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ESA/YCL Contract 8125/NL/FG

The proteolytic performances of each selected strain on MELISSA substrate (rat faeces)

TN 15.4

september 1993

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1 INTRODUCTION

Extensive degradation of proteins is essential in the first compartment of the MELISSA-cycle as the protein-fraction is a pool for nitrogen, sulfur and carbon and the crude protein content of faeces is estimated to be about 20 to 30 % of the dry matter.

Thermophylic organisms are needed to break down the proteins. As Clostridium thermosaccharolyticum and Clostridium thermocellum are not proteolytic, thermophilic proteolytic anaerobic strains were isolated. Strains I8, I12 and I25 were isolated from a Dry Anaerobic Composting process (DRANCO). Strain I29 was isolated from aerobically treated compost. - Strain I8 proved to have the best protein degradation capacities. Therefore this study was focussed on I8; the growth on preconditioned rat faeces (MELISSA test substrate) was examined.

2 MATERIALS AND METHODS

Rat Faeces

The rat faeces used in this study were provided by TNO Leiden via ESTEC. The faeces used in this this study were produced by rats, fed with Spirulina. The C-content of the different samples (varying amount of Spirulina in the diet) was determined.
Before starting the growth experiments, the samples were homogenized (grinded) and stored at - 20°C.

Inoculum

I8 was isolated from DRANCO material and grown under anaerobic conditions at 60 °C. In previous technical notes the isolation procedures were discribed. The composition of the medium used, is presented in Table 1. The medium was selected based on the results of growth experiments (Kersters, 1992).

Analytical techniques

Volatile fatty acids were extracted with diethylether from acidified samples and

determined by gas chromatography using an flame ionisation detector coupled to a glass column containing chromosorb 101.

Protein concentrations were measured using a a modified Lowry dye-binding protein assay (Lowry et al., 1951).

The NH_4^+-N-content was determined by steam destillation in a Kjeltec.1002 apparatus under alkaline conditions. Kjeldahl-N was determined similarly after complete destruction of the sample in strong acid.

The carbon content of the rat faeces was determined after destruction in strong acid and oxidation by bichromate.

Description of the experiments

Homogenized rat faeces were suspended in 100 ml of distilled water. The headspace of the penicillin bottles were flushed with CO_2 in order to remove oxygen from the liquid phase. Before inoculation with I8, the bottles were autoclaved. Ammonium-production and VFA-production were used as a measure for protein degradation. In a first batch test a comparison was made between I8 and Thermobacteroides proteolyticus. Secondly, an experiment was set up to study the effect of the amount of inoculum on the proteolytic performances. In the last test the influence of the amount of rat faeces on the protein degradation was examined.

Table 1. Composition of the medium used for the cultivation of I8

NaOH		4 g/l
yeast extract		2 g/l
trypticase pepton		2 g/l
resazurin solution (0.2 %)		0.5 ml/l
coenzyme M		0.5 g/l
Solution A		10 ml/l
NH_4Cl	100 g/l	
$MgCl_2.6H_2O$	100 g/l	
$CaCl_2.2H_2O$	40 g/l	
pH	4	
Solution B		2 ml/l
$K_2HPO_4.3H_2O$		
Mineral solution		10 ml/l
$Na_2EDTA.2H_2O$	500 mg/l	
$CoCl_2.6H_2O$	150 mg/l	
$MnCl_2.4H_2O$	100 mg/l	
$FeSO_4.7H_2O$	100 mg/l	
$ZnCl_2$	100 mg/l	
$AlCl_3.6H_2O$	40 mg/l	
$Na_2Wo_4.2H_2O$	30 mg/l	
$CuCl_2.2H_2O$	20 mg/l	
$NiSO_4.6H_2O$	20 mg/l	
H_2SeO_3	10 mg/l	
H_3BO_3	10 mg/l	
$NaMoO_4.2H_2O$	10 mg/l	
Na_2S (2.5 %)		20 ml/l
gelatin (3 %)		100 ml/l
final pH		7

3 RESULTS

3.1 Characterisation of rat faeces

Dry matter, carbon content and kjeldahl-nitrogen of six types of faeces were determined. The diet of the rats determined the composition of the faeces. In Table 2 the results of the analyses are presented.

