

THESIS OF DOCTORAL (PhD) DISSERTATION

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2012

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**DEVELOPMENT OF A BIOLOGICAL PRESERVATIVE OF GOOD
EFFICACY FOR THE PRESERVATION OF MEDIUM AND HARD
FERMENTABLE FORAGES**

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1. INTRODUCTION

Roughages are the most natural feedstuffs of ruminants. Our climatic endowments imply that the annual quantity of feed for the farm animals has to be produced in the period from April to November. However the majority of feeds at harvest are not in air-dry state, so it is necessary to preserve them until use. The majority of feedstuffs to preserve are forages which play an important role in the feeding of ruminants both in terms of nutrition physiology and economics.

One possible way of preservation is the fermentation. However, for this the natural fermenting ability of some forages shall be improved by using additives (e.g. biological preservative). The use of biological preservatives is spreading nowadays, to improve the natural fermentation capability of forages. The third generation of biological preservatives is now on the market, which in addition to lactic acid producing bacteria contain any enzyme preparation, too. Experiences with the third generation biological preservatives are quite contradictory. The efficiency of these preservatives are often unsatisfactory, which is in most cases due to the fact, that the prevailing conditions in the silo during fermentation do not meet in all respects the optimal operation requirements of the carbohydrate degrading enzymes.

This primarily relates to the temperature within the silo, but the silage pH is also not always within the range, which would be necessary for optimal operation of the cellulose and hemicellulose degrading enzymes. This problem could be most easily remedied by increasing the enzyme concentration in the additive, but in turn it is limited by the economy.

A possible way of the improvement of bio-preservatives is searching for enzyme complexes, of which the operating conditions in terms of temperature and pH are closer to those prevailing in the silo, than the currently used enzyme preparations. To this option gives the hope, that there is a significant variation in the optimum operating conditions of the various micro fungus enzymes.

Another way of the development may be finding and production of carbohydrate sources by which the natural fermenting ability of moderate and hard fermentable crops could be improved substantially. In my work I have chosen the latter option. I was trying to produce a carbohydrate substrate by enzymatic decomposition of starch, occurring in large quantities in corn grain, but not fermentable for the lactic acid bacteria, that could be a component of a second generation bio-preservatives.

2. OWN EXPERIMENTS

2.1. The aim of the experiments

In recent years several fermentation dynamic experiments were conducted with third generation biological preservatives, used for making alfalfa and grass silage on the domestic farms, at the Department of Animal Nutrition of the University of West Hungary, Faculty of Agriculture and Food Sciences. The performed studies have shown that the effectiveness and impact safety of circulated biological preservatives are far from satisfactory. The amount of enzymes contained therein is not sufficient to produce as much fermentable carbohydrates from the raw fibre and starch content of the feed as many is required for the production of lactic acid ensuring adequate stability of the silage. In cases, when by the manufacturer guaranteed enzyme concentration of the in Hungary circulated biological preservatives was increased the pH of the silages, which is an important quality indicator of the silage, decreased significantly. A substantial increase of enzyme concentration of these preservatives would in turn make the already not cheap biological preservatives more expensive. In fact, the third generation biological preservatives are not effective with the current enzyme concentration, but they are not economic with an increased enzyme concentration.

Using the experiences of several decades of the Animal Nutrition Department on fermentation dynamic experiments, we set as a target of our research and development work, to develop an efficient carbohydrate-based biological preservative, that is suitable for the production of good quality and stable silages from moderate and hard fermentable green forages, with a high security, low losses and a good lactic acid:acetic acid ratio.

The choice of topic was motivated by the fact, that all the necessary conditions for silage experiments, including fermentation dynamic experiments, climatic chambers, laboratory as well as experimental animals in its entirety were available.

Because feedstuffs, rich in fermentable carbohydrates are not available in sufficient quantities in our country, we designed to provide the necessary carbohydrates by the enzymatic breakdown of corn starch. We planned to do it not "in situ" in the silo, but under controlled conditions (temperature, pH). Later on the hydrolyzed and dried corn meal product shall be added to the green alfalfa or grass before ensiling.

Our experiments aimed to answer the following questions:

1. To what extent the corn starch can be broken down into reducing sugars with alpha amylase and amyloglucosidase enzymes.
 - How affect the DM content of the medium the starch degradation?
 - What is the impact of the lenght of hydrolysis and of the enzyme dose on the starch degradation?
2. What is the value of hydrolyzed corn meal, as fermentable corbohydrate source for lactic acid bacteria?
3. How much hydrolyzed corn meal is needed to produce a stable silage depending on the DM content of the ensiled green forage?

