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# Effect of coculture of anaerobic fungi isolated from ruminants and non-ruminants with methanogenic bacteria on cellulolytic and xylanolytic enzyme activities

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**Abstract.** *Neocallimastix* strain N1, an isolate from a ruminant (sheep), was cocultured with three *Methanobacterium formicicum* strains, *Methanosarcina barkeri*, and *Methanobrevibacter smithii*. The coculture with *Methanobacterium formicicum* strains resulted in the highest production of cellulolytic and xylanolytic enzymes. Subsequently four anaerobic fungi, two *Neocallimastix* strains (N1 and N2) from a ruminant and two *Piromyces* species from non-ruminants (E2 and R1), were grown in coculture with *Methanobacterium formicicum* DSM 3637 on filter paper cellulose and monitored over a 7-day period for substrate utilisation, fermentation products, and secretion of cellulolytic and xylanolytic enzymes. Methanogens caused a shift in fermentation products to more acetate and less ethanol, lactate and succinate. Furthermore the cellulose digestion rate increased by coculture. For cocultures of *Neocallimastix strains* with *Methanobacterium formicicum* strains the cellulolytic and xylanolytic enzyme production increased. Avicelase, CMCase and xylanase were almost completely secreted into the medium, while 40–60% of the β-glucosidase was found to be cell bound. Coculture had no significant effect on the location of cellulolytic and xylanolytic enzymes.

**Key words:** Anaerobic fungi – Methanogenic bacteria – Coculture *Neocallimastix* – Cellulase secretion – Enzyme location

Fermentation of cellulose and other complex substrates in the rumen proceeds by the interaction of various microorganisms including, bacteria, protozoa and anaerobic fungi (Hungate 1966; Orpin 1988). Pure cultures of rumen bacteria and fungi may produce substantial amounts of lactate, ethanol, formate, hydrogen and succinate but these products are usually not found in high amounts in the rumen. Hydrogen is utilized in the rumen by hydrogenotrophic methanogens and its consumption results in production of more oxidized products by the other microorganisms (Hungate 1982). Consequently methane, acetate, propionate, butyrate, $CO_2$, and minor amounts of $C_5$–$C_7$ fatty acids are the predominant fermentation products in the rumen (Hungate 1966). In the caecum of nonruminants the microbial population is comparable to that of the rumen and methanogens were also found in their faeces (Miller and Wolin 1986).

Coculture of anaerobic rumen fungi, e.g. *Neocallimastix* strains (Bauchop and Mountfort 1981; Marvin-Sikkema et al. 1990), *Piromyces communis*, and *Caecomyces communis* (Marvin-Sikkema et al. 1990), with *Methanobrevibacter* spp. resulted in increased $CO_2$ and acetate formation and decreased ethanol, lactate, and succinate production. Triculture with *Methanobrevibacter* sp. and the acetoclastic *Methanosarcina barkeri*, using the acetate produced by the fungus as substrate, resulted in an almost complete conversion of cellulose into $CH_4$ and carbon dioxide (Mountfort et al. 1982). Compared to monocultures of anaerobic fungi an increased cellulose digestion rate was observed in both cocultures and tricultures with methanogenic bacteria (Bauchop and Mountfort 1981; Mountfort et al. 1982; Marvin-Sikkema et al. 1990).

In the degradation of cellulose by anaerobic fungi an array of enzymes is involved which consist of endoglucanases, β-glucosidases and glucohydrolases (Mountfort and Asher 1985; Teunissen et al. 1991b). Coculture of *Neocallimastix frontalis* with *Methanospirillum hungatei* (Mountfort and Asher 1985) and *Methanobacterium smithii* (Joblin et al. 1990) resulted in an increased production of cellulolytic enzymes. Cocultures of anaerobic fungi isolated from non-ruminant herbivores with methanogenic bacterial have not been studied so far.

This paper describes the effect of coculture of *Neocallimastix* sp. (strain N1), isolated from a ruminant, with several strains of methanogenic bacteria on secretion of cellulolytic and xylanolytic enzymes. Furthermore, the effect of coculture with the *Methanobacterium formicicum* (DSM 3637) on the digestion of filter paper cellulose, release of fermentation products and production of cellulolytic and xylanolytic enzymes by four anaerobic

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fungi were compared. These fungi, two strains from a ruminant (strains N1, N2) and two strains from non-ruminants (strains E2, R1), were studied before in monoculture and selected on basis of their high cellulose digestion capacity (Teunissen et al. 1991a, b).

## Materials and methods

### *Organisms*

*Neocallimastix patriciarum* (N2) was obtained from the fungal collection of the AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge, UK. Isolation and cultivation of *Neocallimastix* sp. (N1) and *Piromyces* strains E2 and R1 were described previously (Teunissen et al. 1991a). *Methanobacterium formicicum* (strains DSM 1535, DSM 3636 and DSM 3637), *Methanosarcina barkeri* (DSM 800), *Methanosarcina barkeri* Fusaro (DSM 804) and *Methanobrevibacter smithii* were obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSM), Braunschweig, FRG.