Table 2. Characterisation of rat faeces according to the diet

Type	Dry Weight (%)	Carbon-content (%)	Kjeldahl-N (%)
1. Pellet Cont.	95	62.3	4.06
2. Purified Cont.	94	60.0	2.75
3. Deficient diet	94	59.0	2.08
4. 5% Spirulina	95	58.7	2.87
5. 10 % Spirulina	94	57.9	3.44
6. 40 % Spirulina	94	59.0	4.09

The carbon-content of the samples was about 60 %. Only small variations were noticed. However the amount of kjeldahl-nitrogen varied from 2 to 4 %. The rats with a diet of 40% Spirulina produced faeces with the highest nitrogen fraction. For that reason this type was selected for further experiments. The dry weight of all the samples was about 95 %.

3.2 Comparison of I8 and Thermobacteroides proteolyticus

Five gram of rat faeces was suspended in 100 ml water and inoculated with 2 ml (2 % inoculum) of a culture of I8 or Thermobacteroides proteolyticus. The penicillin bottles were incubated at 60 °C and at regular intervals, samples were taken for analyses.
It should be remarked that it was difficult to take homogenous samples of the mixed liquor; during incubation the suspended rat faeces sedimented and sticked to the bottom of the penicillin bottles. It was not possible to resuspend all the solids by shaking.
The experiment was terminated after 25 days. The ammonium- and VFA-production is plotted versus time for the two micro organisms in Figure 1. Although Thermobacteroides proteolyticus seemed to produce more fatty acids during the first days, the curves of VFA-production

by the two organisms were quite similar.

The VFA-pattern at the beginning and the end of the experiment is shown in Table 3. It should be remarked that in all the results the amount of capronic acid was not included due to the presence of capronic acid originating from the internal standard (break down product).

Table 3. Comparison of VFA-production patern by I8 and T. proteolyticus

Volatile fatty acids (mg/l)	I8 t=0 d	I8 t=25 d	T. proteolyticus t=0 d	T. proteolyticus t=25 d
Acetic acid	215	1675	214	2316
Propionic acid	66	262	51	185
Isobutyric acid	22	106	9	103
Butyric acid	62	61	46	56
Isovaleric acid	57	288	36	234
Valeric acid	22	24	11	13
Isocapronic acid	16	15	0	0
Total	460	2434	367	2907

The higher amount of fatty acids formed by T. proteolyticus (1974 mg/l vs. 2540 mg/l) was mainly due to the higher production of acetic acid. Acetic acid was the main metabolite for both strains. Propionic acid, isobutyric acid and isovaleric acid were also important fractions.

There was almost no difference in the ammoniumproduction for the two strains. I8 produced 271 mg NH_4^+-N/l while T. proteolyticus produced 241 mg N/l.

Protein- and kjeldahl-N-analyses proved to be inaccurate, probably due to the difficulties of sampling (results not shown).

3.3. Effect of inoculum volume of I8 on ammonification and VFA production

Hundred milliliters of mixed liquor (water + 5 g rat faeces) were inoculated with respectively 0.5, 2, 5, and 10 ml of a culture of I8 and incubated at 60 ° C during 21 days. Fig. 2 shows the production of volatile fatty acids versus time. In Fig. 3 the ammonification of proteins is presented.

Fig.1. Fermentation of rat faeces (RF) by I8
I8 vs T. proteolyticus
Ammonium-production/VFA-production