4. Whether the efficiency of the developed preservative can be improved by supplementing it with ricotta whey, a dairy by-product that increases its reducing sugar content?

2.2. Materials and methods

2.2.1. Corn hydrolysis experiments

We have chosen corn as carbohydrate source of the preservative to be developed, because corn is in the largest amount available carbohydrate source in our country and among the grains it has the largest starch content. We aimed to break down corn starch by enzymatic technology. To do this, such as grain-based alcohol production technology, the NOVO products (NOVO Nordisk A/S, Denmark) α -amylase (BAN 480) and amyloglukosidase (SPIRIZYME) enzymes were used. Hydrolysis parameters were as follows:

| | BAN 480 | SPIRIZYME |
|-----------------|------------------|------------------|
| Enzyme dose | 1 g/kg of starch | 1 g/kg of starch |
| pH | 5.6-6.0 | 4.5 |
| Temperature | 80 °C | 60 °C |
| Hydrolysis time | 20 minutes | 20 hours |

Finally ground corn meal, average grain size of 0.5 mm, was used in the experiment. Given that the optimum operating temperature and pH requirement of the two enzymes were substantially different, a two stage hydrolysis was performed. The first phase was done with amylase. The duration of the hydrolysis was measured from the time, when the corn-water mixture reached a temperature of 80 °C. Having finished the first phase of

the hydrolysis, the hydrolysate was cooled down to 60 °C, and the pH was set by 6 molar hydrochloric acid to pH 4.5. Then was added the required amount of amyloglucosidase. The corn-water mixture was continuously mixed in both phases of the hydrolysis.

The outcome of the hydrolysis - the amount of starch degraded - is influenced by a great extent from the DM content of the medium. Therefore we also investigated that at what DM content the highest reducing sugar yield can be obtained. This issue was also important from the point of view of economics, because of the hydrolyzed corn meal has to be dried and the cost of it greatly affects the economics of the production of the new preservative. We studied the correlation between the DM content of the medium and the amount of the reducing sugars in a DM range of 10-30%.

2.2.2. Increasing the reducing sugar content of hydrolyzed corn with ricotta whey

The energy demand of enzymatic hydrolysis is greatly affected by the amount of energy, used for heating up the water to 90°C, added prior to the corn meal for setting the DM content of the medium. Most of this energy can be saved if we use e.g. hot whey (80-85 °C), a by-product of the ricotta cheese manufacturing, instead of water for adjusting the DM of the medium. In addition to energy efficiencies, this also has the advantage that the fermentable sugar content of the hydrolysate can be further increased. When determining the whey proportion of the mixture we assumed that the lactose content of the ricotta whey should increase substantially the reducing sugar content of the preservative, but the DM content of the mixture should not

exceed 30 %. A proportion of 1 kg corn meal and 2.5 kg whey proved to be favorable.

2.2.3. Microbiological tests

2.2.3.1. Radical definition of the inoculant composition

Next to the carbohydrate components (hydrolyzed corn meal and ricotta whey) another essential component of the preservative designed is the freeze-dried bacteria culture, which is responsible by changing the racial composition of the epifita microflora for the control of the fermentation process.

When compiling the starter culture, 4 species of bacteria were taken into account. One of them is the *Lactobacillus plantarum*, that has a number of good features (homofermentative, good tolerance to acids, low proteolytic activity, less sensitive to changes in conditions of the water activity), consequently it is to be found in bacterial cultures of all current biological preservatives. Any claim to argue against him is that its reproductive capacity decreases above pH 5. To correct this disadvantage we put also *Enterococcus faecium* bacteria into the culture, which well replicates between pH 5-6, produces a lot of lactic acid already at the beginning of the fermentation, thus creating more favorable conditions (mainly by rapid pH decrease) for the *Lb. plantarum*. Using this two bacterium species in the starter culture has also the advantage, that both are produced in Hungary (Medipharm Ltd.) which makes the aquisition of the bacterium cultures easier and cheaper.

Two more species of bacteria was inserted into the starter culture, as well. These were the *Lb. buchneri*, and the *Propionibacterium*

freudenreichii subsp. *shermanii*. The intention of the inclusion of both bacteria was to increase the aerobic stability of the silage. The microbiological tests were performed at our Faculty by the Institute of Food Science, Department of Microbiology.

In order to determine the favorable proportion of the four bacterial species within the starter culture, we tested the maximum specific growth rate (μ_{\max}) and generation time (t_g) for the applied 4 species at three different temperatures and three different pH values.

