Arch Microbiol (1991) 156:290–296
0302893391001332

Archives of
Microbiology

Fermentation of cellulose and production of cellulolytic and xylanolytic enzymes by anaerobic fungi from ruminant and non-ruminant herbivores

M. J. Teunissen [1], A. A. M. Smits [1], H. J. M. Op den Camp [1], J. H. J. Huis in 't Veld [2], and G. D. Vogels [1]

[1] Department of Microbiology, Faculty of Science, University of Nijmegen, Toernooiveld, NL-6525 ED Nijmegen, The Netherlands
[2] Department of Microbiology, TNO-CIVO Food Analysis Institute, Utrechtseweg 47, NL-3704 HE Zeist, The Netherlands

Received March 11, 1991/Accepted May 31, 1991

Abstract. Four anaerobic fungi were grown on filter paper cellulose and monitored over a 7–8 days period for substrate utilisation, fermentation products, and secretion of cellulolytic and xylanolytic enzymes. Two of the fungi (N1 and N2) were *Neocallimastix* species isolated from a ruminant (sheep) and the other two fungi were *Piromyces* species (E2 and R1) isolated from an Indian Elephant and an Indian Rhinoceros, respectively. The tested anaerobic fungi degraded the filter paper cellulose almost completely and estimated cellulose digestion rates were 0.25, 0.13, 0.21 and 0.18 $g \cdot l^{-1} \cdot h^{-1}$ for strains E2, N1, N2, R1, respectively. All strains secreted cellulolytic and xylanolytic enzymes, including endoglucanase, exoglucanase, β-glucosidase and xylanase. Strain E2 secreted the highest levels of enzymes in a relatively short time. The product formation on avicel by enzymes secreted by the four fungi was studied. Both in the presence and absence of glucurono-1,5-δ-lactone, a specific inhibitor of β-glucosidase, mainly glucose was formed but no cellobiose. Therefore the exoglucanase secreted by the four fungi is probably a glucohydrolase.

Key words: Anaerobic fungi – Cellulase secretion – Xylanase secretion – Glucohydrolase – *Neocallimastix*

Anaerobic chytrid fungi inhabit the alimentary tract of herbivores, ruminants as well as non-ruminants (Milne et al. 1989; Orpin 1988; Teunissen et al. 1991). Anaerobic fungi with monocentric growth patterns have been assigned to three genera: *Neocallimastix, Piromyces*, and *Caecomyces* (Gold et al. 1988; Orpin 1988). Recently anaerobic fungi with a polycentric growth pattern were assigned to the genera *Orpinomyces* and *Ruminomyces* (Barr et al. 1989; Ho et al. 1990). So far, many Piromyces species (Orpin 1981; Teunissen et al. 1991) and one Caecomyces species (Gold et al. 1988) have been isolated from non-ruminants, whereas species from all genera have been isolated from ruminants (Barr et al. 1989; Milne et al. 1989; Orpin 1988).

Anaerobic fungi isolated from ruminant and non-ruminant herbivores have a high digestion capacity for cellulose (Teunissen et al. 1991). Species isolated from ruminants have been shown to produce extracellular enzymes with exoglucanase, endoglucanase, β-glucosidase and xylanase activities in vitro, when grown on cellulose (Gordon and Phillips 1989; Lowe et al. 1987b; Mountfort and Asher 1985; Williams and Orpin 1987). The secretion of cellulolytic and xylanolytic enzymes by anaerobic fungi isolated from non-ruminants has not yet been studied.

Anaerobic fungi can digest high crystalline cellulose. For fungi this characteristic is often associated with the ability to produce an exoglucanase which acts as a cellobiohydrolase (Wood 1989). No direct evidence has been presented for the production of cellobiohydrolase by anaerobic fungi.

In this paper the digestion of filter paper cellulose, release of fermentation products and extracellular cellulolytic and xylanolytic enzymes by four anaerobic fungi are compared. These fungi, two strains from a ruminant and two strains from non-ruminants, were selected on basis of their high cellulose digestion capacity (Teunissen et al. 1990). Furthermore the exoglucanase of the extracellular enzyme preparations was studied in detail with avicel as a substrate.