### *Culture medium and growth conditions*

Anaerobic fungi and methanogenic bacteria were grown at 39 °C in defined medium M2. The composition and preparation of this medium was reported previously (Teunissen et al. 1991b).

Stock cultures of anaerobic fungi were maintained on 0.1 g milled wheat straw in 19 ml medium M2. Cultures were inoculated with 1 ml of culture fluid (containing zoospores), and subcultured every 3 to 4 days. (Teunissen et al. 1991a)

Stock cultures of methanogenic bacteria were maintained in 19 ml medium M2 supplemented with 20 mM sodium formate for *Methanobrevibacter smithii* and *Methanobacterium formicicum* species and with 20 mM sodium acetate for both *Methanosarcina barkeri* spp. Cultures were inoculated with 1 ml of a growing culture, and subcultured every 2 to 4 months. Before combining with the fungus, the methanogens were grown for two weeks in medium M2 with $H_2$–$CO_2$ (80/20, v/v) in the gas phase and in the absence of formate or acetate. Methanogens growing on $H_2$–$CO_2$ (80/20, v/v) were incubated at 39 °C in a New Brunswick (New Brunswick Scientific, New Brunswick, N.J., USA) controlled environment incubator shaker at 100 rpm. To obtain cocultures 1 ml of methanogen culture and 1 ml of a three days old culture of an anaerobic fungus grown on cellobiose were inoculated in 18 ml of medium M2 with 0.5% (w/v) filter paper cellulose (Whatman no 1) as a substrate. To achieve stable fungus-methanogen interactions, co-cultures were subcultured every 3 to 4 days on 0.5% (w/v) cellulose in 19 ml medium M2.

Experimental cultures were inoculated by transferring 1 ml of a 3 days old culture of fungus alone or fungus plus methanogen, grown on 0.5% (w/v) filter paper cellulose. During growth and degradation experiments three or four culture bottles were opened at each sampling time and the pH was measured immediately. All incubation were performed at 39 °C. Cultures of anaerobic fungi and fungus-methanogen cocultures were incubated without shaking.

### *Sample collection and determination of dry matter and remaining cellulose*

The amounts of dry matter remaining in four parallel cellulose cultures were determined by filtration over Whatman (Whatman, Maidenstone, Kent, UK) glass-fibre filters (grade C). Subsequently, filters were washed twice with 20 ml of detonised water and dried overnight at 80 °C. Residual cellulose was determined by the method of Updegraff (1969). Samples of the culture filtrate were stored at −20 °C or directly used for analysis of water soluble fermentation products and determination of enzyme activities.

### *Sonication*

To determine the extracellular, intracellular and cell wall bound enzymatic activities three parallel cellulose cultures were harvested by filtration over Whatman glass-fibre filters (grade C) and the filtrates were assayed for various enzyme activities. Residues were resuspended in 5 ml of 0.1 M citrate-phosphate buffer, and subsequently sonicated with an ultrasonic disintegrator (Branson Sonic Power Company, Sonifier B-12, Danberg Connecticut, NY, USA) (60 W; 6 × 30 s; 0 °C). After centrifugation (20,000 × g; 20 min; 4 °C) the supernatants were removed by pasteur pipette and assayed for enzyme activities (intracellular fraction). The residual pellets were resuspended in 10 ml 0.1 M citrate-phosphate buffer (pH 6.0), and assayed for enzyme activities (cell wall fraction). Microscopic examination of the cell wall fraction showed fragmented rhizoids and sporangia (absent in the intracellular fraction). Enzyme activities in supernatants were determined after each sonication cycle, and values did not increase after five cycles.

### *Fermentation product analysis*

Analysis of fermentation products, except methane, was performed as described by Teunissen et al. (1989, 1991a). Methane in 0.5 ml of head space gas samples, was analyzed with a Pye (Pye Unicam, Cambridge, UK) gas chromatograph fitted with a flame ionisation detector and a packed column with 80 – 10 mesh Porapack Q (Supelco, Bellefonte, USA). Ethane was used as internal standard and methane as external standard.

### *Enzyme assays*

Enzyme assays with culture filtrate, intracellular fractions and cell wall fractions were performed in duplicate at optimum pH and temperature. All enzyme reactions were linear over the period of the assays. Enzyme and substrate controls were included in all assays. Endoglucanase, β-glucosidase and xylanase were assayed in 0.1 M citrate-phosphate buffer (pH 6.0) at 50 °C with carboxymethylcellulose (CM-cellulose sodium salt, low viscosity, Sigma C-8758), *p*-nitrophenol-β-D-glucopyranoside (PNPG) and washed xylan (from oat spelt, Sigma X-0376), respectively, as described before (Teunissen et al. 1991b). Exoglucanase was assayed in the same buffer at 40 °C with Avicel (microcrystalline cellulose; type PH 105; Serva, Heidelberg, FRG) as described before Teunissen et al. 1991b). Units of activity are defined as micromoles of product released per min per ml of culture filtrate.

