = \frac{AmN\ (\%)_{silage\ juice} * \left(\frac{weight\ (\%\ of\ sample)_{dilution}}{corr.DM\ (\%)_{silage}} - 1\right)}{Total\ N\ (\%\ of\ DM)_{forage}} * 100$$

The expression in brackets is the dilution factor, relating AmN in silage juice to the corrected silage DM. *weight* is the total dilution in relation to the silage sample. By withdrawing -1 from the quotient, the dilution factor expresses the ratio of solution to sample DM.

3.2.4 IVOMD

IVOMD was estimated for both forage and silage samples, as described by Lindgren (1983). The method uses a 50-ml mixture of rumen fluid to buffer (1:49) to digest 0.5 g of dried and milled sample anaerobically at rumen temperature. The mixture and samples are incubated in 50 ml glass filter crucibles (porosity 1), situated in a water bath at 38°C for 96 hours. After incubation, residues are rinsed with a standardized procedure, using hot de-ionized water and acetone. The rinsed crucibles are dried at 103°C overnight and weighed, followed by combustion at 500°C for 1½ hour and a second weighing. Finally, IVOMD is calculated as the disappearance of OM in comparison to the initial sample.

3.3 In vitro gas production kinetics

For this part of the trial, equipment for evaluation of biogas potential was used. The idea was to supplement the IVOMD method (section 3.2.4), which gives data about the digestibility of a sample but not any information on the fermentation kinetics over time. As rumen microorganisms are fermenting feeds, gas is produced as a by-product. The amount of gas produced and when it is produced, gives a good hint on how the feed is digested over time in the rumen. The AMPTS II system from Bioprocess Control, Lund, Sweden is built to measure gas production over time, maximum 15 individual gas collection channels can be used

simultaneously. The system is designed to be used with modified 500 ml Kimax bottles with an extra opening and screw-on caps with two connectors, one for gas sampling and the other for optional sampling. There is also an individual stirring device for each bottle that can be controlled for different stirring speeds and stirring intervals. The system measures accumulated gas production and gas flow over time.

3.3.1 Trial setup and preparations

The trial was designed as three consecutive runs, running each set of treatment replicates as separate runs. Observations were made in duplicates by using two bottles per sample. The order within the runs were randomized for treatments by using the Random function in Excel 2016. The order is shown in table 2, observational duplicates were kept together to avoid unnecessary errors.

Table 2 – Randomized trial setup for each treatment replicate with the AMPTS II system.

Bottle	**Run 1**	**Run 2**	**Run 3**
1, 2	FAE 3	FAE 1	Hem & Cell
3, 4	Hem & Cell	FAE 3	FAE 1
5, 6	Control	Pect	Control
7, 8	FAE 2	Hem & Cell	FAE 2
9, 10	*	FAE 2	FAE 3
11, 12	Pect	*	*
13, 14	FAE 1	Control	Pect
15	**	**	**

* No sample added in these bottles, considered as a treatment in the randomization.
** Bottle 15 was always run without sample.

Approx. 100 g of frozen silage from each silo was grinded through a 4.5 mm die with a MR 9 TC 22 meat grinder (Nima AB, Tyresö, Sweden), bagged and re-frozen until the run commenced. Frozen silage was used to avoid loss of volatile (fermentable) compounds from drying. The meat grinder was used to produce a more homogenous sample, chopping with a household scissor did not prove to be consistent enough in our previous pilot trials (unpublished data).

Buffer was prepared in a 10L Duran bottle, situated in a water bath at 38°C with a magnetic stirring device underneath. Ingredients were added together with hot, de-ionized water, according to Table 3. After properly dissolving all ingredients, flushing with CO_2 was applied and continued together with stirring, overnight. The buffer was then completed by addition of $NaHCO_3$ one hour before the run commenced.

Table 3 – Buffer preparation, according to the VOS-method by Lindgren (1983).

