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TECHNICAL NOTE 43.1

Improvement of the biodegradation efficiency by enzymatic treatment

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1. Introduction

The biodegradation efficiency of faecal material by an inoculum of autochtonous bacteria at thermophilic conditions (55°C) and pH 6.5 was equal to about 30%. It appeared that proteins were biodegraded for about 70% and fibrous material for only 10% (TN41.2). A major part of the non-biodegradable fraction of human faecal material consists of fibrous components. The most recalcitrant components are cellulose, xylan and lignin. Those components are plant material taken up by food and difficult to biodegrade by anaerobic bacteria.
Cellulose is biodegradable by a wide range of organisms, but the biodegradation efficiency is strongly dependent on the structure of the cellulose and the linkage with lignin and hemicellulose. It is known that in anaerobic conditions lignin is hardly biodegraded (TN 41.3).
The pretreatment of faecal material with enzymes can promote the biodegradation of those recalcitrant components.
Enzymes are catalysts and are used to accelerate the rate of a chemical reaction. All enzymes are specific which means they catalyse only one reaction. Therefore in most cases only one specific enzyme can biodegrade one particular substrate.
This technical note reports the results of the improvement of the cellulose- and xylan degradation by anaerobic bacteria after the addition of cellulase and xylanase. These enzymes were selected because they were commercial available.

2. Set-up of enzyme test

2.1 Incubation of substrates with their enzymes

2.1.1 Introduction

The concentration of an enzyme, used to attack a substrate, is in most cases much lower than the concentration of the substrate. Temperature, pH and incubation time are important factors for the activity of the enzyme. The optimal temperature for both enzymes is about 37°C. At this temperature high activities and reaction rates are measured. Enzymes are only active at small pH ranges. The pH, at which the highest activity is reached, is called the optimal pH.

2.1.2 Set-up

Two artificial substrates were separately incubated with their corresponding enzyme. All substrates and enzymes were obtained from Fluka (Sigma-Aldrich company).

Cellulose: powder; medium fibrous

Cellulase: powder from Aspergillus niger

Xylan: powder from oat -spelt

Xylanase: powder from Trichoderma viride

* cellulose / cellulase solution: 30 ml cellulose solution (4.35g/l) was incubated with 20 ml cellulase solution (4mg/l) at a temperature of 37°C and an optimal pH of 4.8.

* xylan / xylanase solution: 30 ml xylan solution (4.35g/l) was incubated with 20 ml xylanase solution (4mg/l) at a temperature of 37°C and an optimal pH of 5.4.

Both solution were buffered with 30 ml acetate buffer 0.2M to obtain a constant pH during the 4-days incubation.

To obtain a final substrate concentration of 1 g/l a begin concentration of 4.35 g/l was necessary taking into account the dilutions. This final concentration is realistic with Melissa substrate, where 30 % of the organic matter (average value of 3g/l) consists of cellulose.

2.2 Incubation of substrate / enzyme solution with anaerobic medium and inoculum

2.2.1 Introduction

The effect of the enzymes on the biodegradability of cellulose and xylan was investigated using the pretreated substrate, an anaerobic thermophilic inoculum and an anaerobic medium (composition is represented in addendum 1). The inoculum was taken from the MELISSA demonstration reactor 2 (pH 8). An anaerobic medium was used to provide the bacteria the necessary elements for surviving and growth. Also blanc tests and a test with starch, which can be considered as 100% biodegradable by the inoculum, were set-up in order to compare all the results.

2.2.2 Description of the experiment

Separate tests were carried out for xylan and cellulose. The tests were performed in small flasks of 110 ml. To avoid contamination the tests were prepared under sterile conditions. Flasks, pipettes, water and needles were autoclaved. Each test was incubated at a temperature of 55°C and the pH was set at 7. The same temperature and pH are used in the MELISSA demonstration reactor. Several tests were set-up in order to compare the results. The composition of the different tests are shown in Table 2-1.