Materials and methods

Organisms

Neocallimastix patriciarum (N2) was obtained from the fungal collection of the Institute of Animal Physiology and Genetics Research of the Agriculture & Food Research Council (AFRC), Babraham, Cambridge, U.K. *Neocallimastix* sp. (N1) isolated from rumen fluid of a sheep (*Ovis aries*) and *Piromyces* strains E2 and R1 isolated from faeces of an Indian elephant (*Elephas maximas*) and a Black Rhinoceros (*Diceros bicornis*), respectively, were described previously (Teunissen et al. 1991).

Offprint requests to: M. J. Teunissen

Culture medium and growth conditions

Anaerobic fungi were cultivated at 39° C in defined medium M2 (consisting a carbonate buffer, minerals, vitamins and haemin) as reported previously (Teunissen et al. 1991) except that the amount of $NaHCO_3$ was doubled to 12 g · l^{-1}. The medium contained 0.5% (w/v) cellobiose or filter paper cellulose (Whatman no 1) as carbon source.

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. Stock cultures were preserved anaerobically at −80° C or in liquid nitrogen in medium M2 with 5% (v/v) dimethylsulfoxide (DMSO) as cryoprotectant (Teunissen et al. 1991).

Experimental cultures were inoculated by transferring 1 ml of a 3 days old culture grown on cellobiose. During growth and degradation experiments four culture bottles were opened at each sampling time and the pH was measured immediately. All incubations were at 39° C.

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 (Maidenstone, UK) glass-fibre filters (grade C). Subsequently, filters were washed twice with 20 ml deionised water and dried overnight at 80° C. Residual cellulose was determined by the method of Updegraff (1969). Samples of the culture fluid (filtrate) were stored at −20° C until used for analysis of water soluble fermentation products and determination of enzyme activities.

Fermentation product analysis

Formic acid was determined by the colorimetric method of Sleat and Mah (1984). Malate and succinate were determined enzymatically (Möllering 1974; Williamson 1974). Acetate, ethanol and lactate were determined by gas liquid chromatography (G.L.C.) using the method of Teunissen et al. (1989) with a Hewlett Packard (Cupertino, Calif., USA) 5890A gas chromatograph. The column was packed with 80−100 mesh Chromosorb WAW with liquid phase GP 10% SP-1200/1% H_3PO_4 (Supelco, Bellefonte, USA). Hydrogen in 0.5 ml of head space gas samples, was quantified with a Hewlett Packard 5890A gas chromatographed fitted with a thermal conductivity detector and a column packed with 80−100 mesh Porapack Q (Supelco). Ethane was used as internal standard and hydrogen as external standard.

Soluble sugar analysis

Total soluble sugars were analysed according to the method of Updegraff (1969). Glucose was determined using glucose oxidase and peroxidase (Bergmeyer and Bernt 1974). The same assay was used to determine cellobiose after enzymatic hydrolysis with β-D-glucoside glucohydrolase (EC 3.2.1.21) (Russell and Baldwin 1978). Reducing sugars were determined with dinitrosalicylic acid (DNS) reagent (Miller 1959); the ratio sample to DNS was 1:2 and after boiling for 15 min the absorbance at 575 nm was measured against glucose or xylose standards treated in the same way.

Enzyme assays

Enzyme assays were performed in duplicate. The optimum pH and temperature of the enzymes were determined for each strain. All enzyme reactions were linear over the period of the assays. Enzyme and substrate controls were included in all assays.

Cellulase activity was assayed with Avicel (microcrystalline cellulose; type PH 105; Serva, Heidelberg, FRG) and carboxymethylcellulose (CMC sodium salt, low viscosity, Sigma C-8758; Sigma St. Louis, MO., USA). With Avicel, 0.25 ml of culture filtrate was incubated with 10 mg of the substrate in 0.75 ml of 0.133 M citrate-phosphate buffer (pH 6.0) at 40° C for 2 h. The reaction was stopped by placing the reaction tubes in boiling water for 5 min. The latter step was followed by centrifugation (5 min, 12,000 × *g*) to pellet residual Avicel, and the supernatant was analysed for reducing sugars and/or glucose or total soluble sugars (TSS). With CMC as substrate, 0.025 ml of culture filtrate was incubated for 20 min at 50° C in 0.975 ml of 0.10 M citrate-phosphate buffer (pH 6.0) containing 10 mg of CMC. The reaction was terminated by addition of 2 ml DNS reagent. Avicelase and CMCase activities are expressed as micromoles of glucose equivalents released per min per ml culture filtrate.