Chemical	**g/liter**	**Order**
K_2HPO_4	5,80	1. Dissolve in water
$(NH_2)HPO_4$	0,50	
NaCl	1,00	
$MgSO_4 \cdot 7H_2O$	0,50	
$FeSO_4 \cdot 7H_2O$	0,01	2. Dissolve in water (20 % of total mixture) and add
$CaCl_2$	0,10	
De-ionized water		3. Fill up to final volume
$NaHCO_3$	8,50	4. Add one hour before usage

Before running, the AMPTS II system was tried out to make sure that everything works as planned. The optimal filling volume of the bottles was evaluated to 350 ml, larger volumes risked contaminating the gas collection device with fluids. We also found that the stirring device did not work well with silage, as an inaccessible cake was formed, floating on the top of the bottle. Therefore, automatic stirring was skipped in favor for manual shaking, two times per day at ~12-hour intervals. Moreover, keeping the environment saturated with CO_2 showed to be of high importance for comparable results between the bottles (unpublished data). For this purpose, a CO_2 flushing system was constructed, maintaining a constant and standardized flushing of all bottles at the same time, as seen in Figure 5. Tubes were also attached to the bottle top connectors, reaching down to the bottle bottom, securing saturation with CO_2 throughout the whole volume.

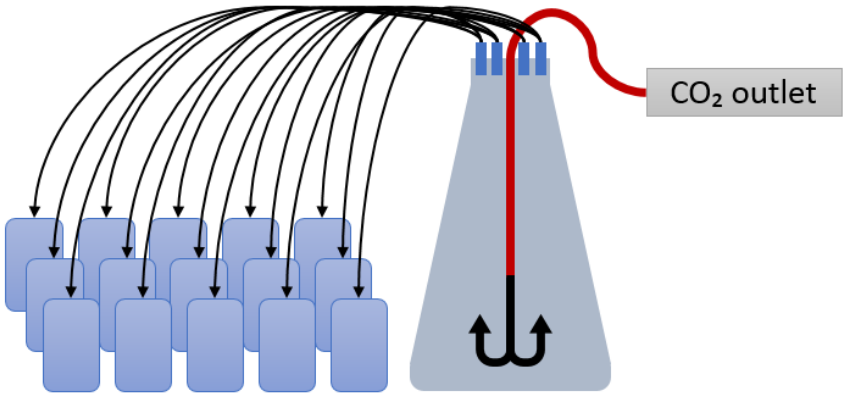

Figure 5 – Standardized method for CO_2 flushing of AMPTS II bottles.

Finally, the optimal sample size and rumen fluid concentration was evaluated. The conclusion was to use 4 g DM of sample and a rumen fluid to buffer ratio of 1:9. This setup gave the most consistent and comparable results between and within test runs (unpublished data).

3.3.2 Running the AMPTS II

The previously grinded frozen silage samples were thawed at room temperature and 4 g of DM was weighed into plastic trays. Each tray was then emptied into each bottle, according to Table 2, by rinsing with 50 ml of the buffer solution to make sure that the whole sample is added. The bottles were placed in a pre-heated water bath with the temperature set to 38°C. The screw-on caps were attached, and tubes were connected to the AMPTS II gas collection device and the CO_2 flushing system (figure 5). Flushing with CO_2 commenced as bottles were connected.

Rumen fluid from a Swedish Red cow (fed at maintenance level) at Lövsta Research Center, SLU, Uppsala was collected and delivered in a thermo-bottle to the laboratory within three hours after sampling. The rumen fluid was filtered through a 1 mm sieve into a measuring cylinder under flushing with CO_2 and was directly mixed with the buffer to reach a final concentration of 10 % of rumen fluid in the AMPTS II bottles. The mixture was thoroughly mixed and flushed with CO_2, and 300 ml of the buffered rumen fluid was added to each bottle through the extra opening. The bottles were then left flushing with CO_2 for 5 minutes before closing. The AMPTS II software was started and recording of gas production commenced. The bottles were then shaken by hand two times per day. Figure 6 shows the system assembled and running.

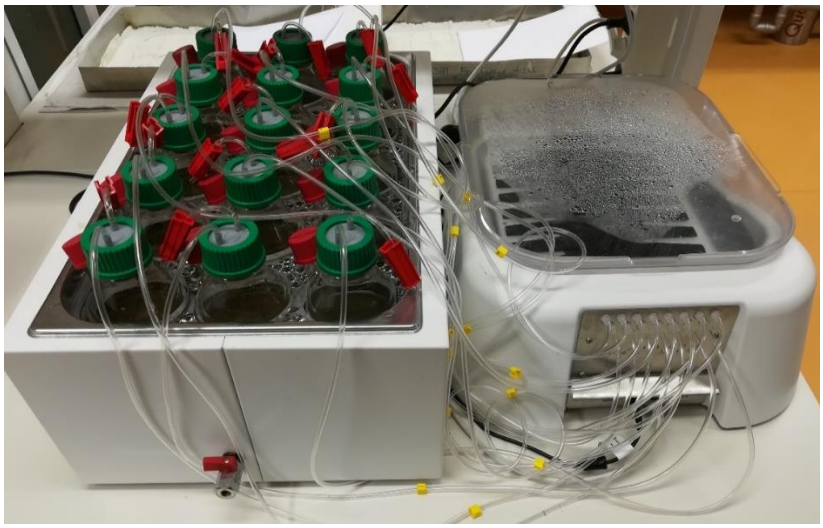

Figure 6 – The AMPTS II system assembled and running, computer not shown in picture.