### *Protein assay*

The protein concentrations of supernatant culture were assayed with Bio-Rad protein reagent with bovine gamma globuline as standard.

## Results

### *Stability of cocultures*

Stable cocultures of *Neocallimastix* strains N1 and N2 were obtained with *Methanobrevibacter smithii* and all tested *Methanobacterium formicicum* strains but not with

*Methanosarcina barkeri*. Piromyces strain R1 formed stable cocultures with *Methanobrevibacter smithii* and *Methanobacterium formicicum* strains DSM 3636 and 3637, whereas *Piromyces* strain formed only stable cocultures with *Methanobacterium formicicum* strain DSM 3636 and 3637.

### *Coculture of Neocallimastix sp. (strain N1) with several methanogenic bacteria*

*Neocallimastix* sp. (strain N1) was cocultured with several methanogenic bacteria to select the coculture with highest enzymatic activity. For this experiment methanogenic bacteria were combined with fungi (first generation cocultures) and growth on 0.5% (w/v) cellulose (filter paper) was monitored by measurement of methane production. After the production of methane stopped the fermentation products and enzyme activities were determined in culture filtrates. In cocultures with *Methanobacterium formicicum* DSM 1535 and *Methanosarcina barkeri* formate was not used by the methanogenic bacteria. For these first generation cocultures of *Neocallimastix* strain N1 only the coculture with *Methanosarcina barkeri* showed some transitory accumulation of hydrogen (2.9 mM). In the coculture of *Neocallimastix* strain N1 with the acetoclastic *Methanosarcina barkeri* the amount of acetate (22.9 mM) was comparable to other cocultures indicating that no acetate was used by the methanogen during the incubation period (results not shown).

The effect of coculture on extracellular cellulolytic and xylanolytic activities and protein is given in Table 1. All cocultures of strain N1 with methanogenic bacteria showed enhanced enzyme activities with exception of CMCase activity in cocultures with *Methanobrevibacter smithii* and *Methanosarcina barkeri*. Coculture with *Methanobacterium formicicum* DSM 3636 showed the highest increase in specific activity of all extracellular enzymes. Specific activities of avicelase, endoglucanase, $\beta$-glucosidase and xylanase in this coculture were respectively 0.21, 10.8, 0.90 and 28.3 IU · mg protein$^{-1}$. For the coculture with *Methanobrevibacter smithii* specific activity of extracellular enzymes exept CMCase increased. For the coculture with *Methanosarcina barkeri* no significant increase of specific enzyme activities was found. On average, cocultures of the four tested anaerobic fungi with *Methanobacterium formicicum* DSM 3637 showed the highest increase in cellulolytic and xylanolytic enzyme activities (results not shown).

### *Growth and cellulose utilization by stable cocultures of four anaerobic fungi with Methanobacterium formicicum (DSM 3637)*

The time courses of cellulose degradation by stable cocultures of four anaerobic fungi with *Methanobacterium formicicum* (DSM 3637) are shown in Fig. 1A. After a lag period of 20 to 48 h the fungi started to grow and all cellulose was degraded within 24 h. Cellulose digestion rates were estimated from the maximal declines of the digestion curves and were 0.33, 0.26, 0.23 and 0.21 g · l$^{-1}$ × h$^{-1}$ for cocultures with strains E2, N2, R1 and N1, resprectively. The fungal and bacterial dry matter, remaining after all the cellulose was digested was respectively, 0.38, 0.76, 0.55, and 0.76 g · l$^{-1}$ for cocultures with E2, N2, R1 and N1.

### *Transitory accumulation of hydrogen and formate in stable cocultures of four anaerobic fungi*

Transitory accumulations of hydrogen and formate in stable cocultures of four anaerobic fungi with *Methanobacterium formicicum* (DSM 3637) are shown in Fig. 1B and C. For both *Neocallimastix* strains N1 and N2 a transitory accumulation of hydrogen was found of 0.7 and 1.3 mM which accounts for respectively 5.5 and 10% of the total production (after 144 h) found for the monocultures (Table 2). For both *Piromyces* strains (E2 and R1) a considerably higher transitory accumulation of hydrogen (3.4 mM) was found which is about 30% of the total production found for monocultures. A small transitory accumulation of 1.8 mM formate was found for the coculture of *Neocallimastix* strain N1. For *Neocallimastix patriciarum* (strain N2) and *Piromyces* strains E2 and R1 a transitory accumulation of 19.7, 18.0 and 20.4 mM formate was found, respectivcly. This corre-