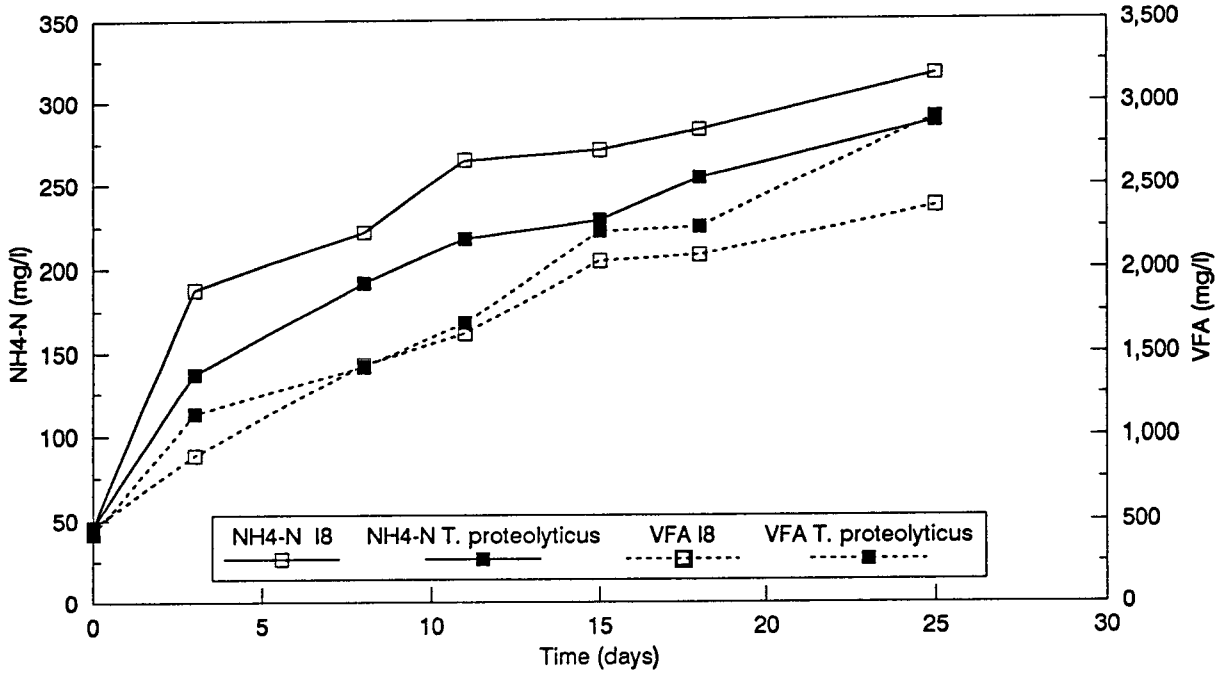
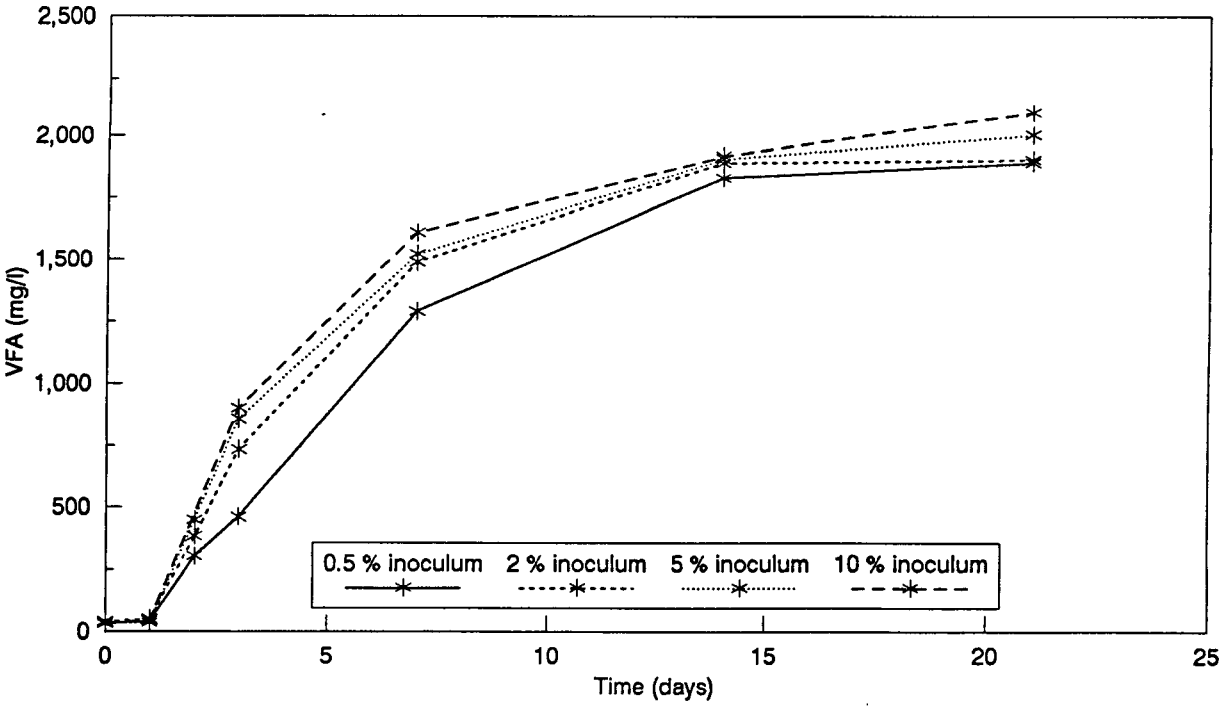


Fig.2. Fermentation of rat faeces (RF) by I8
Effect of inoculation volume on VFA-production



In all experiments a lag-phase of a day could be noticed. The VFA-production increased suddenly after one day incubation at 60 °C. The amount of inoculum volume seemed to have little influence on the fermentation pattern. The initial VFA-production rate increased (not linearly) with the volume of inoculum used. The VFA production rate was 261.3 mg/l.d, 331.8 mg/l.d, 415 mg l/d and 431.7 mg/l.d for 0.5 %, 2 %, 5 % and 10 % inoculum volume respectively. In Table 4 the net VFA-production between the start-up and day 21 of the different series is presented. There was only a 10 % difference of VFA-production between 0.5 and 10 % inoculum.