Table 2-1 Flask content of the different tests expressed as amounts (ml) of different solutions used

Test	*Total volume*	*Inoculum*	*Anaerobic medium*	*Pretreated substrate*	*substrate 1 g/l*	*Starch 1g/l*	*Buffer solution*	*H_2O*
	ml	ml	ml	Ml	ml	ml	ml	ml
. Blanc: inoculum $^-$, pretreated substrate$^+$	60	0	20	35	0 (2)	0	0 (1)	5
. Blanc: inoculum $^+$, pretrated substrate $^-$	60	5	20	0	0	0	13	22
. Blanc: inoculum$^-$, pretreated substrate$^-$, substrate$^-$	60	0	20	0	0	0	13	27
. inoculum $^+$, substrate$^+$	60	5	20	0	22	0	13	0
. inoculum $^+$, pretreated substrate$^+$	60	5	20	35	0 (2)	0	0 (1)	0
. inoculum $^+$, starch $^+$	60	5	20	0	0	22	13	0

with substrate: cellulose or xylan
enzyme: cellulase or xylanase
(1) Buffer solution already in pretreated substrate
(2) Substrate already in pretreated substrate

Each test was set-up in quadruple. All the flasks were flushed with a gas containing 70% N_2 and 30% CO_2 to obtain anaerobic conditions. All flasks were regularly shaken. The total reported test period was 18 days.

During this period pressure in the flasks was frequently measured and at day 18 pH, VFA and gas composition were analysed.

3. Results

3.1 Pressure

3.1.1 Cellulose / cellulase

The evolution of the pressure built-up during the experiment is represented in Figure 3-1. The gas production of the test ' **inoculum$^+$, pretreated cellulose$^+$**' was high until day 11. From day 13 the pressure of the test '**inoculum$^+$, cellulose$^+$** ' increased with high rates. The increase of gas at day 13 was due to the transformation of acetic acid, present in the buffer, into gas. This fenomenon can also be noticed in the test '**blanc inoculum$^+$, pretreated cellulose$^-$**'. Both ' **blanc inoculum$^-$, pretreated cellulose$^+$**' and '**blanc inoculum$^-$, pretreated cellulose$^-$, cellulose$^-$**' had low pressure values. The gas production found in those two tests was of chemical origin. To obtain a chemical equilibrium between the gas- and liquid phase, CO_2 will evaporate and therefore the pressure in the bottles will increase.