In the second phase of the experiments next to hydrolyzed corn we also included ricotta whey as carbohydrate source in the preparation. Therefore in an experiment we also tested how efficiently utilize the 4 selected bacteria species the lactose, the carbohydrate component of whey. This was determined by measuring the growth rate of the species on lactose. In addition to lactose glucose was also included in the study as a control.

Of the two substrates tested, culture mediums were prepared, containing of the same amount of reducing sugars ($4.7 \text{ g}/100 \text{ cm}^3$), which were supplemented with yeast extract and peptone. Propagation tests lasted for 25 hours on both substrates, during which we tested the pH of the medium every 3 hours and samples were also taken at the same time to determine the number of bacteria in cm^3 by the plate count method. Based on the experimental data the maximum specific growth rate (μ_{\max}) and the generation time (t_g) of the four bacteria species for the tested substrates could be calculated.

2.2.3.2. Determining the microbial stability of the developed preservative

In our experimental work we also wanted to determine the form of packaging that maintains the microbial stability of the product during storage. In further experiments we tested how long the final product (carbohydrate additives + bacterial culture) preserves the pre-colony number, which is needed for the production of the high quality stable silage. The studies were also extended to the impact of storage temperature on the microbiological stability of the preservative.

In the experiment as many $1.5 \times 10^8 / \text{cm}^3$ CFU *Lb. plantarum* and $3.6 \times 10^9 / \text{cm}^3$ CFU *Lb. buchneri* culture were administered to the 93 % DM hydrolyzed corn meal, that shall meet the inoculation concentration of 10^5 CFU/g green forage. Both bacterial cultures were homogeneously mixed to hydrolyzed corn, and then the prepared samples were closed and placed into sterile glass flasks. Half of the flask was stored in refrigerator of 4 °C, the other half at room temperature. On the 0th, 1st, 2nd, 4th, 6th and 8th weeks of the storage we determined the acid producing, non acid producing microorganisms, yeast and mould count of the samples.

2.2.4. Ensiling experiments

2.2.4.1. Model ensiling experiments

The fermentation dynamic experiments were performed with green alfalfa and grass wilted of varying degrees. In these experiments we examined the effect of bacterial inoculation combined with hydrolyzed corn meal supplement and with hydrolyzed corn meal and ricotta whey supplement on the fermentation parameters in the function of fermentation time (Table 1 and 2). As a positive control, a commercially available third-

generation bio-preservative was used. In the fermentation dynamic experiments we also aimed to examine, that what kind of impact has the DM content of the ensiled forage on the required dose of the hydrolyzed corn meal as well as the hydrolyzed corn meal and ricotta whey mixture, to produce a stable silage.

The fermentation dynamic tests were performed in model silos of 850 ml capacity, in which 400-430 g wilted alfalfa or grass was ensiled. Silos were stored in a climatic chamber at 25 ± 1 °C. 25 model silos were filled for each treatment. 5-5 of them was opened on the defined day of fermentation. The pH, lactic acid, volatile fatty acids, alcohol and ammonia content of the silages were determined. On the last opening day (120 or 180 days) we also determined the dry matter and energy losses occurred during fermentation.

Table 1: Treatments and days of fermentation in the fermentation dynamic studies with alfalfa

| | |
|----------------------|---|
| DM | 31.7 % |
| Treatments | <ol style="list-style-type: none"> 1. Control 2. Bacterial inoculation 3. 1% hydrolyzed corn meal + inoculant |
| Days of fermentation | 3., 7., 15., 30., 180. |
| DM | 23.3 % |
| Treatments | <ol style="list-style-type: none"> 1. Control 2. Bacterial inoculation 3. 1.5 % hydrolyzed corn meal + inoculant 4. 2.0 % hydrolyzed corn meal + inoculant 5. 2.5 % hydrolyzed corn meal + inoculant 6. Goldzym |
| Days of fermentation | 7., 15., 30., 60., 120. |
| DM | 32.3 % |
| Treatments | <ol style="list-style-type: none"> 1. Control 2. Bacterial inoculation 3. 1.0 % hydrolyzed corn meal + inoculant 4. 1.5 % hydrolyzed corn meal + inoculant 5. 2.0 % hydrolyzed corn meal + inoculant 6. Goldzym |
| Days of fermentation | 7., 15., 30., 60., 120. |
| DM | 38.1 % |
| Treatments | <ol style="list-style-type: none"> 1. Control 2. Bacterial inoculation 3. 0.5 % hydrolyzed corn meal + inoculant 4. 1.0 % hydrolyzed corn meal + inoculant 5. 1.5 % hydrolyzed corn meal + inoculant 6. Goldzym |
| Days of fermentation | 7., 15., 30., 60., 120. |
| DM | 30.0 % |
| Treatments | <ol style="list-style-type: none"> 1. Control 2. 1% hydrolyzed corn meal + inoculant 3. Goldzym 4. Bactozym 5. Lalsil PS |
| Days of fermentation | 7., 15., 30., 60., 120. |
| DM | 28.8 % |
| Treatments | <ol style="list-style-type: none"> 1. Control 2. 1.0 % hydrolyzed corn meal+Ricotta whey+ inoculant 3. 0.83 % hydrolyzed corn meal+Ricotta whey+ inoculant 4. Lalsil PS |
| Days of fermentation | 7., 15., 30., 60., 120. |

