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MELISSA
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TN15.5
Protein degradation in the MELISSA substrate (rat faeces)
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INTRODUCTION

In TN15.4 was described what the influence was of the inoculum volume and the concentration of rat faeces on the growth of I8 on a suspension of rat faeces in anaerobic thermophilic conditions. The efficiency of breakdown proved to be quite low.
In this study we tried to improve the degradation and to learn more about the behaviour of the degradation of the proteins.

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- and N-content of the faeces produced by rats which were fed with Spirulina (40 % of total diet) were 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 described. MS-medium (Boone, 1987) was used as a cultivation medium. 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 ionization detector coupled to a glass column containing chromosorb 101.
Soluble protein concentrations were measured using a modified Lowry dye-binding protein assay (Lowry et al., 1951).
Total protein concentrations were determined by acid hydrolysis (decomposition into

amino acids) and a colorimetic measurement (Hattingh et al., 1967).
The NH_4^+-N-content was determined by steam distillation in a Kjeltec.1002 apparatus under alkaline conditions. Kjeldahl-N was determined similarly after complete destruction of the sample in strong acid.

Description of experiments

After thawing of the rat faeces, the particle size was reduced even more by grinding to improve the availability of the substrate. To test the degradation of rat faeces 1 g, 2.5 g or 5 g were suspended in 100 ml of distilled water. The medium was flushed with N_2-gas and the initial pH was measured. The bottles with the suspensions were autoclaved and inoculated with 2 ml of a full grown culture of I8 (2 % inoculation). The experiment was set up in a waterbath/shaker at 60 °C in order to avoid sedimentation and compactation of the faecal material. The degradation was monitored during 17 days.

3 RESULTS AND DISCUSSION

3.1 Effect of concentration of rat faeces on degradation

Concentrations of 10 g/l, 25 g/l and 50 g/l were tested. In Figure 1 the amount of ammonium-nitrogen versus time is presented. In Figure 2 the amount of fatty acids produced is plotted versus time.

Ammonium production took place during the first week of incubation. The highest production rate was observed between day 2 and 3, and was respectively 60 mg NH_4^+-N/l, 96 mg NH_4^+-N/l and 131 mg NH_4^+-N/l on 10, 25 and 50 g rat faeces/l. This substantial increase could be due to substrate limitation. About 20 % of the original organic nitrogen was converted to ammonium. The final ammonium-nitrogen concentration in the medium on day 17 is presented in Table 1. The amount of NH_4^+-N in the liquid was proportional to the initial concentration of rat faeces (Fig. 3).

The initial fatty acids concentration of the faeces was low. Some acetic acid and butyric acid was present. The fastest fatty acid production took place between day 2 and 3. On 10 g RF/l it was 229 mg/l.d. On 25 and 50 g RF/l it was respectively 365 mg/l.d and 807 mg/l. So the same phenomenon as for the ammonium production was observed (substrate limitation). There was a correlation between the initial rat faeces concentration and the amount of fatty acids produced. This is shown in Fig. 3. The fatty acids, present in the suspension on day 17 are presented in Table 1.
Comparing the degradation in the bottle with 5 g RF/100 ml with the results in TN15.4, it is clear that there was an improvement of 30 % by shaking the bottles (1872 mg VFA/l =>- 2425 mg VFA/l). This illustrates clearly that there is a problem with the availability of the substrate. Initially about 30 g C/l was present in the suspension. About 1100 mg C/l was converted to fatty acids (= ± 4 %). It should be mentioned that the pH of the suspension which was originally about 6.7 dropped to 5.5, which is the lower level for growth.

An increasing production of CO_2 with the increase of the concentration of rat faeces was detected. This corresponded with the other results (ammonium, VFA).

Table 1 Net ammonium and VFA concentration on day 0 and day 17

		1 g rat faeces per 100 ml		2.5 g rat faeces per 100 ml		5 g rat faeces per 100 ml	
		Day 0	Day 17	Day 0	Day 17	Day 0	Day 17
NH_4^+-N	(mg/l)	24	113	35	232	53	392
Kj-N	(mg/l)	420	432	965	944	1820	1806
Acetic acid	(mg/l)	0	52	0	1433	185	1950
Propion. acid	(mg/l)	0	59	0	140	0	275
Isobutyr. acid	(mg/l)	0	32	0	61	0	116
Butyric acid	(mg/l)	0	0	0	0	45	46
Isovaler. acid	(mg/l)	0	77	0	138	0	269
Total	(mg/l)	0	720	0	1572	230	2655