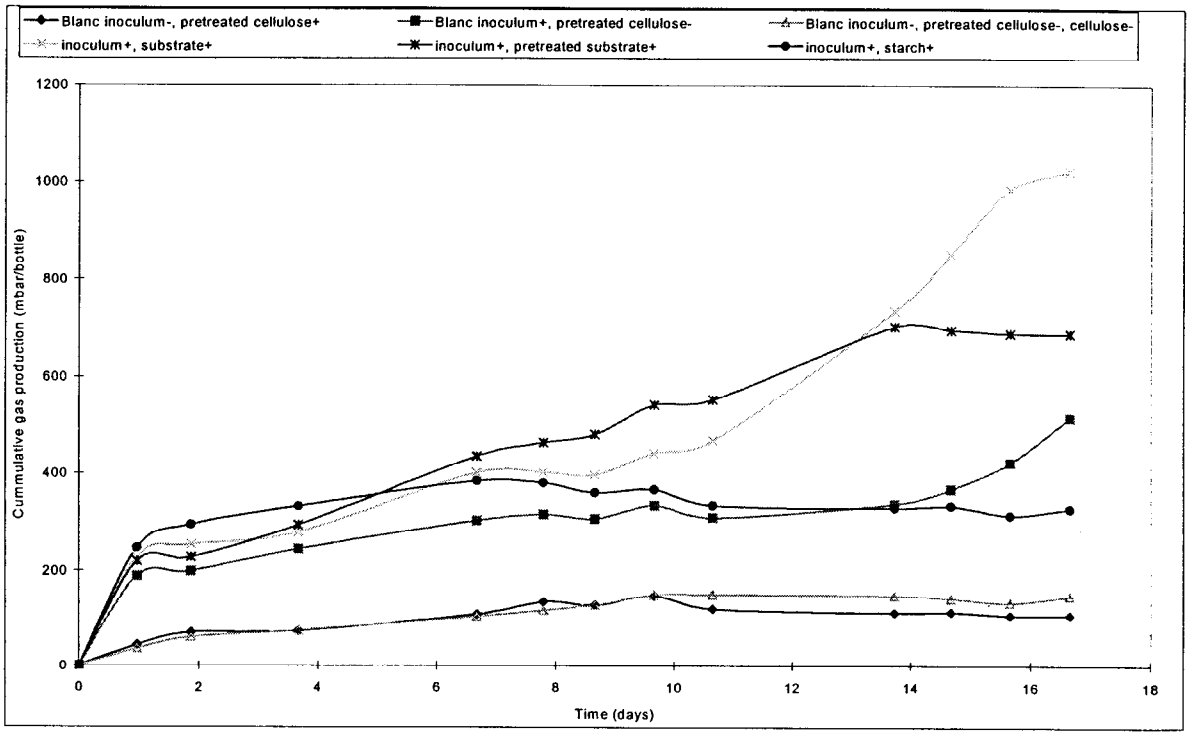

Figure 3-1Cumulative gas production of cellulose / cellulase experiment

3.1.2 Xylan / xylanase

The evolution of the pressure built-up is shown in Figure 3-2. The gas production of the test '**inoculum$^+$, pretreated xylan$^+$**' was significant higher than the other tests. Both '**blanc inoculum$^-$, pretreated xylan$^+$**' and test '**blanc inoculum$^-$, pretreated xylan$^-$, xylan$^-$**' had low pressure readings. The gas production found in those two tests was of chemical origin. To obtain a chemical equilibrium CO_2 evaporated.

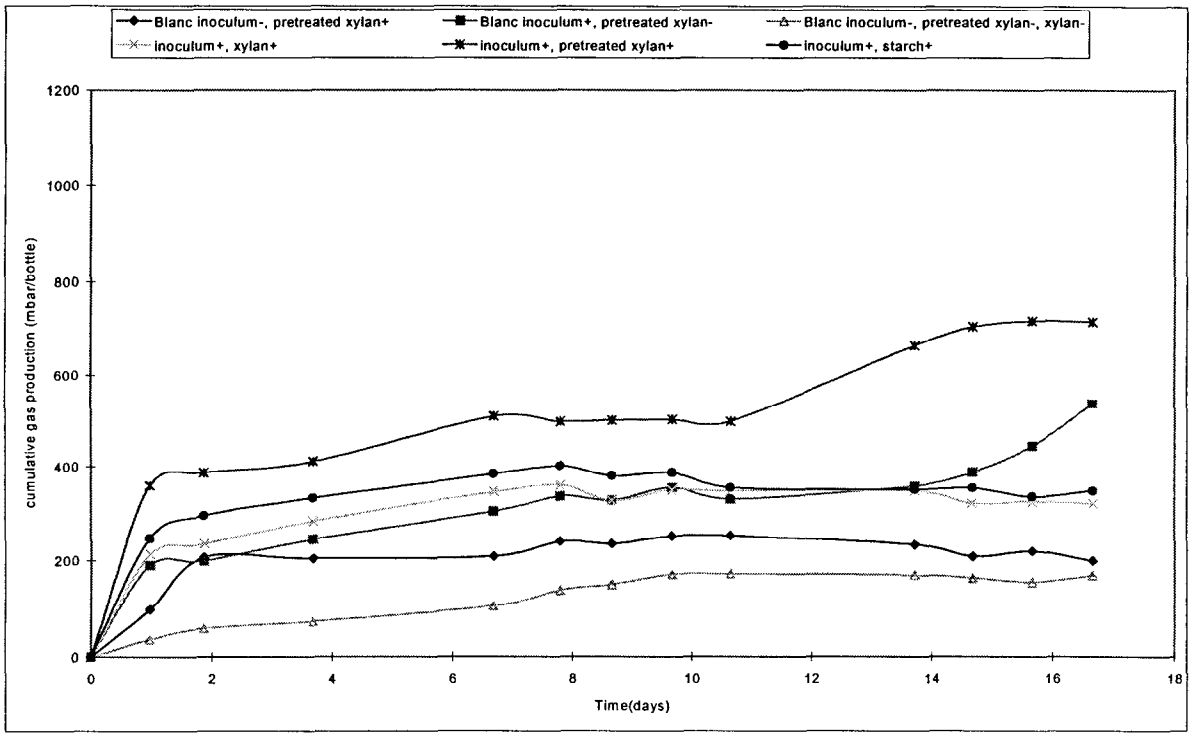

Figure 3-2 Cumulative gas production of the xylan / xylanase experiment

3.2 Volatile fatty acids production

The buffer present in every flask consisted of 2600 mg/l acetic acid. This means that volatile fatty acids were already present from the start. This value needed to be taken into account for further calculations.

3.2.1 Cellulose / cellulase

The composition of the volatile fatty acids are represented in Figure 3-3. VFA were found in all the different tests. Those VFA were acetic acid, propionic acid, butyric acid, isobutyric acid and isovaleric acid. A concentration of 2600 mg/l acetic acid from the buffer was present from the start and therefore the VFA concentrations found for the test '**blanc inoculum$^-$, pretreated substrate$^+$**' and '**blanc inoculum$^-$, pretreated substrate$^-$, substrate$^-$**' can be explained.