Estimation of DM in the buffered rumen fluid was carried out for each run. This was done to evaluate if there were any DM differences in rumen fluid between runs. From the rumen fluid and buffer mixture, ~100 g was poured into aluminum trays, and dried. First in a forced draught oven at 60°C overnight, followed by 6 hours in a 103°C oven. Weight was recorded before and after drying and average DM was calculated from all trays.

3.3.3 End fermentation procedure

Incubation was terminated after 96 hours and bottles were disconnected. pH was measured directly after disassembly of the bottles, to make sure that fermentation had proceeded properly. Estimation of IVOMD was done by filtering the bottle residues through 50 ml glass filter crucibles (porosity 1). To avoid clogging of the filter, four crucibles were used per bottle. The filtering procedure was started with emptying of residues into the crucibles, hot de-ionized water was added simultaneously to avoid clogging. Each crucible was then treated by following the standardized procedure for the IVOMD-method, as described in section 3.2.4. Finally, DM disappearance was calculated by comparing rinsed and dried residues with initial DM. Then OM disappearance was calculated by correcting for initial and end ash.

3.4 Statistical analyses

The effect of treatment was evaluated for DM loss, chemical composition and IVOMD in the silage by using the GLM procedure of SAS (v. 9.4; SAS Institute Inc., Cary, NC, USA). The statistical model used was as follows:

$$y_{ij} = \mu + \alpha_i + e_{ij}$$

Where y_{ij} is the response variable, μ is the overall mean and α_i is the fixed treatment effect. Significant differences were declared as $p < 0.05$. Multiple comparisons between significant factors were done by using the Tukey adjustment. Data was reported as least square means together with standard error of the mean (SEM).

For evaluation of the in vitro gas production kinetics and DM and OM disappearance, each treatment replicate was handled as a separate run. Therefore, the statistical model was modified to also consider the effect of runs (β_j), as shown in the new model below.

$$y_{ij} = \mu + \alpha_i + \beta_j + e_{ij}$$

4 Results

4.1 Silage quality

DM loss during ensiling was calculated for each silo as described in section 3.2, estimated mean values and SEM for each treatment are presented in table 4. There were no significant differences between treatments.

Table 4 – Evaluation of the ensiling process, data presented as estimated means and SEM for each treatment. DM loss is expressed as % of DM and AmN as % N of total N.

	Treatment							
Variable	**Control**	**Hem & Cell**	**Pect**	**FAE 1**	**FAE 2**	**FAE 3**	**SEM**	***p*-value**
DM loss	3.4	3.3	3.3	3.3	3.2	3.4	0.05	0.1074
pH	4.75[a]	4.68[b]	4.75[a]	4.73[ab]	4.77[ac]	4.82[c]	0.012	<.0001
AmN	8.1	8.0	8.5	8.0	8.1	8.3	0.12	0.1344

[abc] Values sharing the same superscripts are not significantly different from each other.

Estimated mean values of pH and AmN for each treatment are shown in Table 4, together with the SEM. The *Hem & Cell* treatment reached a significant lower pH during ensiling than the control. On the contrary, *FAE 3* showed a significantly higher pH after ensiling. There were no significant differences in AmN concentration between treatments.

Data from the aerobic stability testing of silage is shown in Figure 7. For a better overview, average daily temperatures for each treatment were calculated from all individual silo readings.