**Table 1.** Cellulolytic and xylanolytic enzyme activities[a] in culture filtrates of *Neocallimastix* sp. strain N1 grown in monoculture or in coculture with several methanogenic bacteria

| Coculture with methanogenic bacterium[b] | Enzyme activity | | | | Protein |
|---|---|---|---|---|---|
| | Avicelase (IU · ml$^{-1}$) | CMCase (IU · ml$^{-1}$) | $\beta$-Glucosidase (IU · ml$^{-1}$) | Xylanase (IU · ml$^{-1}$) | (mg · ml$^{-1}$) |
| – | 0.018 ± 0.002 | 1.05 ± 0.10 | 0.075 ± 0.006 | 2.41 ± 0.16 | 0.119 ± 0.013 |
| *Mbr. smithii* (DSM 861) | 0.026 ± 0.004 | 1.23 ± 0.02 | 0.112 ± 0.007 | 3.78 ± 0.09 | 0.141 ± 0.008 |
| *Mb. formicicum* (DSM 1535) | 0.025 ± 0.002 | 1.31 ± 0.02 | 0.112 ± 0.011 | 3.51 ± 0.25 | 0.128 ± 0.008 |
| *Mb. formicicum* (DSM 3636) | 0.026 ± 0.001 | 1.36 ± 0.04 | 0.114 ± 0.007 | 3.57 ± 0.26 | 0.126 ± 0.007 |
| *Mb. formicicum* (DSM 3637) | 0.027 ± 0.002 | 1.39 ± 0.11 | 0.118 ± 0.008 | 3.59 ± 0.19 | 0.134 ± 0.009 |
| *Ms. barkeri* (DSM 804) | 0.022 ± 0.004 | 1.03 ± 0.02 | 0.097 ± 0.010 | 2.93 ± 0.03 | 0.113 ± 0.010 |

[a] Enzymatic activites were determined after 144 h of growth on 0.5% (w/v) filter paper cellulose
[b] *Mbr.* is *Methanobrevibracter*, *Mb.* is *Methanobacterium* and *Ms.* is *Methanosarcina*. Each value represents the mean ±SD ($n$ = 3)