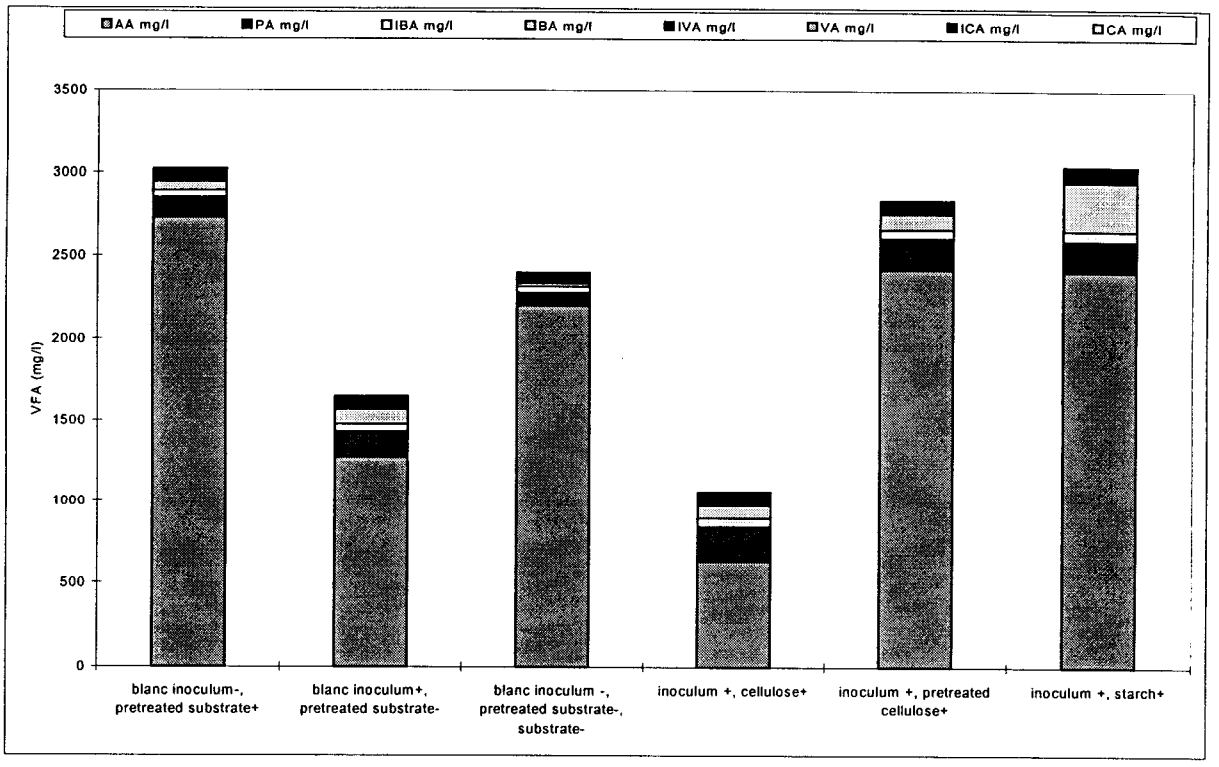

Figure 3-3 VFA composition of the cellulose / cellulase experiment

3.2.2 Xylan / xylanase

The volatile fatty acids composition of the xylan / xylanase test is shown in Figure 3-4. The amount of VFA, except for AA which was present from the start, was higher for the tests '**inoculum$^+$, xylan$^+$**' , '**inoculum$^+$, pretreated xylan$^+$**' and '**inoculum$^+$, starch$^+$**'.