For determination of the xylanase activity xylan (from oat spelt, Sigma X-0376) was washed with deionised water to remove soluble sugars which are present in the substrate. A 2% (w/v) suspension was made and centrifuged for 10 min at 5,000 × *g*. The supernatant was discarded and the pellet was resuspended in deionised water. The washing was repeated two times, and the xylan was freeze-dried. Xylanase activity was measured by incubating 0.025 ml of culture filtrate with 10 mg of washed xylan in 0.975 ml 0.10 M citrate-phosphate buffer (pH 6.0) for 20 min. The reaction was terminated by placing the reaction tubes for 5 min in boiling water. Samples were centrifuged to pellet residual xylan, and the filtrate was analysed for reducing sugars. Units of activity are defined as micromoles of xylose equivalents released per min per ml of culture filtrate.

β-Glucosidase and β-cellobiosidase (cellobiohydrolase) activities were determined by measuring the absorbance at 420 nm of the *p*-nitrophenol released from *p*-nitrophenol-β-D-glucopyranoside (PNPG) and *p*-nitrophenol-β-D-cellobioside (PNPC), respectively. A sample (0.025 ml) of culture filtrate was incubated with 5 mM PNPG at 50° C for 20 min in 0.475 ml 0.10 M citrate-phosphate buffer (pH 6.0). Another sample (0.1 ml) of culture filtrate was incubated with 3.5 mM PNPC at 40° C for 45 min in 0.40 ml 0.125 M citrate-phosphate buffer (pH 6.0). Reactions were stopped by the addition of 1 ml 1 M Na_2CO_3. Units of activity are defined as micromoles of *p*-nitrophenol released per min per ml of culture filtrate.

Protein assay

The protein concentration of culture filtrate was assayed with the Bio-Rad (Bio-Rad Laboratories, Richmond, Calif., USA) protein reagent with bovine gamma-globulin as standard.

Results

Growth and degradation of filter paper cellulose

The degradation of cellulose and growth of the four strains was followed during a time course experiment and the results are presented in Fig. 1A and 1B. Growth was calculated by subtracting the amount of undegraded cellulose from the dry matter (DM). After a lag period of 8 to 48 h growth started and reached a maximum within another 40 h. The maximum fungal DM for strains E2, N1, N2, and R1 were 0.45, 1.08, 0.92, and 0.90 g · l^{-1}, respectively. After growth stopped the DM of fungal strains E2, N1, N2, and R1 decreased to about 70% of the maximal value and remained stable thereafter.

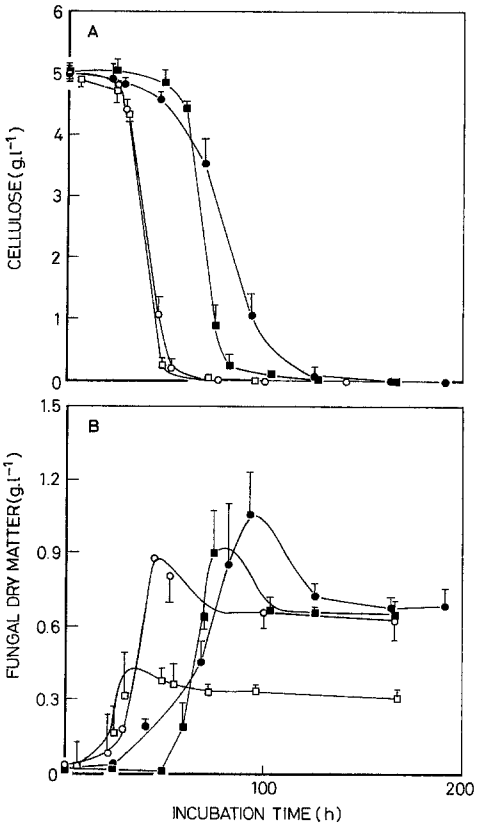

Fig. 1. Digestion of filter paper cellulose **A** and biomass formation **B** by four anaerobic fungi during growth in defined medium M2. Each value represents the mean ± SD ($n = 4$). *Symbols*: □, E2; ●, N1; ■, N2; ○, R1

