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Eco Process Assistance

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TN 22.3

Breakdown of artificial medium (cellobiose and gelatine) and pig manure by co-cultures and breakdown of pig manure enriched with gelatine by single cultures.

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1 INTRODUCTION

In TN 22.1 the biodegradation of pig manure by *Clostridium thermocellum*, *Clostridium thermosaccharolyticum*, *Coprothermobacter proteolyticus* and *Coprothermobacter proteolyticus* I8 was reported. Only the strain *Clostridium thermocellum* produced a small amount of volatile fatty acids (355 mg VFA/l in 14 days).

This experiment was repeated with the addition of gelatine to the pig manure in order to stimulate the deterioration of the manure (Item 2.4.1).

TN 22.2 mentioned that by the biodegradation of artificial human faeces during 21 days, a co-culture of *Clostridium thermocellum* and *Clostridium thermosaccharolyticum* I8 produced 352 mg/l volatile fatty acids and 20 mg/l ammonia-nitrogen. A co-culture of *Clostridium thermocellum*, *Clostridium thermosaccharolyticum* and *Coprothermobacter proteolyticus* I8 produced 364 mg/l volatile fatty acids and 32 mg/l ammonia-nitrogen. Compared to the blank, the concentration of volatile fatty acids and ammonia-nitrogen increased only with about 10%.

In order to investigate if this low production of volatile fatty acids and ammonia was related to the use of artificial human faeces, a second biodegradation experiment was set up. Pig manure and pig manure enriched with gelatine were used as substrates for the co-cultures (Item 2.4.2).

To evaluate the capability of the strains *Clostridium thermocellum*, *Clostridium thermosaccharolyticum* and *Coprothermobacter proteolyticus* I8 to cooperate in an optimal way, the degradation of cellobiose and gelatine by a co-culture of these strains was tested (Item 2.4.3).

2 MATERIALS AND METHODS

2.1 Collection and characteristics of the pig manure

The pig manure used for this experiment was obtained from a pig farm. Table 2.1 shows the characteristics of the pig manure.

Table 2.1 Characteristics of the pig manure used in the biodegradation test with co-cultures

Parameter	Mean value mg/l	Standard error$^{n=3}$ mg/l	Mean value mg/g DM	Standard error$^{n=3}$ mg/g DM
Dry matter	35402	1539	-	-
Organic matter	19294	1646	544	52
Ash	16108	586	456	32
Suspended solids	24647	2696	699	100
Volatile suspended solids	15564	1832	441	66
COD_{tot}	24359	1643	688	45
COD_{sol}	6401	865	181	30
TOC	ND	-	-	-
NH_4^+-N	2316	104	65	3
NO_3^--N	0	0	-	-
Kjeldahl N	3202	246	90	11
Organic N	882	321	25	10
Protein content	5512	306	156	11
VFA	1117	110	32	3
pH*	7.9	-	-	-

* no unit
ND: not determined

In the second experiment, pig manure enriched with gelatine was used. The characteristics of the manure are shown in Table 2.2.

Table 2.2 Characteristics of the pig manure enriched with gelatine used in the biodegradation test with single cultures

Parameter	Mean value mg/l	Standard error$^{n=3}$ mg/l	Mean value mg/g DM	Standard error$^{n=3}$ mg/g DM
Dry matter	31098	264	-	-
Organic matter	20900	374	672	13
Ash	10198	265	327	9
Suspended solids	12081	248	388	9
Volatile suspended solids	7645	155	245	5
COD_{tot}	25463	850	818	28
COD_{sol}	15167	232	487	9
TOC	ND	-	-	-
NH_4^+-N	1494	38	48	1
NO_3^--N	0	0	0	-
Kjeldahl N	3480	31	111	1
Organic N	1986	49	63	2
Protein content	12412	306	399	10
VFA	854	95	27	3
pH	7.9	-	-	-

* no unit
ND: not determined

Table 2.3 shows the characteristics of the mineral solution medium with 4 g/l gelatine and 4 g/l cellobiose used as a substrate in the third biodegradation experiment.