Fig. 1 Effect of amount of rat faeces on ammonium production by I8

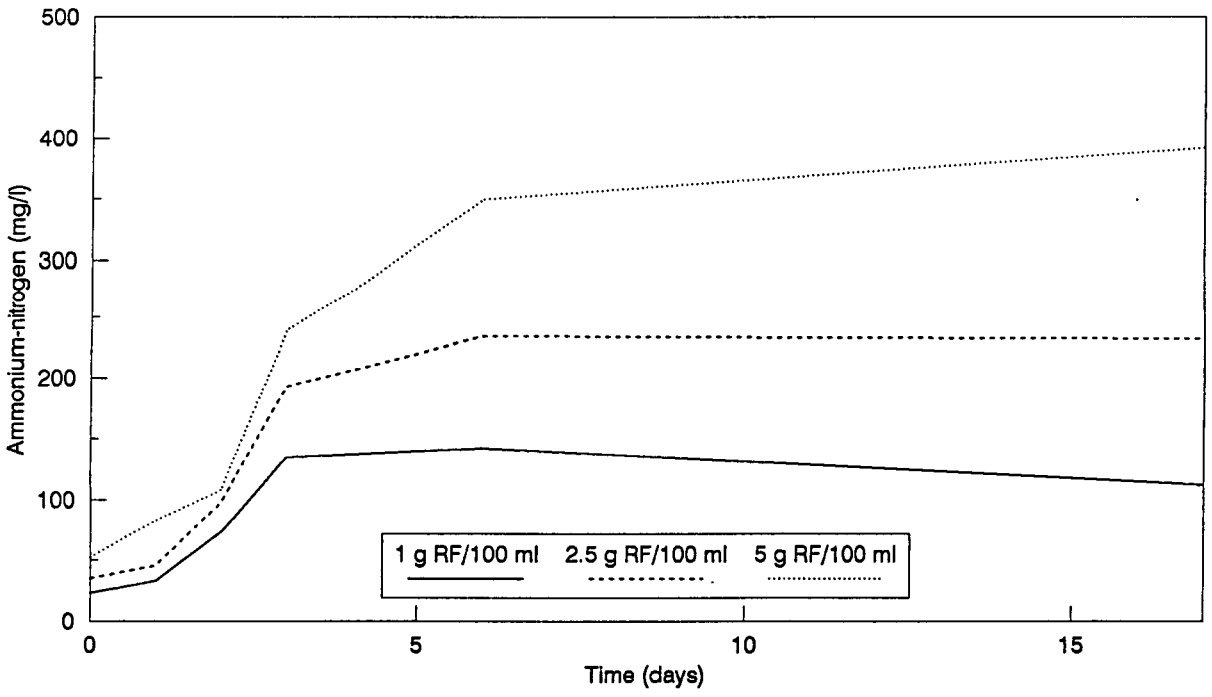


Fig. 2 Effect of amount of rat faeces on VFA-production by I8

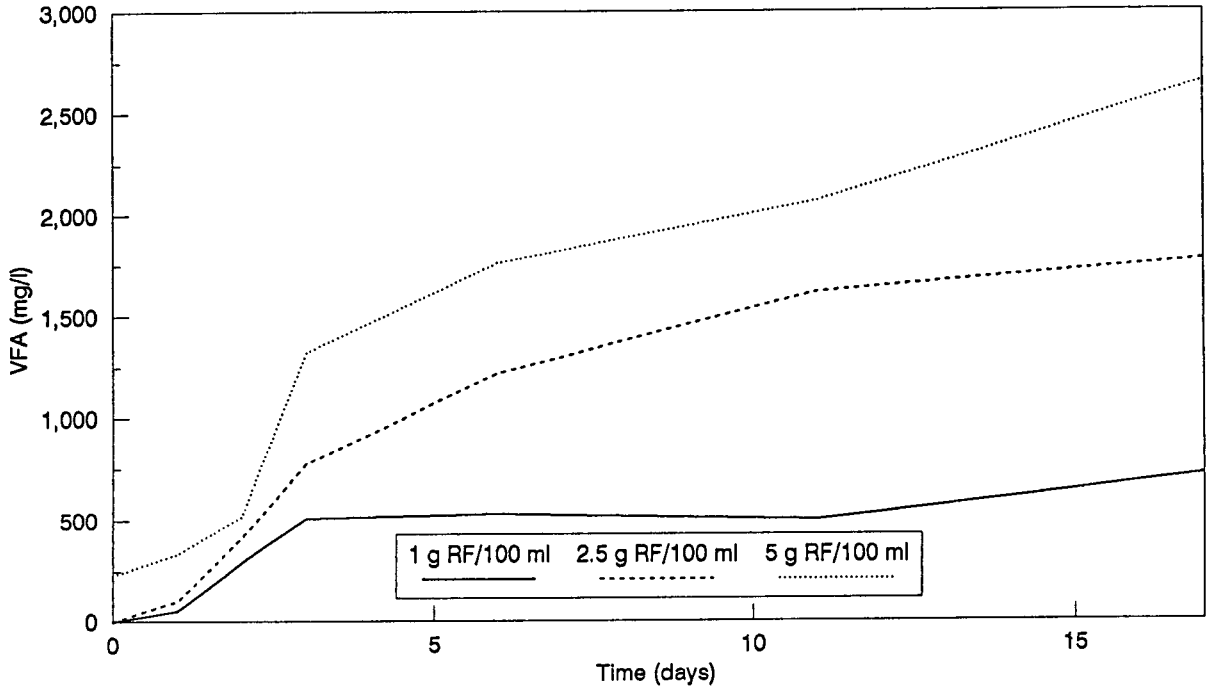
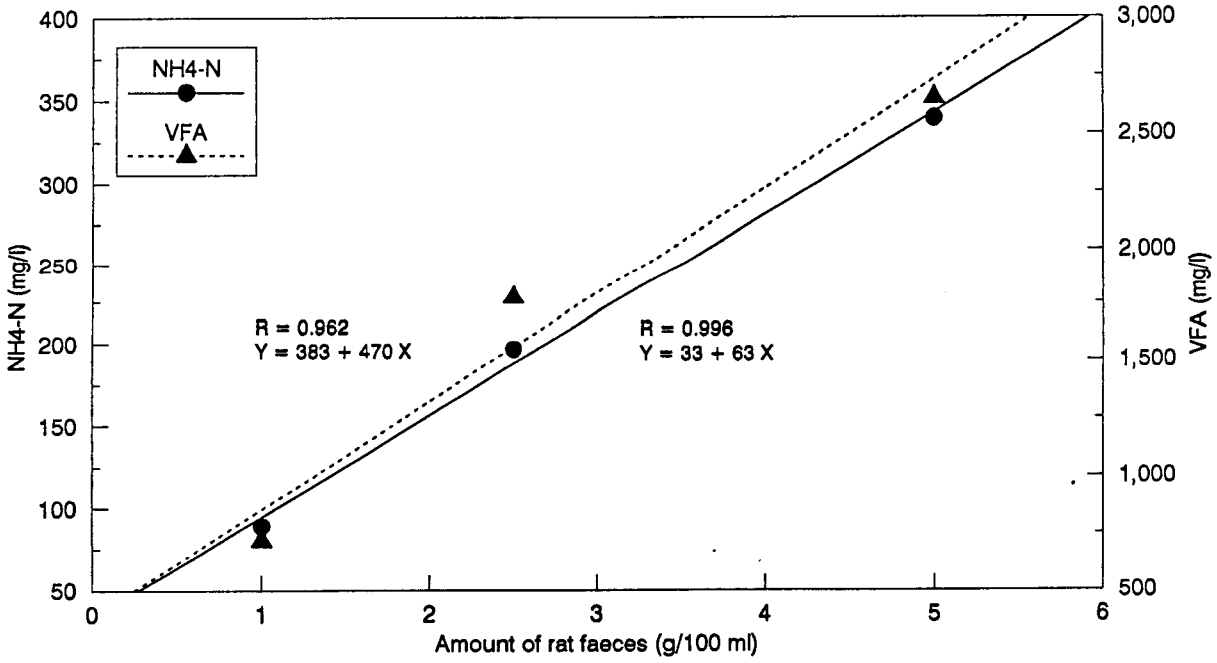


Fig. 3 Correlation between the amount of rat faeces in suspension and the amount of NH4-N and VFA production



3.2 Degradation of proteins

In rat faeces two types of proteins are present: soluble and insoluble proteins. The soluble proteins were determined on the filtrate by the Lowry method. Insoluble proteins were determined after acid destruction of the suspended solids to amino acids and removal of ammonium nitrogen. The amino acids were determined colorimetically.
The test was set up similarly to the previous one: 5 g of rat faeces were suspended in 100 ml water, flushed with N_2, autoclaved and inoculated with 5 ml of a culture of I8. The bottles were incubated during 7 days.
In Table 2 the results of the protein and nitrogen analyses are presented.