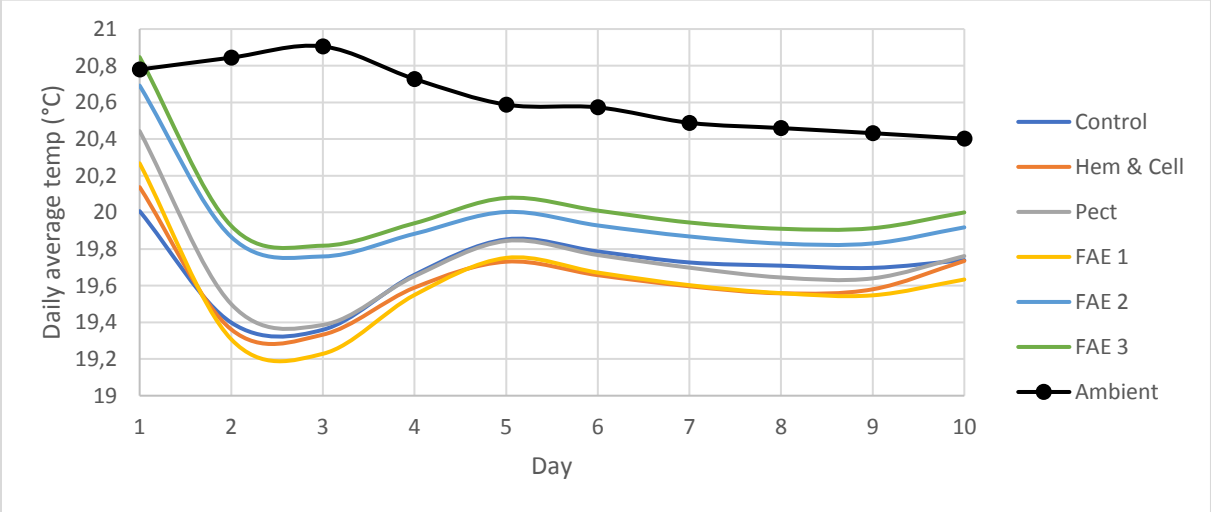

Figure 7 – Daily average silage temperature for each treatment during aerobic stability testing at 20°C.

The first drop between day 1 and 2 shows how silage is equilibrating with the room temperature, the silage was prepared in another room with higher room temperature. Thereafter, there were no noticeable changes in temperature, silages were stable for 10 days of exposure to oxygen.

4.2 Chemical analyses

Forage DM was determined to 31.4 % and OM to 90.3 % of the DM. WSC were determined to 3.54 % and CP to 16.9 % of the forage DM. The forage fiber fraction comprised 54.1 % NDF and 29.0 % ADF on DM basis. The OM digestibility was determined to 71.0 % by the IVOMD-method. Estimated means of all analytical values for each treatment are shown in Table 5 together with the SEM.

Table 5 – Chemical analysis of the silage, presented as estimated means and SEM for each treatment. DM is expressed as a % of wet weight and OM as % of DM. NDF, ADF and Hemi-cellulose is expressed as a % of DM and IVOMD as % of OM.

	Treatment							
Variable	**Control**	**Hem & Cell**	**Pect**	**FAE 1**	**FAE 2**	**FAE 3**	**SEM**	***p*-value**
DM*	31.9	32.0	31.9	32.2	31.9	32.1	0.10	0.2902
OM	90.4	90.4	90.3	90.5	90.5	90.5	0.05	0.1003
NDF**	48.4	47.8	47.8	47.9	47.3	47.5	0.31	0.2549
ADF**	28.9^{a}	27.9^{b}	27.8^{b}	28.6^{ab}	28.0^{ab}	28.3^{ab}	0.22	0.0201
Hemi-cellulose	19.4^{a}	19.9^{a}	20.0^{a}	19.3^{a}	19.3^{a}	19.2^{a}	0.20	0.0479
IVOMD (VOS-method)	71.1	70.6	70.8	70.5	71.2	72.0	0.76	0.7627

^{abc} Values sharing the same superscripts are not significantly different from each other.
* Corrected for volatile losses at drying, as described in section 3.2.3.
** Presented without ash.

There were no significant differences between treatments for DM, OM and NDF. Further analysis of the fiber fraction showed significant differences for ADF and hemi-cellulose. The *Hem & Cell* and *Pect* treatments reduced ADF significantly compared to the control. However, by pairwise comparison of treatments for hemi-cellulose, no significant differences were found. Finally, IVOMD did not show and significant differences between treatments.

4.3 Evaluation of the in vitro gas data

Gas flow over time from each bottle was re-calculated to a proportion of the final cumulative gas production, presented as mean values of each treatment per hour in Figure 8. The graph is scaled to the first 48 hours for a more detailed view of the major part of the gas flow.