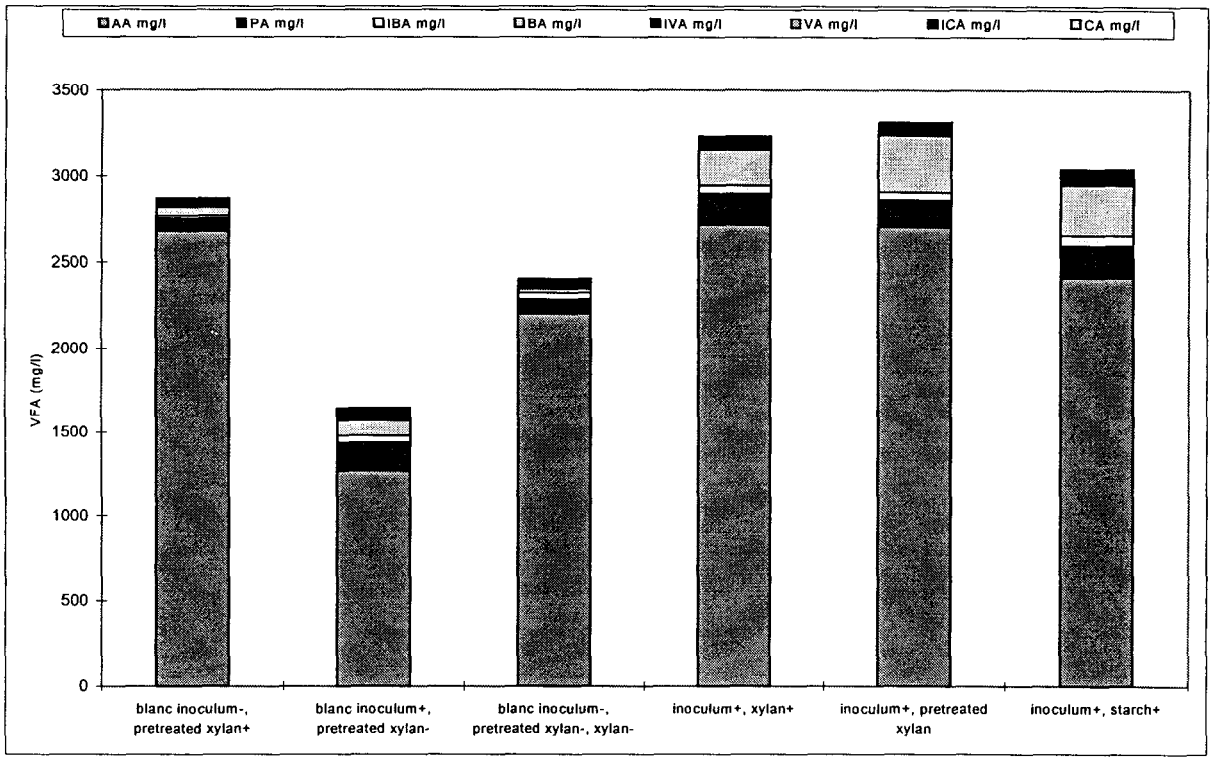

Figure 3-4 VFA composition of the xylan / xylanase experiment

3.3 Gas production

From the results with the gas analyser can be concluded that no methane was found in the tests '**blanc inoculum$^-$, pretreated substrate$^+$**' and '**blanc inoculum$^-$, pretreated substrate$^-$, substrate$^-$**'. In the tests '**inoculum $^+$, substrate $^+$**' and '**inoculum$^+$, starch$^+$**' 100% methane was found. The test '**inoculum$^+$, pretreated substrate$^+$**' contained 65% methane and 35% CO_2 for the cellulose tests and 53% methane and 47% CO_2 for the xylan test.

When pH decreases, due to acidification, occur, the CO_2 in the solution evaporates. The acidification is of non-biological nature and is caused by the enzymes. It was most pronounced in the xylan test ' **blanc inoculum$^-$, pretreated substrate$^+$**'. Therefore it can be concluded that the pressure built-up in the test '**blanc inoculum$^-$, pretreated substrate$^+$**' was of non-biological nature. The results of the test '**blanc inoculum$^-$, pretreated substrate$^+$**' needed to be subtracted from the results of the test '**inoculum$^+$, pretreated substrate$^+$**' to avoid false results due to gas production of non- biological nature.

Table 3-1 Final pH values of both tests

Test (pH set-point:7)	*Cellulose Test*	*Xylan Test*
	pH	**pH**
Blanc inoculum$^-$, pretreated substrate$^+$ 1	6.1	5.5
Blanc inoculum$^-$, pretreated substrate$^+$ 2	6.1	5.5
Blanc inoculum$^-$, pretreated substrate$^+$ 3	6.0	5.5
Blanc inoculum$^+$, pretreated substrate$^-$ 1	6.5	6.5
Blanc inoculum$^+$, pretreated substrate$^-$ 2	6.5	6.5
Blanc inoculum$^+$, pretreated substrate$^-$ 3	6.4	6.4
Blanc inoculum$^-$, pretreated substrate$^-$, substrate$^-$ 1	6.2	6.2
Blanc inoculum$^-$, pretreated substrate$^-$, substrate$^-$ 2	6.1	6.1
Blanc inoculum$^-$, pretreated substrate$^-$, substrate$^-$ 3	6.1	6.1
inoculum$^+$, substrate$^+$ 1	6.5	5.8
inoculum$^+$, substrate$^+$ 2	6.5	5.9
inoculum$^+$, substrate$^+$ 3	6.6	5.8
inoculum$^+$, pretreated substrate$^+$ 1	6.1	5.8
inoculum$^+$, pretreated substrate$^+$ 2	6	5.9
inoculum$^+$, pretreated substrate$^+$ 3	6.1	6
inoculum$^+$, starch$^+$ 1	5.7	5.7
inoculum$^+$, starch$^+$ 2	5.7	5.7
inoculum$^+$, starch$^+$ 3	5.8	5.8