If only 0.5 % inoculum was used, the lag-phase for ammonium production amounted to two days. The maximum production rate (34.6 mg NH_4^+-N/l.d) occurred after day three. For the series with 2 and 5 % inoculum, the lag-phase was only one day while the maximum production-rate occured between day two and three (53.8 mg NH_4^+-N/l.d and 66.2 mg NH_4^+-N/l.d). In the bottle with 10 % inoculum there was only a small increase in production rate from day one to day three.

Tabel 4. Effect of inoculum volume (I8) on net VFA-production

Volatile fatty acids (mg/l)	0.5 % I8 t=21 d	2 % I8 t=21 d	5 % I8 t=21 d	10 % I8 t=21 d
Acetic acid	1356	1382	1467	1545
Propionic acid	199	146	201	198
Isobutyric acid	76	75	77	86
Butyric acid	20	59	21	44
Isovaleric acid	215	210	205	205
Valeric acid	0	0	0	0
Isocapronic acid	0	0	0	0
Total	1866	1872	1971	2078

3.4. Effect of concentration of rat faeces on ammonification and VFA-production

Three different amounts of homogenized rat faeces (1 g, 2.5 g and 5 g) were suspended in 100 ml water. After sterilisation, the mixed liquor was inoculated with 2 ml of a culture of I8 and incubated during 27 days at 60° C.

In Fig. 4 the VFA-production versus time in the different samples is plotted. In Fig. 5 the ammonium production is shown. In Table 5 the net VFA production at day 27 is presented.

Fig.3. Fermentation of rat faeces (RF) by I8
Effect of inoculation volume on ammonification

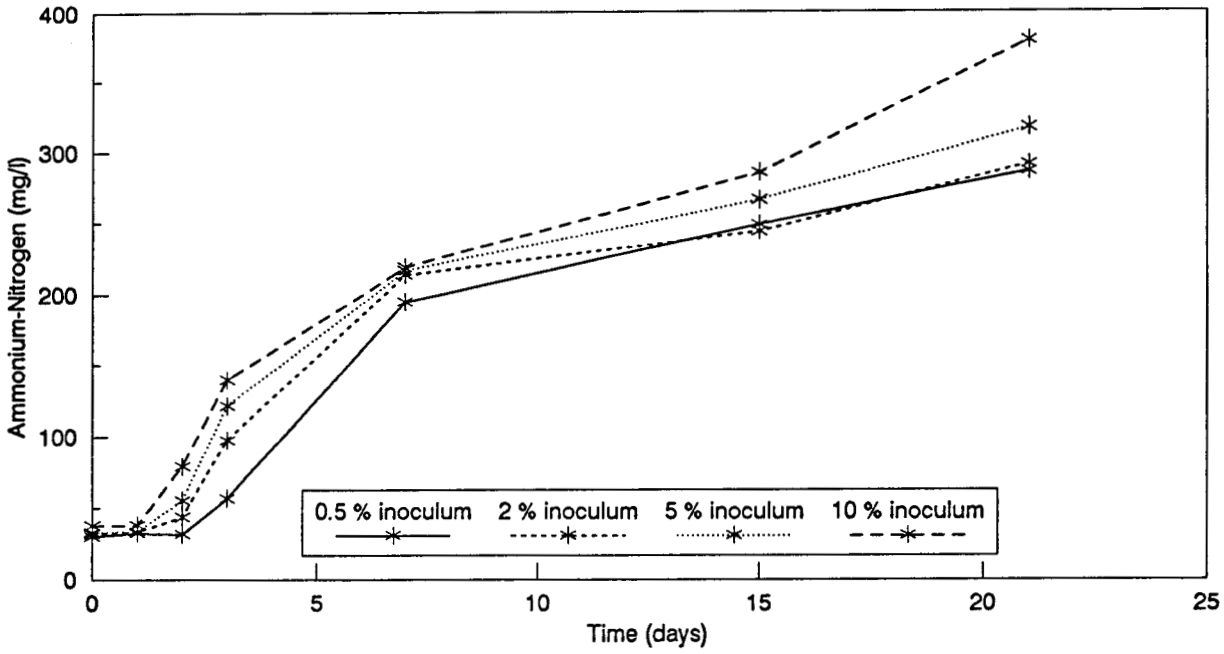


Fig. 4. Fermentation of rat faeces by I8
Effect of concentration on VFA-production