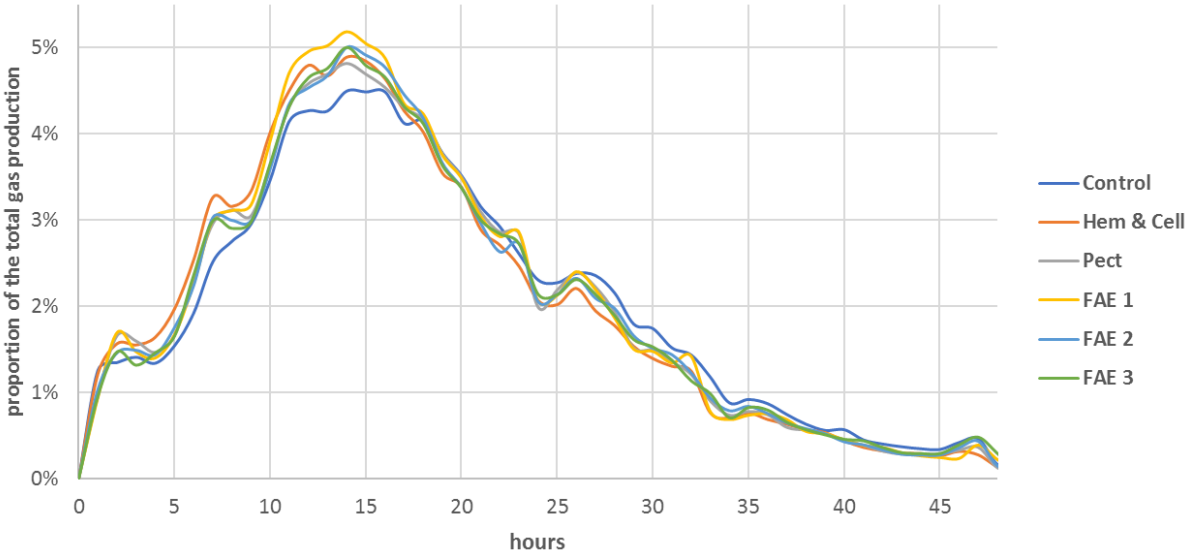

Figure 7 – Gas flow per hour for each treatment, presented as a proportion of the final cumulative gas production.

The graph shows a slight difference between the control and the treatments. This was further investigated by summing up the proportion of gas produced at different intervals, comparisons were then made between each treatment at different intervals, presented as mean values and SEM in Table 6. The total gas production from each bottle was re-calculated to represent ml of gas produced per g DM of sample, data is presented as mean values and SEM for each treatment in Table 6.

Table 6 – Total gas produced and summarized gas flows at different time intervals, presented as estimated means and SEM for each treatment. Total gas is express as ml of gas per g DM of sample and gas flow is expressed as a proportion, in %, of the total gas produced.

	Treatment							
Variable	**Control**	**Hem & Cell**	**Pect**	**FAE 1**	**FAE 2**	**FAE 3**	**SEM**	***p*-value**
Total gas	53.7	50.6	48.6	47.2	50.6	49.0	1.55	0.1490
0 – 6 h	8.8	10.5	9.7	9.2	9.4	9.2	0.36	0.0980
6 – 12 h	20.1	23.1	21.7	22.3	21.5	21.5	0.68	0.1498
12 – 18 h	26.0	27.3	27.2	28.1	28.0	27.7	0.57	0.2126
18 – 24 h	18.3	17.0	18.1	17.6	17.4	17.7	0.39	0.3436
24 – 36 h	19.5[a]	16.3[b]	17.4[ab]	16.9[ab]	17.6[ab]	17.4[ab]	0.54	0.0333
36 – 72 h	7.3	5.9	6.0	5.9	6.2	6.5	0.73	0.6085

[abc] Values sharing the same superscripts are not significantly different from each other.

There were no significant differences between treatments for total gas produced. For the interval data, a significantly lower gas flow was observed at 24 – 36 h for *Hem & Cell* compared to the control. The 72 – 96 h interval was excluded due to that no gas flow was observed during this interval. The block effect was significant for both total gas and the gas flow intervals.

4.3.1 Rumen fluid and residues

The rumen fluid buffer mixture used in each run had a DM content of 1.32 ± 0.01 % DM/kg mixture, indicating a consistent sample of rumen fluid for each run. The average end-pH of fermentation was 6.79 ± 0.02 for bottles with sample and 7.05 ± 0.03 for bottles with only rumen fluid and buffer. Results from the DM and OM disappearance estimation with the AMPTS II system is presented as means with SEM for each treatment in Table 7.

Table 7 – Disappearance of DM and OM during fermentation with the AMPTS II system, presented as means with SEM for each treatment and expressed as % of the initial sample.