4. Mass balance Calculations and Discussion

4.1 Volatile fatty acids + CH_4 production

4.1.1 Introduction

The biodegradation of organic material such as cellulose results in a production of CH_4, CO_2 and volatile fatty acids. The sum of those components is necessary in order to have an idea about the amount of biodegraded material. VFA and gas production needed to be transformed into mg/l VFA produced.

At the beginning of the tests buffer (0.2 M or 12g/l acetic acid) was added. After dilution, caused by the addition of the other components (Table 2-1), the final concentration of the buffer in the different flasks was 2600 mg/l. This value was taken into account when mass balance calculations for final volatile fatty acids production were made.

4.1.2 Calculations in mg/l volatile fatty acids

Based on the stochiometric model by Anglidaki, it is assumed that the biodegradation of 1 g OM results in a production of 1 g volatile fatty acids and 1 g of VFA can be converted into 1 g of biogas (TN34.1; Anglidaki, 1993). When this occurs it is assumed that 100% degradation is obtained.

4.1.2.1 Cellulose / cellulase

In Figure 4-1 can be seen that the VFA and CH_4 production could be neglected for the tests '**blanc inoculum$^-$, pretreated substrate$^-$, substrate$^-$**'. This means that no methane and VFA were produced. The negative amount found was due to small measurement-errors. The amount of VFA and CH_4 found for the test '**blanc inoculum$^+$, pretreated substrate$^-$**' was due to the transformation by the inoculum of acetic acid, present from the start, into gas. Therefore this 181 mg/l VFA needed to be subtracted from the results of the tests '**inoculum$^+$, substrate$^+$**', '**inoculum$^+$, pretreated substrate$^+$**' and '**inoculum$^+$, starch$^+$**'. This taken into account, a final 93% starch degradation and 96% cellulose degradation when cellulose was pretreated with cellulase was found. Without the addition of cellulase a cellulose biodegradation efficiency of 65% was obtained.