Table 2.3 Characteristics of the mineral solution medium with gelatine and cellobiose used in the biodegradation test with co-cultures

Parameter	Mean value mg/l	Standard Error$^{n=3}$ mg/l
COD_{tot}	8839	63
NH_4^+-N	228	1
Kjeldahl N	822	17
Organic N	602	17
VFA	114	29
pH*	6.8	-

* pH: no unit

2.2 Inoculum

Table 2.4 gives an overview of the three strains used for the degradation tests. The strains were grown in the cultivation media during five days.

Table 2.4 Medium and substrate used to grow the strains

Strain	Reference	Medium	Substrate	Activity
Clostridium thermocellum	ATCC 27405	MS-medium	Cellobiose (3 g/l)	Cellulolytic
Clostridium thermosaccharolyticum	LMG 2811	MS-medium	Cellobiose (3 g/l)	Cellulolytic
Coprothermobacter proteolyticus I8	DRANCO-isolate	MS-medium	Gelatine (3 g/l)	Proteolytic

MS-medium (see addendum 1)
DRANCO-isolate (Kersters, 1992)

2.3 Analytical techniques

The *dry matter (DM)* of the sample was determined after 24 hours drying at 105° C. The *ash content* was determined after incineration at 450°C during a period of 3 hours.

A sample was filtered and the residue was dried for 24 hours at 105°C to determine the *suspended solids* (SS). The *volatile suspended solids (VSS)* were determined by incineration of the dried residue at 450°C during 3 hours.

Volatile fatty acids (VFA) were extracted with diethylether from acidified samples and determined by gas chromatography using a flame ionization detector coupled to a glass column containing chromosorb 101.

Total protein concentrations were determined by acid hydrolysis (decomposition into amino acids) and a colorimetric measurement (Hattingh et al., 1967).

The NH_4+-N content was determined by steam distillation in a Kjeltec 1002 apparatus under alkaline conditions. *The (NO_3- + NO_2-)-N content* was determined by steam distillation in a Kjeltec 1002 apparatus after reduction to NH_3 by the addition of Devarda alloy. *The Kjeldahl-N content* was determined similarly after complete destruction of the sample in strong acid.

The *chemical oxygen demand (COD)* corresponds to the amount of oxygen necessary for complete oxidation of all organic matter present in a given volume of sample. The organic content of the sample was subjected to oxidation by potassium dichromate in a strong acid medium (sulphuric acid plus silver sulphate) at a temperature of 150° C for two hours. The excess dichromate was then measured by back titration with ferrous ammonium sulphate. The *total COD (COD_{tot})* was determined on the total sample, whereas *soluble COD (COD_{sol})* was determined on a centrifuged sample.

2.4 Experiments

2.4.1 Biodegradation of pig manure enriched with gelatine by single cultures

Bottles of 250 ml were filled with 120 ml pig manure and 60 ml of a 3% gelatine solution and flushed with nitrogen gas. The pH was set at 7.5 and the bottles were autoclaved during 20 minutes at 121°C. Afterwards 0.8 ml of a 2.5% Na_2S - solution was added to ensure anaerobic conditions.

Next, 5 ml of an inoculum of the strains *Clostridium thermocellum*, *Clostridium thermosaccharolyticum*, *Coprothermobacter proteolyticus* and *Coprothermobacter proteolyticus* I8 were injected in separate bottles. The blank bottle was injected with 5 ml MS-medium.

At the end of the experiment (after 14 days) the concentration of ammonia and volatile fatty acids was determined in treble. The DM, COD_{tot}, COD_{sol} and Kjeldahl-nitrogen content were determined in single.

2.4.2 Biodegradation of pig manure by co-cultures

Bottles of 250 ml were filled with 120 ml pig manure and flushed with nitrogen gas. The pH was set at 7.5 and the bottles were autoclaved during 20 minutes at 121° C. Afterwards 0.8 ml of a 2.5% Na_2S - solution was added to ensure anaerobic conditions.