	Treatment							
Variable	**Control**	**Hem & Cell**	**Pect**	**FAE 1**	**FAE 2**	**FAE 3**	**SEM**	***p*-value**
DM disappearance	81.7	81.2	83.2	82.4	81.8	81.6	0.64	0.5268
OM disappearance	80.8	80.9	82.4	81.4	81.0	80.7	0.61	0.4622

No significant differences were observed for either DM or OM disappearance.

5 Discussion

In a study evaluating silage treatments, appropriate evaluation of the ensiling process is of high importance. If the treatment affects general silage quality negatively, it is better to not treat the silage at all. Moreover, if the ensiling process does not proceed as expected, important effects of the treatments might be foreseen. Average DM-loss for all silos were 3.3 % with no significant differences between treatments. McDonald et al. (1991) suggests 2 – 4 % as a normal loss of DM during the fermentation process at ideal conditions. Indicating that no major losses occurred due to inadequate filling and packing of the silos or from oxygen leaking in during the ensiling process.

The final silage pH is important for storage stability and gives a hint on how well the ensiling process succeeded, as described in section 2.3. According to Pahlow et al. (2003), sufficient reduction in pH in combination with anaerobiosis is important for inhibition of spoilage bacteria, such as clostridia and enterobacteria. According to Spörndly (2003), a normal value for silage with a DM of ~32 % should be ~4.5. The average pH for all silos was 4.75, which is above the normal. Though this did not affect the aerobic stability of the silage. The insufficient reduction in pH can probably be explained by the low content of WSC in the forage, as it is the primary source of carbon for lactic acid production by lactobacilli (Rooke and Hatfield, 2003). Interestingly, the *Hem & Cell* treatment showed a significantly lower pH than the control. This might be explained by a partial hydrolysis of both cellulose and hemi-celluloses into glucose and other WSC by the added enzymes. By providing more WSC for fermentation by the *Lactobacilli* into lactic acid, pH is reduced further. On the contrary, treating the silage with *FAE 3*, resulted in a significantly higher final pH than the control. Indicating that an inhibition of fermentation might have occurred by the treatment, though, no clear reason for this was found. For further interpretation of the pH data, analysis of organic acids would be required.

The average AmN concentration was 8.2 g/kg total N for all silos. The limit for a good silage is below 8, according to Spörndly (2003). This might indicate a slightly increased presence of spoilage bacteria during the ensiling process. As mentioned in section 2.3, an insufficient reduction in pH might leave room for spoilage bacteria (e.g. enterobacteria and clostridia) that break down amino acids and peptides into ammonia. Though, no significant differences between treatments or correlations to pH was found. Finally, the relatively high CP (16.9 %) content of the forage might also influence the slightly elevated AmN content in the silage.

The general aim for this thesis was to improve fiber digestibility of a late harvested crop by addition of fibrolytic enzymes to the forage before ensiling. If there was any effect of the enzymes, it should be observed in the fiber fraction. For NDF (total fiber), there was no significant difference between treatments. Though, there is a tendency of total fiber reduction seen for the *FAE 2* and *FAE 3* treatments. The ADF (cellulose and lignin) fraction was significantly reduced by the *Hem & Cell* and *Pect* treatments. This might be explained by a disruption of the lignocellulose complex. Cellulases split the cellulose fibrils and hemi-cellulases break up the structure binding together the cellulose fibrils (Gupta et al., 2016). Pectinase breaks down pectin in middle lamellas and primary cell walls, possibly de-attaching cells from each other (Voragen et al., 2009). Thereby, suspending more of the cell wall to digestion during the ensiling process and further in the rumen. However, the IVOMD did not show any significant differences or tendencies of improvement by the treatments. Though, an issue with the IVOMD-method is the usage of dried samples. Volatile compounds are partly lost in drying, not contributing to the rumen fluid fermentation. If the significant reduction in

ADF means a release of substrate for LAB, part of their fermentation products during the ensiling process are lost during sample preparation for the IVOMD analysis and is therefore not reflected fully in the results.

Evaluation of the in vitro gas production kinetics was done to evaluate if there were any differences in digestion over time. Figure 7 indicates that the control treatment seemd to produce slightly less gas than the treatments up to ~16 hours, then catching up from ~24 hours and forward. There were no significant differences found for total gas between treatments. However, for time interval gas flows, the *Hem & Cell* treatment showed a significantly lower gas flow during 24 – 36 h compared to the control. Though, this alone does not strengthen the theory described above. As a final attempt to evaluate the gas data, cumulative gas production was also fitted to the degradability function described by Ørskov and McDonald (1979) and the Gompertz sigmoid curve (data not presented), though without any success in relating the variables to something relevant for comparison. From the rinsed and dried fermentation residues, no significant differences were seen for either DM or OM disappearance. Both values were reported due to discrepancies in the ash analysis of residue, it seemed like a part of the ash was lost during the rinsing process (described in section 3.3.3).