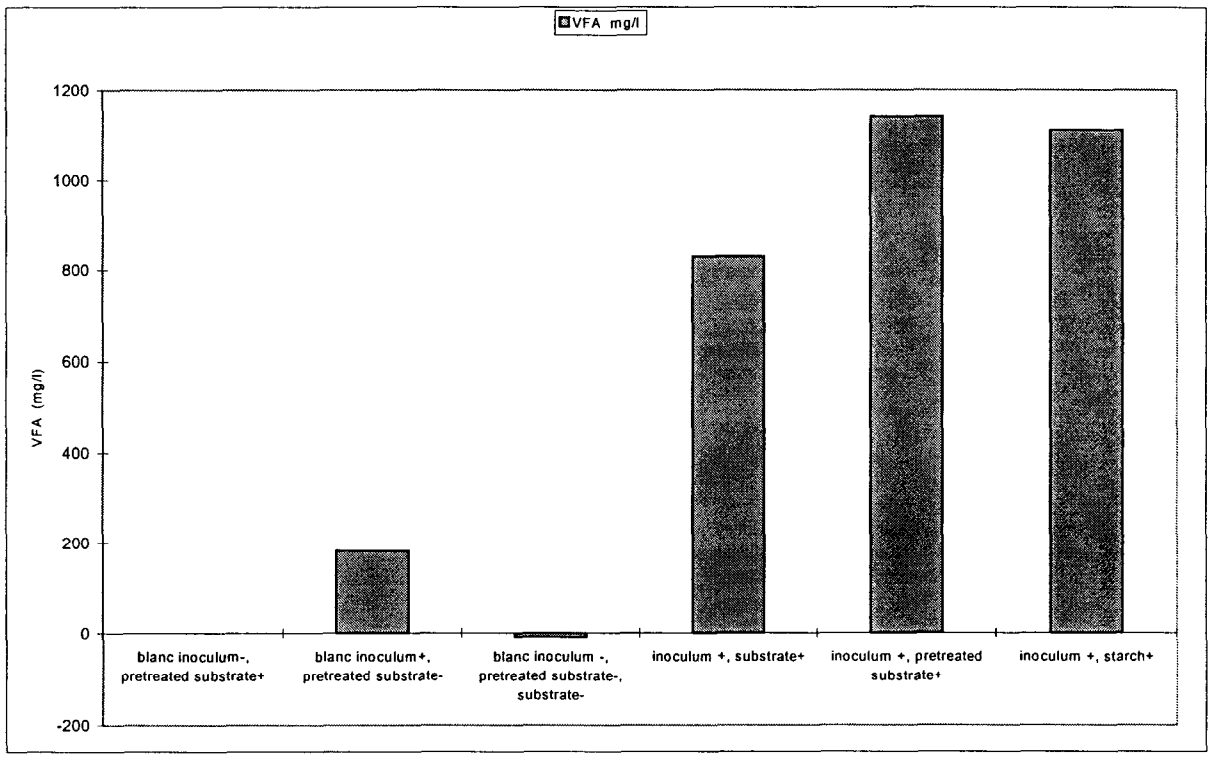

Figure 4-1 Cellulose / cellulase: Biological VFA and CH_4 production (in mg/l VFA)

4.1.2.2 Xylan / xylanase

The results from the tests '**blanc inoculum$^-$, pretreated substrate$^-$, substrate$^-$**' represented in Figure 4-2 can be neglected. The same reasoning as previous can be made for the test '**blanc inoculum$^+$, pretreated substrate$^-$**'. Therefore 181 mg/l found for this test need to be substracted from the results of the tests '**inoculum$^+$, substrate$^+$**', '**inoculum$^+$, pretreated substrate$^+$**' and '**inoculum$^+$, starch$^+$**'. The degradation of xylan in the test '**inoculum$^+$, substrate$^+$**' was 100% which means that without the addition of enzymes powdered xylan was fully biodegraded by the inoculum.

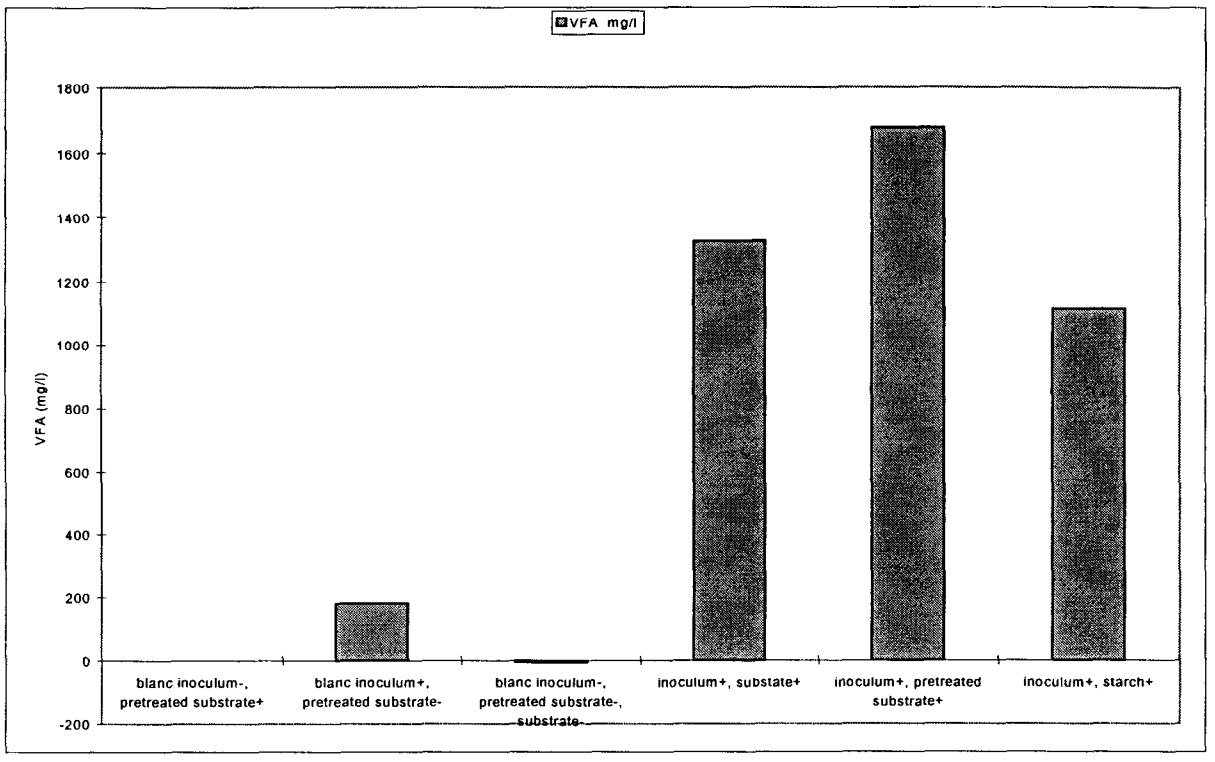

Figure 4-2 Xylan / xylanase: Biological VFA and CH4 production (in mg/l VFA)