Table 2 Nitrogen balances in rat faeces (protein, Kjeldahl-nitrogen, ammonium nitrogen)

	Day 0	Day 7
Soluble protein (mg/l)	3029	1747
Insoluble protein (mg/l)	5100	12900
Kj-N (mg/l)	1820	1806
NH_4^+-N (mg/l)	36	

From the results on day 0, an average protein/protein-N ratio of 4.46 could be calculated, this is lower than the 6.25 for human proteins. In one week time about 42 % of the initial soluble protein fraction is broken down. There was a discrepancy between the insoluble protein on day 0 and day 7. An increase of 7800 mg/l was measured. Part of that increase can be due to the production of cell biomass. The rest can be explained by the method which seems to be not precise. Hattingh et al. (1967) didn't give any indication about that. It can be concluded that probably only a part of the soluble protein was broken down.

4 CONCLUSIONS

The tests with increasing amounts of rat faeces indicate that there is a problem with the availability of the substrate.
After comparing the present results with previous tests it can be concluded that shaking improves the degradation. The final fatty acids concentrations, with and without shaking (2 % inoculum, 5 g rat faeces/100 ml, are presented in Table 3.

Table 3 Effect of shaking on fatty acids production

	Without shaking (21 days)	With shaking (17 days)
Final VFA-concentration (mg/l)	1872	2655
Final Carbon-concentration (mg/l)	820	1160
Efficiency of carbon breakdown (%)	2.7	3.9

In an attempt to make a nitrogen balance an alternative method for determination of protein was tested. These protein measurements are not very precise. About 42 % of the soluble substrate in rat faeces can be broken down in 7 days.

We have to bear in mind that rat faeces are not very similar to human faeces, a lot of lignin (woody substance) is present which can be the cause of the limited availability of substrates.
In order to obtain more realistic results, future research should focus on the break down of synthetic human faeces. These can be produced in the lab in a reproducible manner (Molly et al., 1993).

It would be interesting to see what the performances are of co-cultures of the three strains, which have been the subjects of our studies. Our knowledge of these stains are summarized in the appendix on the next page.

5 REFERENCES

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Knowledge of three selected strains for the liquefying compartment of MELISSA

Incubation at 60 °C, in anaerobic conditions

Clostridium thermocellum

growth: measured by gas production (ml/50 ml medium) or acetic acid production
C-sources: glucose, cellobiose, cellulose (best growth on cellobiose)
no growth on starch

N-sources: best growth on organic N, worst growth on ureum

no proteolysis
no growth on rat faeces

end products: CO_2, H_2, acetic acid, isovaleric acid, lactic acid, ethanol

Clostridium thermosaccharolyticum

growth: measured by gas production (ml/50 ml medium) or volatile fatty acids production
C-sources: glucose, starch, cellobiose (no big differences in efficiency)
N-sources: best growth on organic N, worst growth on ureum

no proteolysis
no growth on rat faeces

end products: CO_2, H_2, acetic acid, butyric acid, ethanol, lactic acid

growth and product formation was generally better for this strain than for the previous one

Coprothermobacter proteolyticus I8 (Thermobacteroides proteolyticus I8)

properties:
-straight rods, sometimes slightly curved;
-non-motile;
-non-sporeforming;
-single cells or in pairs;
-width: 0.2-0.5 µm;
-length: 3-5 µm.

growth: measured by optical density (610 nm), growth curves
proteins: growth rate from about 0.3 (casein, trypton) to about 0.6 d^{-1} (gelatin, BSA, bacto-pepton)
efficiency of hydrolysis was determined

carbohydrates:
no growth on cellulose
almost no growth on raffinose
slower growth (< 1 d^{-1}) on maltose, xylose, starch)
faster growth (1.2-1.4 d^{-1}) on glucose, cellobiose, sucrose, mannose, fructose

pH optimum for growth: 6.8 (5.4-7.9)

temperature optimum for growth: 60 °C (40–70 °C)

end products: proteins: CO_2, H_2, acetic acid, propionic acid, isobutyric acid, isovaleric acid, lactic acid (no ethanol)
carbohydrates: CO_2, H_2, acetic acid, propionic acid, isobutyric acid, (butyric acid), lactic acid, ethanol

remark: yeast extract is essential for growth and can not be substituted by coenzym M, Na-acetate or trypticase

chemical composition of the strain (grown in gelatin medium):

carbon: 47.4 %
hydrogen: 6.9 %
nitrogen: 12.3 %
oxygen: 21.3 %
sulfur: 0.7
phosphor: 0.8 %