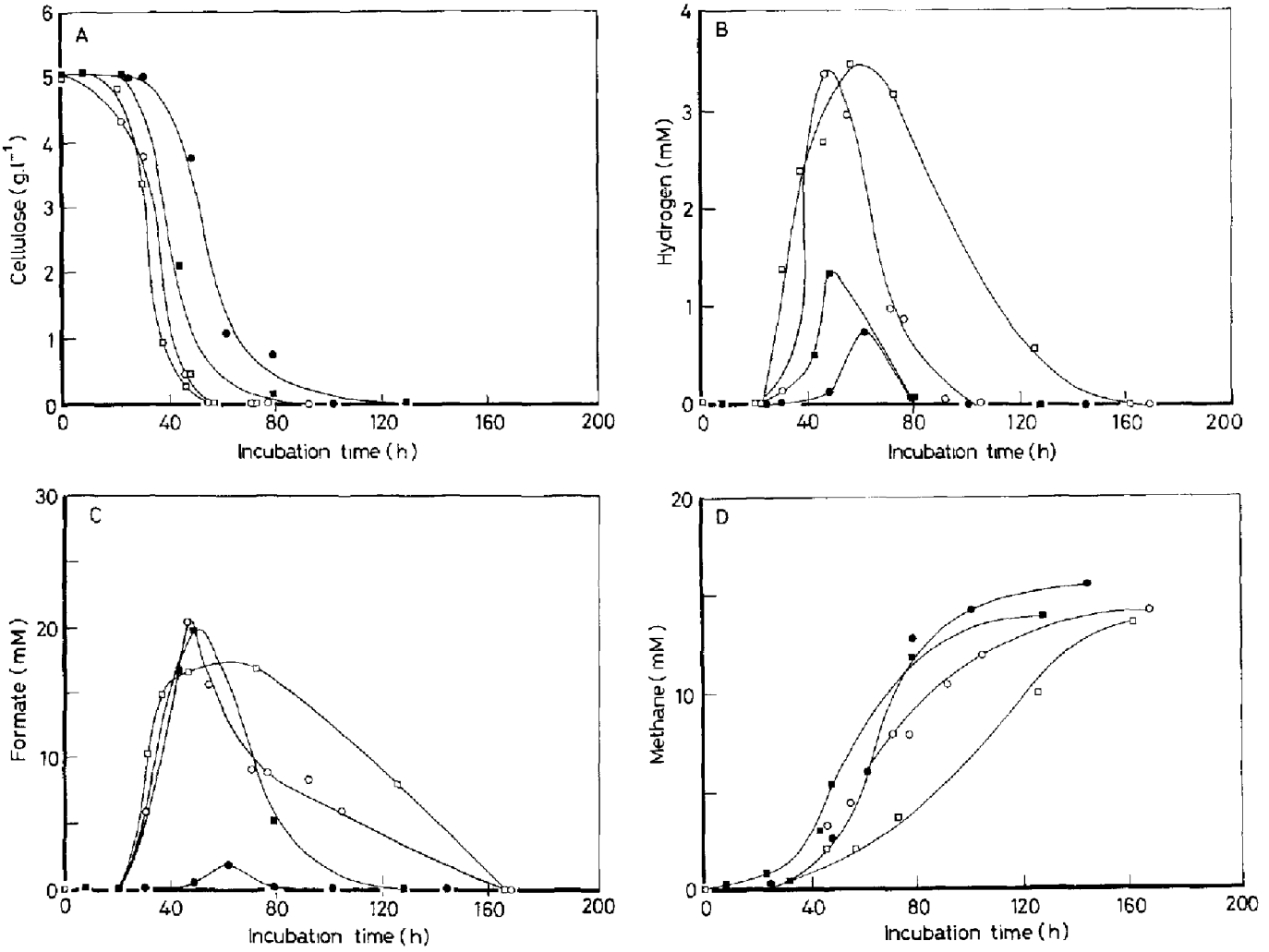


**Fig. 1.** The degradation of cellulose (**A**) and formation of hydrogen (**B**), formate (**C**) and methane (**D**) by cocultures of four anaerobic fungi with *Methanobacterium formicicum* (DSM 3637). Each value represents the mean of four replicates. Generally the S.D. did not exceed 10% of the mean.
*Symbols:* □, E2; ●, N1; ■, N2; ○, R1

sponds to 84%, 66% and 62%, respectively, of the total production found for the monocultures. The accumulation of hydrogen and formate took place during the period of fast degradation of cellulose and were completely used by the methanogens thereafter.

### *Fermentation products of the cocultures of four anaerobic fungi with Methanobacterium formicicum (DSM 3637)*

The methane production by stable fungus-methanogen cocultures is shown in Fig. 1D. Total methane produced by cocultures varied from 13.5 mM for coculture of *Piromyces* strain E2 to 17.6 mM for coculture of *Neocallimastix* strain N1. The amount of fermentation products for the four strains in monoculture or coculture after growth for 144 h on 0.5% filter paper cellulose is shown in Table 2. The fermentation of cellulose by the anaerobic fungi alone resulted in the formation of hydrogen, formate, acetate, lactate, ethanol and succinate. The acetate production increased substantially in the cocultures of the *Neocallimastix* strains whereas for the *Piromyces* strains no significant increase in acetate production was found. The ethanol and lactate production in all cocultures decreased compared to monocultures, especially in cocultures of both *Neocallimastix* species. Only small amounts of malate ($\leq$0.4 mM) were found in the culture filtrates of both monocultures and cocultures.

### *Comparison of cellulolytic and xylanolytic enzyme production by monocultures and cocultures of four anaerobic fungi*

Changes in enzyme activities of the culture filtrates coincided with the observed accelerated cellulose degradation (results not shown). Enzymatic activities in culture filtrates from monocultures and cocultures of four anaerobic fungi are shown in Table 3. A significant increase of avicelase, of about 60%, was only found for the coculture *Neocallimastix* strain N1. The CMCase and $\beta$-glucosidase activities were higher for all cocultures. The

**Table 2.** Fermentation products[a] of four anaerobic fungi in monoculture or coculture with *Methanobacterium formicicum* (DSM 3637)

| Fungal strain | Coculture | $H_2$[b] (mM) | $CH_4$[b] (mM) | Formate (mM) | Acetate (mM) | Ethanol (mM) | Lactate (mM) | Succinate (mM) |
|---|---|---|---|---|---|---|---|---|
| *Neocallimastix* N1 | − | 12.6 ± 0.5 | − | 29.2 ± 0.2 | 22.9 ± 1.0 | 6.6 ± 1.0 | 10.8 ± 2.2 | 2.5 ± 0.1 |
| *Neocallimastix* N1 | + | N.D.[c] | 17.9 ± 0.9 | N.D. | 31.2 ± 2.4 | 2.4 ± 0.1 | 2.8 ± 0.5 | 0.8 ± 0.2 |
| *Neocallimastix* N2 | − | 13.0 ± 0.4 | − | 23.4 ± 1.4 | 17.5 ± 0.7 | 5.1 ± 0.6 | 18.7 ± 0.5 | 1.7 ± 0.2 |
| *Neocallimastix* N2 | + | N.D. | 14.3 ± 1.2 | N.D. | 23.6 ± 2.0 | 2.8 ± 0.6 | 9.3 ± 0.6 | 0.9 ± 0.0 |
| *Piromyces* E2 | − | 11.1 ± 0.4 | − | 27.4 ± 2.4 | 20.0 ± 3.8 | 9.0 ± 0.8 | 5.2 ± 0.8 | 6.2 ± 0.6 |
| *Piromyces* E2 | + | N.D. | 13.5 ± 1.0 | N.D. | 25.7 ± 2.4 | 7.7 ± 0.8 | 4.3 ± 1.0 | 5.4 ± 0.7 |
| *Piromyces* R1 | − | 12.3 ± 0.8 | − | 33.0 ± 1.0 | 22.6 ± 0.9 | 11.1 ± 0.6 | 6.1 ± 1.0 | 6.9 ± 1.4 |
| *Piromyces* R1 | + | N.D. | 14.2 ± 1.2 | N.D. | 24.9 ± 1.6 | 10.5 ± 0.5 | 3.6 ± 0.4 | 5.7 ± 0.5 |