Table 2: Treatments and days of fermentation in the fermentation dynamic studies with grass

| | |
|----------------------|---|
| DM | 31.6 % |
| Treatments | <ol style="list-style-type: none"> 1. Control 2. Bacterial inoculation 3. 0.33 % hydrolyzed corn meal + inoculant 4. 0.66 % hydrolyzed corn meal + inoculant 5. Goldzym |
| Days of fermentation | 3., 7., 15., 30., 180. |
| DM | 23.3 % |
| Treatments | <ol style="list-style-type: none"> 1. Control 2. Bacterial inoculation 3. 0.5 % hydrolyzed corn meal + inoculant 4. 1.0 % hydrolyzed corn meal + inoculant 5. 1.5 % hydrolyzed corn meal + inoculant 6. Goldzym |
| Days of fermentation | 7., 15., 30., 60., 120. |
| DM | 29.6 % |
| Treatments | <ol style="list-style-type: none"> 1. Control 2. Bacterial inoculation 3. 0.2 % hydrolyzed corn meal + inoculant 4. 0.6 % hydrolyzed corn meal + inoculant 5. 1.0 % hydrolyzed corn meal + inoculant 6. Goldzym |
| Days of fermentation | 7., 15., 30., 60., 120. |
| DM | 33.7 % |
| Treatments | <ol style="list-style-type: none"> 1. Control 2. Bacterial inoculation 3. 0.1 % hydrolyzed corn meal + inoculant 4. 0.4 % hydrolyzed corn meal + inoculant 5. 0.7 % hydrolyzed corn meal + inoculant 6. Goldzym |
| Days of fermentation | 7., 15., 30., 60., 120. |

The effect of hydrolyzed corn and ricotta whey combination on the fermentation was also investigated in larger plastic silos of 100 liter. Two silos per treatment were filled with slightly wilted (28.7 % DM) alfalfa. The following treatments were applied:

1. Control
2. 0.5% hydrolyzed corn meal

3. 0.41% hydrolyzed corn meal + 0.09 % ricotta whey supplement
4. Lalsil PS of 10 g/t green alfalfa

The main parameters of the third generation bio-preservatives used in the fermentation dynamic experiments were as follows:

Goldzym: bio preservative containing the bacteria strains *Lb. plantarum*, *Enterococcus faeium*, *Lb. casei* és *Pediococcus pentosaceus*, as well as cellulase and hemicellulase enzymes. Inoculation colony count was 1.5×10^5 /g green alfalfa. Enzyme activity of the preparation was: 0.17 IU/g.

Bactozym: it contained the same bacterial strains as the Goldzyme, but in addition to cellulase and hemicellulase it contained glucoseoxidase, too. Enzyme activity was 0.22 IU/g, inoculation colony count 1.5×10^5 /g alfalfa.

Lalsil PS: next to the bacterial strains *Lb. plantarum* and *Pediococcus acidilactici* it contained cellulase and hemicellulase. Proposed dose 10 g/t, inoculation colony count: 2.5×10^5 /g green alfalfa. Declared enzyme activity: 10^4 CMC/g.

2.2.4.2. Methodology of the farm ensiling trial

In the framework of the preservative development we also performed farm scale ensiling experiments at the dairy farm of the Agrár Co., Darnózseli.

Slightly wilted alfalfa (32.7-33.8 % DM) was ensiled using hose foil technology. The control silage was treated with the third generation bio-preservative Lalsil in a dose of 10 g/t green fodder, used already at the plant. The experimental silage was treated with 1 % hydrolyzed corn supplement,

which included the freeze-dried bacteria culture. Inoculation rate was 1.5×10^5 /g green alfalfa. The volume of the ensiled green alfalfa was 50.5 t and 50.0 t for the control and experimental silage, respectively.