Next, the cultivated strains were injected in the bottles. Table 2.4 shows the three different set-ups. The bottles were incubated at 60°C and shaken manually several times per day. Each set-up was carried out in treble.

Table 2.5 Amount of inoculum (ml) injected in the 250 ml bottles for the different set-ups

Strains	Application		
	Blank	2S	3S
Clostridium thermocellum	-	5	5
Clostridium thermosaccharolyticum	-	-	5
Coprothermobacter proteolyticus I8	-	5	5
MS-medium (without strain)	15	5	-

2S: two strains
3S: three strains

At the end of the experiment (after 14 days) the volatile fatty acids, ammonia, Kjeldahl-nitrogen, the COD_{tot} and the dry matter (DM) were determined.

2.4.3 Biodegradation of gelatine and cellobiose by a co-culture

Bottles of 100 ml were filled with 40 ml pig manure and flushed with nitrogen gas. The pH was set at 7.5 and the bottles were autoclaved during 20 minutes at 121° C. Afterwards 0.3 ml of a 2.5 % Na_2S-solution was added to ensure anaerobic conditions.

Next, 3 ml of an inoculum of *Clostridium thermocellum*, *Clostridium thermosaccharolyticum* and *Coprothermobacter proteolyticus* I8 was injected in the bottles. The blank was injected with 9 ml MS-medium. Each experiment was carried out in treble.

The bottles were incubated at 60°C and shaken manually several times per day. At the end of the experiment (after 7 days) the volatile fatty acids, ammonia, kjeldahl-nitrogen and the COD_{tot} were measured.

At the end of the experiment a sample was evaluated microscopically to see if a strain was outcompeted.

3 RESULTS

3.1 Biodegradation of pig manure enriched with gelatine by single cultures

The results of the biodegradation test are summarised in Table 3.1. The bottles inoculated with the strains *Clostridium thermocellum* and *Coprothermobacter proteolyticus* I8 contained a significant (0.05 level) higher amount of volatile fatty acids compared to the blank. The concentration of ammonia and COD_{sol} did not significantly (0.10 level) change during the test.

Although a slight production of volatile fatty acids was noticed in the two cases, it couldn't be confirmed that notable liquefaction of the insoluble matter in the manure occurred.

No significant decrease (0.10 level) of dry matter and COD_{tot} was measured during the test. This indicates that no biodegradation of soluble material to gaseous compounds occurred.

Table 3.1 Values (± standard error$^{n=3}$) of the measured parameters at the beginning and at the end of the biodegradation test (pig manure enriched with gelatine + single cultures)

	pH	DM mg/l	COD_{tot} mg/l	COD_{sol} mg/l	Kj-N mg/l	NH_4+-N mg/l	VFA mg/l
t = O days :							
	7.5	31098 ±264	25463 ±850	15167 ±232	3480 ±31	1494±38	854±95
t = 14 days :							
Blank	7.6	32780	25000	14961	3254	1677±28	1222
Cl. thermocellum	7.5	30590	25490	15804	3401	1645±14	1631
Cl. thermosach.	7.6	30040	25784	14922	3454	1531±17	1098
Copr. Prot.	7.6	29210	23725	14784	3464	1654±18	1326
Copr. Prot. I8	7.6	32460	26765	15686	3479	1595± 5	1056

3.2 Biodegradation of pig manure by co-cultures

Table 3.2 shows the values of the measured parameters. After an incubation period of 14 days, no significant differences (level 0.10) in volatile fatty acids were detected between the blank at the beginning and at the end of the experiment and the two applications 2S and 3S.

The ammonia concentration was significantly (0.10 significance level) higher after 14 days compared to the initial concentration (blank t=0) for every set-up.

No significant decrease (0.10 level) of the dry matter or COD_{tot} was measured during the test. This indicates that no biodegradation of soluble material to gaseous compounds occurred.