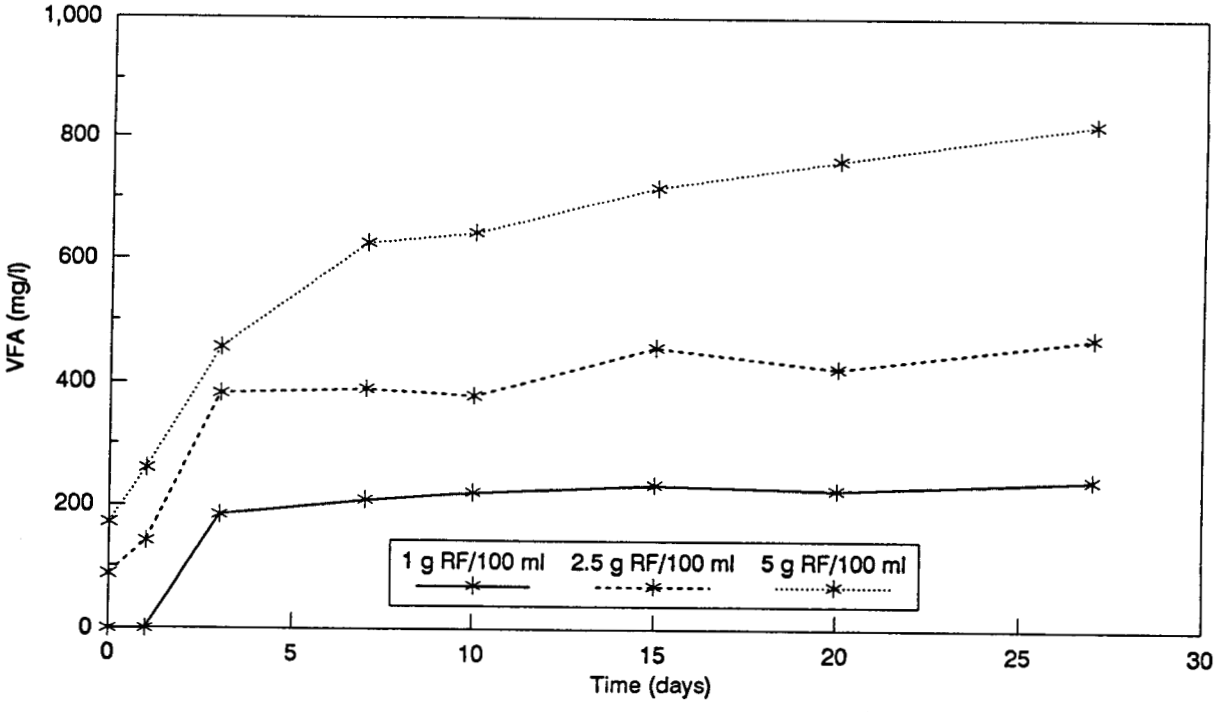


Table 5. Net VFA-production over 27 days in a suspension of rat faeces at different solids concentrations (1g/100 ml, 2.5 g/100 ml, 5 g/100 ml). The concentration of the different fatty acids is given.