5. Conclusions

The biodegradation of fibrous components in faecal material during anaerobic conditions is difficult. Especially cellulose, lignin and xylan are hard to biodegrade. It is assumed that the pretreatment of faecal material with enzymes in aerobic conditions stimulates the degradation of the recalcitrant components.
Enzymes were separately incubated with their corresponding artificial substrates for 4 days at a temperature of 37°C and a pH dependent on the enzyme. The artificial substrates were, unlike the cellulose and xylan in the faecal material, powders. The pH and temperature are of major importance for the enzyme. Deviations can inactivate the enzyme. After this 4-days of incubation the enzyme/ substrate solution was incubated with inoculum from the MELISSA reactor and anaerobic medium. Powdered cellulose was biodegraded for 65%. The addition of cellulase stimulated the cellulose degradation, what resulted in a biodegradation of 96%. This resulted in a higher VFA and gas production in comparison with the test without the enzymes. The xylan test was less pronounced. Powdered xylan was biodegraded for 100% by the inoculum and without the addition of enzymes. The gas production in both '**blanc inoculum-, pretreated substrate+**' and '**blanc inoculum-, pretreated substrate-, substrate-**' was CO_2 and due to adjustment of the chemical equilibrium.

The same test procedures will be carried out with both cellulase and xylanase at the same time in order to find out if there actions are compatible. The same concentrations of enzymes and artificial substrate will be used. For the incubation of the enzymes with there substrates an average pH of 5.1 and a temperature of 37°C will be applied.

Lignin, one of the most recalcitrant components, can be biodegraded by laccase and peroxidase. Laccase is an enzyme which can directly oxidize phenolic substrates and lignin units. The peroxidase can be replaced with a Fetons reagent, containing $FeSO_4$ and H_2O_2. This reagent reacts with the non-biodegraded organic matter. The fetons reagent will be incubated with lignin, from woody material, at a pH of 3 to 4 in order to prevent sedimentation. After this incubation laccase will be added at a pH of 6.5.

Fibrous material containing cellulose and xylan are biodegraded for only 10 % in the MELISSA demonstration reactor. The cellulose and xylan are, unlike the commercial powders, more persistent. The addition of cellulase and xylanase to the MELISSA demonstration reactor will investigate if the enzymes will stimulate the biodegradation of those recalcitrant components and if higher biodegradation efficiencies will arise. Therefore a second reactor will be combined with the first MELISSA reactor. Effluent form the first reactor will be centrifuged. The cake will be pretreated with enzymes for 2 days at a temperature of 37°C and a pH of 5.1. After this incubation the pH will be set at 6.8 and the treated cake will be fed into the second reactor. VFA and gas production will be followed up.

6. References

Anglidaki, I. and Ahring, B.K. (1993). A mathematical model for the dynamic simulation of anaerobic digestion of complex substrates: focusing on ammonia inhibition. Biotechnology and Bioengineering, 42, 159-166.

Addendum 1 Composition of anaerobic medium

Anaerobic medium (1 litre)	
Yeast extract	0.4g
Tripticase peptone	0.4g
Resazurine	0.5 ml
0.2 g in 100 ml a.d.	
Solution A in 1 litre a.d.	10 ml
100 g NH_4Cl	
100 g $MgCl_2.2H_2O$	
40 g $CaCl_2.2H_2O$	
Final pH: 4	
Solution B in 1 l a.d.	2 ml
200 g $K_2HPO_4.3H_2O$	
Trace elements in 1 litre a.d.	10 ml
500mg Na2EDTA.2H2O	
150 mg CoCl2.6H2O	
100 mg MnCl2.4H2O	
100 mg FeSO4.7H2O	
100 mg ZnCl2	
40 mg AlCl3.6H2O	
30 mg Na2Wo4.2H2O	
20 mg CuCl2.2H2O	
20 mg NiSO4.6H2O	
10 mg H3BO3	
10 mg NaMoO4.2H2O	
Na_2S (2.5%)	5 ml
2.52 g/50 ml NaHCO3	16.6 ml
Final pH: 7	