[a] Fermentation products were determined after 144 h of growth on 0.5% (w/v) filter paper cellulose
[b] Hydrogen and methane production were normalized to the culture fluid
[c] N.D., not detectable (0.05 and 0.005 mM for formate and methane, respectively)

**Table 3.** Cellulolytic and xylanolytic enzyme activities[a] in culture filtrates of ruminant and non-ruminant fungi in monoculture or in coculture with *Methanobacterium formicicum* (DSM 3637)

| Fungal strain | Coculture | Enzyme activity | | | | Protein |
|---|---|---|---|---|---|---|
| | | Avicelase ($IU \cdot ml^{-1}$) | CMCase ($IU \cdot ml^{-1}$) | β-Glucosidase ($IU \cdot ml^{-1}$) | Xylanase ($IU \cdot ml^{-1}$) | ($mg \cdot ml^{-1}$) |
| *Neocallimastix* N1 | − | 0.017 ± 0.002 | 1.10 ± 0.04 | 0.075 ± 0.002 | 1.93 ± 0.07 | 0.117 ± 0.007 |
| *Neocallimastix* N1 | + | 0.029 ± 0.001 | 1.37 ± 0.04 | 0.101 ± 0.002 | 3.14 ± 0.17 | 0.129 ± 0.003 |
| *Neocallimastix* N2 | − | 0.027 ± 0.002 | 1.11 ± 0.04 | 0.091 ± 0.003 | 3.34 ± 0.55 | 0.114 ± 0.003 |
| *Neocallimastix* N2 | + | 0.028 ± 0.001 | 1.38 ± 0.18 | 0.124 ± 0.006 | 4.33 ± 0.17 | 0.128 ± 0.008 |
| *Piromyces* E2 | − | 0.030 ± 0.002 | 1.82 ± 0.05 | 0.066 ± 0.005 | 2.99 ± 0.38 | 0.151 ± 0.011 |
| *Piromyces* E2 | + | 0.027 ± 0.001 | 2.02 ± 0.06 | 0.085 ± 0.010 | 3.30 ± 0.52 | 0.209 ± 0.001 |
| *Piromyces* R1 | − | 0.025 ± 0.001 | 1.25 ± 0.07 | 0.062 ± 0.002 | 2.79 ± 0.51 | 0.160 ± 0.001 |
| *Piromyces* R1 | + | 0.025 ± 0.001 | 1.51 ± 0.05 | 0.083 ± 0.002 | 2.68 ± 0.12 | 0.191 ± 0.009 |

[a] All cultures were grown for 144 h on 0.5% (w/v) filter paper cellulose and the enzyme activities were determined simultaneously to exclude small differences in assay conditions. Each value represents the mean ±SD ($n = 3$)

**Table 4.** Relative enzymatic activities in culture filtrates, intracellular cell fractions and cel wall fractions of ruminant and non-ruminant fungi in monoculture or coculture with *Methanobacterium formicicum* (DSM 3637)

| Fungal strain | Coculture | Avicelase | CMCase | | | β-Glucosidase | | | Xylanase | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Location[a] I | E | E | I | C | E | I | C | E | I | C |
| *Neocallimastix* N1 | − | >99 | 86 | 3 | 11 | 43 | 4 | 53 | 91 | 1 | 8 |
| *Neocallimastix* N1 | + | >99 | 87 | 3 | 10 | 44 | 5 | 51 | 90 | 1 | 9 |
| *Neocallimastix* N2 | − | >99 | 89 | 3 | 8 | 44 | 7 | 49 | 92 | 1 | 6 |
| *Neocallimastix* N2 | + | >99 | 88 | 3 | 9 | 45 | 9 | 46 | 93 | 1 | 6 |
| *Piromyces* E2 | − | 100 | 92 | 1 | 7 | 63 | 9 | 31 | 100 | 0 | 0 |
| *Piromyces* E2 | + | 100 | 93 | 1 | 6 | 67 | 5 | 28 | 100 | 0 | 0 |
| *Piromyces* R1 | − | 100 | 87 | 1 | 12 | 57 | 4 | 39 | 100 | 0 | 0 |
| *Piromyces* R1 | + | 100 | 91 | 1 | 8 | 59 | 4 | 37 | 100 | 0 | 0 |

[a] E is extracellular, I is intracellular fraction, C is cell wall fraction. The enzyme activities of the extracellular fraction is given in Table 2. Each value is expressed as percentage of total enzyme activity and represents the mean of three replicates

increase in CMCase activity ranged from 11 to 25% and β-glucosidase activity was in all cases about 30% higher. Xylanase activity was only higher (30 to 60%) in culture filtrates of both *Neocallimastix* strains.