Table 3.2 Values (± standard error$^{n=3}$) of the measured parameters at the beginning and at the end of the biodegradation test (pig manure + co-cultures)

	pH	DM g/l	COD_{tot} g/l	Kj-N mg/l	NH4+-N mg/l	VFA mg/l
t = O days :						
	7.5	35.4±1.5	24.3±1.6	3202±346	2316±104	1117±110
t = 14 days :						
Blank	7.4	36.9±2.2	25.9	3263	2578±47	1095±172
2S	7.5	35.9±2.2	23.9	3566	2653±47	1365±172
3S	7.5	36.4±2.2	25.3	3487	2603±47	1418±172

2S: two strains
3S: three strains

3.3 Biodegradation of gelatine and cellobiose by a co-culture

The results of this experiment are summarised in Table 3.3. During the incubation a significant amount (0.05 level) of volatile fatty acids and ammonia was formed compared to the blank. The concentration of the volatile fatty acids increased about 25 times, but the ammonia concentration was only 20% higher compared to the blank.

This indicates that the cellulolytic strains *Clostridium thermocellum* and *Clostridium thermosaccharolyticum* were more active than the proteolytic strain *Coprothermobacter proteolyticus*.

The microscopic evaluation showed that the three strains were present but that the amount of *Clostridium thermosaccharolyticum* cells was approximately ten times higher.

Table 3.3 Values (± standard error$^{n=3}$) of the measured parameters at the beginning and at the end of the biodegradation test (gelatine and cellobiose + co-culture)

	pH	CODtot mg/l	Kj-N mg/l	NH4+-N mg/l	VFA mg/l
t = O days :	6.8	8839±63	822±17	228±1	114± 29
t = 7 days :					
Blank	6.8	8998± 52	831± 4	213±3	94± 31
Co-culture 3S	6.4	8709±128	841± 9	254±1	2600± 2

3S: three strains

4 CONCLUSIONS

The first experiment confirmed that the addition of gelatine to increase the readily biodegradable organic matter did not stimulate the biodegradation of pig manure by single cultures. Earlier TN22.1 mentioned that adding gelatine to artificial human faeces had no effect on the deterioration of the faeces by single cultures.

The second experiment showed that a co-culture of *Clostridium thermocellum* and *Clostridium thermosaccharolyticum* I8 as well as a co-culture of *Clostridium thermocellum*, *Clostridium thermosaccharolyticum* and *Coprothermobacter proteolyticus* I8 weren't capable to degrade the pig manure. In both cases, no significant amount of volatile fatty acids was formed and the concentration of ammonia increased only with about 10% (0.10 significance level).

Several hypotheses can be postulated to explain the inability of the co-cultures to decompose the manure.

The manure was obtained from a pig farm. To prevent diseases antibiotic components are included in the pig diet. These components are excreted together with the faeces. The pig manure used in the experiments could probably contain antibiotic components that prevented the biodegradation of the manure, although TN 22.2 reported that also artificial human faeces were not decomposed by the same co-cultures.

Another possible interpretation follows from the fact that the used co-cultures were not capable to cooperate in an optimal way and no synergistic activity took place. The third experiment showed that the activity of the cellulolytic strain *Clostridium thermosaccharolyticum* was higher than the proteolytic activity. It can be concluded that the combination of the strains did not result in an optimal biodegradation.

So far, artificial faecal material and pig manure were not broken down by single strains, even with addition of gelatine, and co-cultures of these strains.

5 REFERENCES

Hattingh, W.H.J, Thiel, P.G. & Sievert, M.L. (1967). Determination of protein content of anaerobic digesting sludge. Wat.Res., p. 185 - 189.

Kersters, I. (1992). Melissa TN 15.3 ESA/YCL Contract 8152/NL/FG.

Addendum 1: Composition of MS-medium

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		2ml/l
$K_2HPO_4.3H_2O$	200 g/l	
Mineral solution		10ml/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	
$Na_2Mo_4.2H_2O$	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%)		5 ml/l
final pH		7