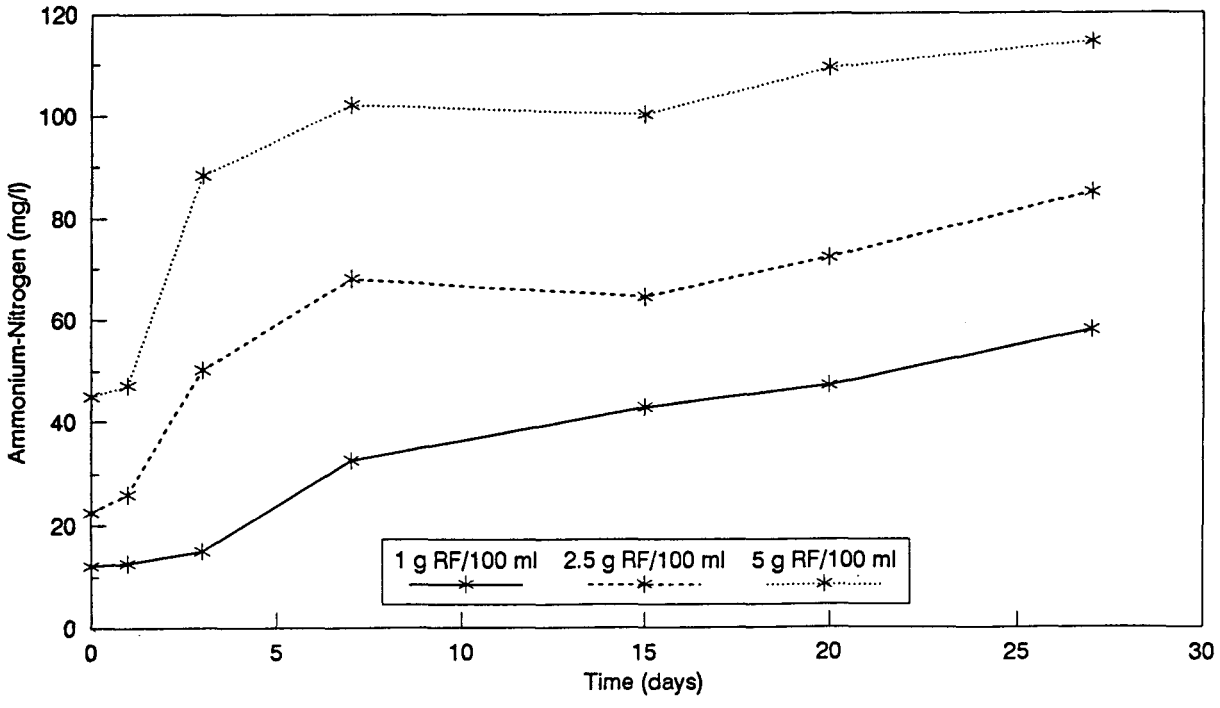
Volatile fatty acids (mg/l)	1 g RF t=27 d	2.5 g RF t=27 d	5 g RF t=27 d
Acetic acid	140	221	365
Propionic acid	33	52	81
Isobutyric acid	19	33	54
Butyric acid	6	2	24
Isovaleric acid	41	68	156
Valeric acid	4	0	0
Isocapronic acid	0	10	0
Total	243	386	680

There was a considerable increase in VFA-production with the amount of RF in the mixed liquor. The concentration of acetic acid after 27 days in a 1 % suspension was 140 mg/l compared to 365 mg/l in a 5 % suspension. The pattern of VFA-production was quite similar in the four tests. From these results the importance of availability of the substrate can be deducted.

The net production of NH_4^+-N varied from 46 mg/l over 63 mg/l to 70 mg/l for a 1 %, 2.5 % and 5 % suspension respectively.

The overall VFA- and ammonium-concentration was low for this experiment compared to the previous results. Thus a repitition of this experiment can be taken in consideration.

Fig 5. Fermentation of rat faeces by I8
Effect of concentration on ammonification



4. DISCUSSION

The experiments show that strain I8 could break down part of the proteins present in RF. There seemed to be not much difference between I8 and T. proteolyticus in ammoniumproduction, although the VFA production of the two strains varied more. This was mainly due to the higher acetic acid production of T. proteolyticus from the start till day three.

By using a larger inoculum the lag-phase was shorter and the initial protein degradation rate was higher. After 21 days there was only a 10 % difference in total VFA-concentration between the bottle with the smallest inoculum (0.5 %) and the bottle with the largest inoculum (10 %).

An increase in solids (rat faeces) in the bottles increased the amount of available substrate which improved the degradation of proteins.

The carbon content of the rat faeces was about 60 % so the initial C-concentration in the penicillin-bottles (5 g RF/100 ml) was about 30 g C/l. At the end of the experiments maximum 2-3 g VFA were produced. The average carbon content of the produced VFA is about 50 % (this can be calculated from the amount of the produced fatty acids produced and the C-content of each fatty acid). So maximum 3-5 % (1 - 1.5 g/l) of the added carbon was converted to fatty acids.

The nitrogen input in the system was 2 g Kj-N/l. During the test period maximum 300 mg NH_4^+-N/l was produced. This comprises 15 % of the total N.

5. FURTHER STUDY OBJECTIVES

The test on the influence of the amount of rat faeces has to be repeated.

6. REFERENCES

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