2.2.5. Methods of chemical analyses

The lactic acid, volatile fatty acid, and alcohol concentration of the silage samples was measured using a liquid chromatograph (Biotronik, Wissenschaftliche Geräte GmbH, Germany). The type of column was Bio-Rad Aminex[®] HPX-874. Size 300 mm x 7.8 mm; Separation temperature 45 °C; Eluent: 0.005M H₂SO₄; Pump: flow rate: 0.85 ml/min.; Pressure: 77 kg/cm².

The pH value of silage samples was measured by electronic pH meter (type: OP-211/1, Radelkis Ltd., Budapest, Hungary) and the NH₃ content of it was determined by ammonium sensitive electrode (type: OP-264/2, Radelkis Ltd., Budapest, Hungary).

Dry matter, crude protein, ether extract, crude fibre and crude ash content of diets were measured according to Hungarian National Standard (Hungarian Feed Codex, 2004). The water soluble carbohydrate content of the above mentioned samples were analysed by the method of Somogyi (1952).

The energy content of the alfalfa, grass and silage samples was measured by bomb calorimeter, typ: C-2000 basic IKA (IKA-WERKE GmbH, Staufen, Germany).

2.2.6. Statistical analyses

The statistical analysis of the experimental results was performed by the single-factor analysis of variance (one way ANOVA) using the program SPSS 12.0 for Windows (SPSS Inc., Chicago, USA). For testing the homogeneity of variances the Levene test was used. Out of in the statistical program selectable post hoc tests, we used in case of homogeneous variances the LSD (if n numbers were identical) or the Scheffe (if the n numbers were different), while in case of heterogeneous variances the Dunnett's T3 test. Level of significance chosen was $P < 0.05$.

3. NEW SCIENTIFIC RESULTS

Based on the results of the investigations the following new scientific results can be stated:

1. Developed a combined enzymatic hydrolysis procedure by which 90 % of the corn starch can be broken down into reducing sugars within 20 hours.

2. Developed a procedure for the use of the ricotta whey, a by product of cheese making, as a silage aid.

3. Based on hydrolyzed corn and ricotta whey developed an efficient biological preservative for the preservation by ensiling of the medium and hard fermentable green forages.

4. In fermentation dynamic experiments determined the dry-matter dependent dose of preservative, by which from green alfalfa or grass a stable silage of low losses and of favorable lactic:acetic acid ratio can be produced.

4. LIST OF PUBLICATIONS IN THE THEME OF THE DISSERTATION

Scientific paper published in Hungarian language

1. Rigó E. – Zsedely E – Tóth T. – Schmidt J. (2010): Lucerna és fű silózása szénhidrát alapú biológiai tartósítószerrel. Állattenyésztés és Takarmányozás. 59. évf. 1.szám 45-56. o
2. Rigó E. – Zsedely E – Tóth T. – Schmidt J. (2010): Lucerna silózása biológiai tartósítószerrel. Takarmányozás. 59. évf. 2-3.szám 205-214.

Scientific paper published in foreign language

1. Rigó E.- Zsedely E.- Toth T. – Schmidt J. (2011): Ensiling alfalfa with hydrolyzed corn meal additive and bacterial inoculant. Acta Agronomica Óváriensis (accepted)
2. Rigó E. - Szigeti J. - Asvanyi B. - Zsedely E. - Schmidt J. (2012): The use of dried permeat powder for ensiling green alfalfa. Journal of Animal and Feed Sciences (submitted)

Oral presentations in Hungarian

1. Rigó E. : Zöldlucerna silózása második generációs biológiai tartósítószerrel. XIII. Ifjúsági Tudományos Fórum, Keszthely, 2007. március 22.(CD issue)
2. Rigó E. – Tóth T. – Schmidt J.: Második generációs biológiai tartósítószer kifejlesztése a zöldlucerna silózással történő tartósításához. XXXII. Óvári Tudományos Nap, Mosonmagyaróvár, 2008.október 09.
3. Rigó E. - Schmidt J.: Zöldlucerna silózása szénhidrátalapú biológiai tartósítószerrel. 51. Georgikon napok, Keszthely, 2009. október 01-02.

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1. Rigó E. -Tóth T. : Erjeszhető szénhidrát szubsztrát előállítása a kukorica enzimes hidrolízisével. – poszter- 50. Georgikon napok, Keszthely, 2008.szeptember 25-26.