The protein concentration of the culture filtrates was about 11% higher for both *Neocallimastix* cocultures and for cocultures of *Piromyces* strains E2 and R1, an increase of 38 and 19% was found, respectively. Specific activities ($IU \cdot mg\ protein^{-1}$) for avicelase, CMCase, β-glucosidase and xylanase ranged from 0.13 to 0.24, 7.8 to 12.1, 0.39 to 0.97, and 14.0 to 29.3, respectively. Only for *Neocallimastix* strain N1 the specific avicelase activity increased by coculture (45%). For the *Neocallimastix* strain the specific enzyme activities were higher than for the *Piromyces* strains. The specific activities of CMCase, β-glucosidase and xylanase increased for both *Neocallimastix* cocultures (10 to 50%) and decreased for the coculture of *Piromyces* strain E2 (5 to 31%). For coculture

of *Piromyces* strain R1 the specific activity of avicelase and xylanase decreased significantly (15 and 20%, respectively) whereas the other specific enzyme activities were comparable to those found for the monoculture.

The increase in enzyme activities in the culture filtrate of the anaerobic fungi could be the result of a change in the ratio of free enzymes to cell associated enzymes. To investigate this, biomass was sonicated after growth on filter paper was complete, and the enzyme activities in the intracellular and cell wall fractions were determined (Table 4). Avicelase activity was exclusively found in the extracellular fractions. Xylanase activity was found only extracellular for both *Piromyces* strains and for the *Neocallimastix* strains a minor part (up to 9%) was cell wall associated. β-Glucosidase activities were found to be present roughly in same amounts in extracellular and cell wall fractions for both *Neocallimastix* strains, while for the *Piromyces* strains the extracellular-cell wall fraction ratio was 60 to 35. CMCase activity was mainly found in the extracellular fractions for all anaerobic fungi (86–93%). Only small amounts of β-glucosidase and CMCase activities could be liberated from the fungal biomass by sonication. No significant effect of coculture on the ratio of extracellular, intracellular and cell wall bound enzymes was found for all anaerobic fungi.

## Discussion

Cocultures of rumen anaerobic fungi with methanogenic bacteria have been described in detail but no cocultures have been described for anaerobic fungi isolated from non-ruminants (Bauchop and Mountfort 1981; Joblin et al. 1990; Marvin-Sikkema et al. 1990; Mountfort et al. 1982). Furthermore in the fermentation studies, little information is given on the effect of coculture on the production of cellulolytic enzymes. This paper described the influence of coculture of four anaerobic fungi, two isolated from ruminants and two from non-ruminants, with hydrogen consuming methanogens on cellulose degradation, and production of cellulolytic and xylanolytic enzymatic activities and fermentation products.

Compared to monocultures (Teunissen et al. 1991b) the rate of cellulose degradation was higher in all cocultures of anaerobic fungi isolated from ruminants and non-ruminants. The increase in the cellulose degradation rate was 60% for the *Neocallimastix* strain N1 coculture and about 30% for all other cocultures. Increase of cellulose digestion rate has also been reported for other *Neocallimastix* species (Bauchop and Mountfort 1981; Mountfort et al. 1982; Marvin-Sikkema et al. 1990). The cellulose digestion rates of the cocultures in these studies varied from 0.09 to 0.14 $g \cdot l^{-1} \cdot h^{-1}$ which is two and a half to four times lower than the digestion rates found for the cocultures of fungi in this study.

Time course studies with stable cocultures (at least four times subcultured) showed in all cases a transitory accumulation of hydrogen and formate. However, for the cocultures of *Neocallimastix* strain N1 the transitory accumulation of these fermentation products was small, and coincided with the lowest digestion rate of cellulose. For cocultures of *Neocallimastix* strains (Bauchop and Mountfort 1981; Mountfort et al. 1982; Marvin-Sikkema et al 1990) and *Piromyces communis* (Marvin-Sikkema et al. 1990) with *Methanobrevibacter smithii* and *Methanobacterium bryantii* no transitory accumulation of formate and hydrogen was found. However in these cocultures the fermentation rate of cellulose was considerable lower as discussed above and other methanogens were used.

The present study has shown that cocultures with five of the six tested methanogenic bacteria secreted more enzymes with β-glycosidic and endoglucolytic activities. Avicelase activities increased only in cocultures of *Neocallimastix* strain N1 and xylanolytic enzyme activities increased only for cocultures of the *Neocallimastix* strains. A cellulose-grown coculture of *Neocallimastix frontalis* with *Methanospirillum hungatei* has been shown to produce more cellulolytic enzymes (Mountfort and Asher 1985). No information on titres of cellulolytic and xylanolytic activities for *Piromyces* cocultures is given in literature. Especially specific enzyme activities of cocultures of *Neocallimastix* strains with all *Methanobacterium formicicum* strains increased. Increased specific cellulolytic and xylanolytic enzyme activities were also found for xylan grown cocultures of *Neocallimastix frontalis* with *Methanobrevibacter smithii* (Joblin et al. 1990). No increased specific enzyme activities were found for both non-ruminal *Piromyces* strains used in this study; in most cases decreased activities were found. In contrast, for cocultures of ruminal *Piromyces communis* with *Methanobrevibacter smithii* specific activities increased significant (Joblin et al. 1990; Joblin and Williams 1991).