5.1 Conclusions and final remarks

There are probably many reasons to why the expected results were not observed. The added concentration of enzymes might be too low for achieving the desired effect. Though, without information on the specific activity of the enzyme, further conclusion cannot be drawn on that matter. Moreover, enzymes are not motile and thereby need to be dispersed in a solution to reach their substrate. Meaning that the water content of the silage or total dilution volume of the enzymes might have been too low. Finally, enzymes chosen for the trial might not be optimal for this type of substrate, especially as pH changes over time in the silage.

Further research is needed to evaluate the effect of adding fibrolytic enzymes in forage production, as way of improving digestibility.

References

Akin, D.E., 1989. Histological and Physical Factors Affecting Digestibility of Forages. Agronomy Journal 81, 17.

Buxton, D.R., Mertens, D.R., Moore, K.J., 1995. Forage Quality for Ruminants: Plant and Animal Considerations. The Professional Animal Scientist 11, 121–131. https://doi.org/10.15232/S1080-7446(15)32575-4

Buxton, D.R., O'Kiely, P., 2003. Preharvest Plant Factors Affecting Ensiling, in: Buxton, D.R., Muck, R.E., Harrison, J.H. (Eds.), Silage Science and Technology, Agronomy. American Society of Agronomy, Inc. : Crop Science Society of America, Inc. : Soil Science Society of America, Inc, Madison, pp. 199–250.

DLG, 2006. DLG-Richtlinien für die Prüfung von Siliermitteln auf DLG-Gütezeichen-Fähigkeit (DLG guidelines for the test of silage additives for approval of DLG quality lables). DLG Commission for Silage Additives, Frankfurt a.M., Germany.

Eurostat, 2017. Cows'milk collection and products obtained - monthly data [WWW Document]. URL http://ec.europa.eu/eurostat/web/agriculture/data/database (accessed 11.4.2017).

Gupta, V.K., Kubicek, C.P., Berrin, J.-G., Wilson, D.W., Couturier, M., Berlin, A., Filho, E.X.F., Ezeji, T., 2016. Fungal Enzymes for Bio-Products from Sustainable and Waste Biomass. Trends in Biochemical Sciences 41, 633–645.

Himmel, M.E. (Ed.), 2008. Biomass recalcitrance: deconstructing the plant cell wall for bioenergy. Blackwell Pub, Oxford.

Hoffman, P.C., Sievert, S.J., Shaver, R.D., Welch, D.A., Combs, D.K., 1993. In Situ Dry Matter, Protein, and Fiber Degradation of Perennial Forages. Journal of Dairy Science 76, 2632–2643.

Jung, H.G., Buxton, D.R., Hatfield, R.D., Ralph, J., Theander, O., Westerlund, E., 1993. Quantitative Analysis of Cell Wall Components, in: ACSESS Publications. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America.

Kalu, B.A., Fick, G.W., 1981. Quantifying Morphological Development of Alfalfa for Studies of Herbage Quality. Crop Science 21, 267.

Kung, L., Shaver, R., 2001. Interpretation and Use of Silage Fermentation Analysis Reports. Focus on Forage, Team Forage, Univertsity of Wisconsin-Extension Vol 3, 5.

Kuoppala, K., Ahvenjärvi, S., Rinne, M., Vanhatalo, A., 2009. Effects of feeding grass or red clover silage cut at two maturity stages in dairy cows. 2. Dry matter intake and cell wall digestion kinetics. Journal of Dairy Science 92, 5634–5644.

Lindgren, E., 1983. Nykalibrering av VOS-metoden för bestämning av energivärde hos vallfoder (New calibration of the VOS-method for determination of energy value in forage feeds). (Working paper). Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, Uppsala, Sweden.

McDonald, P., Edwards, R.A., Greenhalgh, J.F.D., Morgan, C.A., Sinclair, L.A., Wilkinson, R.G., 2011. Animal nutrition, Seventh edition. ed. Pearson Education Ltd, Essex, England.