The results on location of the hydrolytic enzymes are in agreement with those of Reese (1977); cellulolytic and xylanolytic enzyme activities are either extracellular or cell wall bound. Similar results were found for cultures of *Neocallimastix frontalis* (Mountfort and Asher 1985; Pearce and Bauchop 1985). However, Lowe et al. (1987) described a *Neocallimastix* strain (R1) in which β-glucosidase was totally bound. This can be caused by the shorter period of incubation; the fungi were harvested during the period of fast growth. However, for the anaerobic fungi used in this study the amounts of cell-bound β-glucosidase and CMCase enzymes were not more than 10% higher during the period of fast growth compared to the amounts found after growth stopped (results not shown). The ratio of cell wall bound to secreted endoglucanase ranged for the four tested strains, ruminal and non-ruminal, from 12:83 to 6:93. This is similar similar to the ratio of 13:84 reported for *Neocallimastix frontalis* (Pearce and Bauchop 1985). The ratio cell wall bound to secreted β-glucosidase reported for *Neocallimastix frontalis* (44:56; Pearce and Bauchop 1985) is closer to the ratio found for the non-ruminal *Piromyces* strains E2 and R1 (respectively 31:63 and 39:57) than to those found for the *Neocallimastix* strains N1 and N2 (respectively 53:44 and 49:44). Avicelase was only found in the extracellular fraction for the four tested strains whereas for *Neocallimastix frontalis* about 15% was reported to be cell wall bound (Pearce and

Bauchop 1985). For both *Neocallimastix* strains up to 9% of the xylanase is cell wall bound which is lower than the 16–19% reported in literature for *Neocallimastix frontalis* strains (Pearce and Bauchop 1985; Lowe et al. 1997b). In contrast the non-ruminal *Piromyces* strains E2 and R1 secreted all xylanase into the medium. Williams and Orpin (1987) showed that a part of xylanase of ruminal *Piromyces* strains is cell wall bound. No information on location of enzymes of non-ruminal anaerobic fungi is available in literature.

In cocultures of the anaerobic fungi a shift of the fermentation pattern to more acetate and less electron sink products (lactate, ethanol and succinate) was found. This shift was also described for all other anaerobic fungus-methanogen cocultures (Bauchop and Mountfort 1981; Joblin et al. 1990; Marvin-Sikkema et al. 1990; Mountfort et al. 1982). The amount of methane produced in all cocultures was significantly higher than could be expected on basis of production of hydrogen and formate in monocultures. This effect is more pronounced for the cocultures of the *Neocallimastix* strains. The presence of a hydrogenase, which catalyzes the production of hydrogen from reduced pyridine nucleotides at low hydrogen pressure, was demonstrated in *Neocallimastix patriciarum* and was localized in microbodies designated as hydrogenosomes (Yarlett et al. 1986). The presence of hydrogenosomes was shown in all other genera, including *Piromyces*, indicating that a similar system is operative (Munn et al. 1988). By keeping the partial hydrogen pressure low the methanogens facilitate the hydrogen production.

The stimulation of fungal enzyme activities by methanogens is not caused by the removal of formate and hydrogen as indicated by Mountfort and Asher (1985) and Joblin et al. (1990). In the coculture of *Neocallimastix* strain N1 with *Methanobacterium formicicum* DSM 1535 enzyme activities were much higher whereas the formate concentration is comparable to that of the monoculture. No stimulatory effect of formate removal was found when cocultures of anaerobic fungi (including *Neocallimastix*, *Piromyces* species) with hydrogen consuming methanogenic bacteria were compared with cocultures with formate and hydrogen consuming methanogenic bacteria (Marvin-Sikkema et al. 1990). Addition of 12 mM hydrogen to monocultures at the start of the incubation had no significant effect on the production of enzymes and fermentation products (results not shown). Close association of hydrogenotrophic bacteria and anaerobic fungi would even facilitate hydrogen production and cocomitant ATP formation (Marvin-Sikkema et al. 1990). The higher ATP yield in cocultures could be used for increased growth of fungus or higher enzyme production. However the fungal dry matter in cocultures at the end of the growth period was the same as found for fungal monocultures (Teunissen et al. 1991b). Furthermore, the lower concentration of electron sink product, as result of coculture has also been suggested to have a stimulatory effect on enzyme synthesis and cellulose degradation (Joblin et al. 1990); Marvin-Sikkema et al. 1990). The stimulation of fungal enzyme activities by methanogens is likely to be a complex event and to elucidate this problem more research has to be conducted.

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