McDonald, P., Henderson, N., Heron, S., 1991. The biochemistry of silage, 2nd ed. Chalcombe Publications, Marlow.

Moore, K.J., Moser, L.E., Vogel, K.P., Waller, S.S., Johnson, B.E., Pedersen, J.F., 1991. Describing and Quantifying Growth Stages of Perennial Forage Grasses. Agronomy Journal 83, 1073.

Muck, R.E., Moser, L.E., Pitt, R.E., 2003. Postharvest Factors Affecting Ensiling, in: Buxton, D.R., Muck, R.E., Harrison, J.H. (Eds.), Silage Science and Technology, Agronomy. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, pp. 251–304.

Ohlsson, C., Wedin, W.F., 1989. Phenological Staging Schemes for Predicting Red Clover Quality. Crop Science 29, 416.

Ørskov, E.R., McDonald, I., 1979. The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. The Journal of Agricultural Science 92, 499. https://doi.org/10.1017/S0021859600063048

Pahlow, G., Muck, R.E., Driehuis, F., Elferink Oude, S.J.W.H., Spoelstra, S.F., 2003. Microbiology of Ensiling, in: Buxton, D.R., Muck, R.E., Harrison, J.H. (Eds.), Silage Science and Technology, Agronomy. American Society of Agronomy, Inc. : Crop Science Society of America, Inc. : Soil Science Society of America, Inc, Madison, pp. 31–93.

Pettersson, O., Sundberg, M., Westlin, H., 2009. Maskiner och metoder i vallodling (Equipment and methods in forage production), JTI rapport Lantbruk och industri, 377. RISE (www.jti.se), Uppsala, Sweden.

Puniya, A., Singh, R., Kamra, D.N. (Eds.), 2015. Rumen microbiology: from evolution to revolution. Springer, New Delhi, Heidelberg

Rinne, M., Nykänen, A., 2000. Timing of primary growth harvest affects the yield and nutritive value of timothy-red clover mixtures. Agricultural and Food Science in Finland 9, 121–134.

Rooke, J.A., Hatfield, R.D., 2003. Biochemistry of Ensiling, in: Buxton, D.R., Muck, R.E., Harrison, J.H. (Eds.), Silage Science and Technology, Agronomy. American Society of Agronomy, Inc. : Crop Science Society of America, Inc. : Soil Science Society of America, Inc, Madison, pp. 95–139.

SMHI, 2015. Climate indicators – length of vegetation period [WWW Document]. Swedish Meteorological and Hydrological Institute. URL https://www.smhi.se/en/climate (accessed 11.4.17).

Spörndly, R., 2003. Fodertabeller för idisslare 2003 (Feeding tables for ruminants) (No. 257). Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, Uppsala.

Udén, P., 2006. In vitro studies on microbial efficiency from two cuts of ryegrass (Lolium perenne, cv. Aberdart) with different proportions of sugars and protein. Animal Feed Science and Technology 126, 145–156.

Van Soest, P.J., 1994. Nutritional ecology of the ruminant, 2nd ed. Comstock Pub, Ithaca.

Van Soest, P.J., Robertson, J.B., 1979. Systems of analysis for evaluating fibrous feeds, in: Standardization of Analytical Methodology for Feeds: Proceedings... IDRC, Ottawa, ON, CA.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. Journal of Dairy Science 74, 3583–3597.

Vinther, F.P., 2006. Effects of cutting frequency on plant production, N-uptake and N2 fixation in above- and below-ground plant biomass of perennial ryegrass-white clover swards. Grass and Forage Science 61, 154–163.

Voragen, A.G.J., Coenen, G.-J., Verhoef, R.P., Schols, H.A., 2009. Pectin, a versatile polysaccharide present in plant cell walls. Structural Chemistry 20, 263–275. https://doi.org/10.1007/s11224-009-9442-z

Wilson, J.R., 1993. Organization of Forage Plant Tissues, in: Jung, H.G., Buxton, D.R., Hatfield, R.D., Ralph, J. (Eds.), Forage Cell Wall Structure and Digestibility. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, Wisconsin, pp. 1–32.

Wilson, J.R., Hatfield, R.D., 1997. Structural and chemical changes of cell wall types during stem development: consequences for fibre degradation by rumen microflora. Australian Journal of Agricultural Research 48, 165–180.

Wilson, J.R., Kennedy, P.M., 1996. Plant and animal constraints to voluntary feed intake associated with fibre characteristics and particle breakdown and passage in ruminants. Australian Journal of Agricultural Research 47, 199–225.