During the periods of fast growth more than 90% of the cellulose was digested. The remaining cellulose was digested during the period in which fungal DM declined. Cellulose digestion rates were estimated from the maximal declines of the digestion curves and were 0.25, 0.13, 0.21 and 0.18 $g \cdot l^{-1} \cdot h^{-1}$ for strains E2, N1, N2, R1, respectively. The specific cellulose digestion rates were 0.45, 0.12, 0.23 and 0.20 $g \cdot g$ fungal DM^{-1} for strains E2, N1, N2, R1, respectively. The pH of the cultures shifted from 7.3 to 6.6 during growth of all strains.

Fermentation products

Growth of the fungal strains on cellulose resulted in a mixed type of fermentation with the products: formate, acetate, lactate, ethanol, hydrogen, malate and succinate. No C_3-C_6 volatile fatty acids or C_1-C_5 alcohols other than ethanol could be detected. In Table 1 the amounts of fermentation products after 168 h of incubation are given. All strains produced acetate and formate as major endproducts. Lactate was also produced as a major endproduct by strain N2.

A carbon and redox balance was constructed to compare the growth of the strains on defined medium M2 containing cellulose (Table 2). Values for cellulose, CO_2 and biomass were estimated from established stoichiometry (Yarlett et al. 1986; Lowe et al. 1987a). Carbon recoveries varied from 78.4% for strain E2 to 94.6 for strain N1. All strains produced nearly equivalent amounts of oxidized and reduced products.

Table 1. Fermentation products of anaerobic fungi after growth on filter paper cellulose in defined medium M2

Product	Production ($mmol \cdot l^{-1}$)			
	Neocallimastix		*Piromyces*	
	Strain N1	Strain N2	Strain E2	Strain R1
Acetate	21.8 ± 0.5	19.0 ± 0.7	22.0 ± 1.8	22.6 ± 0.9
Ethanol	9.0 ± 0.8	5.2 ± 0.5	9.0 ± 0.8	10.6 ± 0.7
Formate	29.2 ± 1.2	23.4 ± 1.4	28.4 ± 2.4	29.0 ± 1.0
Lactate	8.3 ± 1.2	19.9 ± 0.7	6.1 ± 1.0	5.2 ± 0.8
Malate	0.5 ± 0.1	0.1 ± 0.0[a]	0.0 ± 0.0[a]	1.5 ± 0.7
Succinate	1.0 ± 0.2	0.4 ± 0.3	1.9 ± 1.1	1.5 ± 0.3
Hydrogen[b]	12.8 ± 0.7	13.0 ± 0.4	11.1 ± 0.4	12.3 ± 0.8

[a] Error < 0.05
[b] Hydrogen production was normalized to the medium volume in the culture bottles
Each value represents the mean ± SD ($n = 4$)

Table 2. Carbon and redox balances of the fungal strains grown on medium M2 with filter paper cellulose as carbon source

Product or parameter	Carbon in product (mM)[a]			
	Neocallimastix		*Piromyces*	
	Strain N1	Strain N2	Strain E2	Strain R1
Fermentation products	121.6	131.1	116.3	122.8
Carbon in cells[b]	37.6	32.2	15.6	31.5
Carbon in soluble sugars	9.8	9.2	9.9	7.3
CO_2[c]	2.2	0.3	0.6	1.3
Substrate	185.2	185.2	181.7	184.2
	Carbon/Redox balances			
% C recovery	94.6	94.1	78.4	88.4
Oxidized/ Reduced balance	0.99	0.99	1.02	1.00

[a] At time when maximum growth was found
[b] Carbon content of cells is 42% (Lowe et al. 1987a)
[c] CO_2 = [(acetate + ethanol) − (formate + malate + succinate)]

Enzyme activity

Cellulase and xylanase (Fig. 2) activities from culture filtrates of fungal strains grown on filter paper cellulose (0.5%, w/v) were assayed over a 8-day period. To determine optimal temperature for enzyme activity assays were performed over a range from 20 to 80° C at pH 6.0. The temperature optima for CMCase, β-glucosidase, xylanase were found to be 50° C, while the temperature optimum for avicelase was 40° C. For pH optima assays were performed at optimum temperatures over the pH range

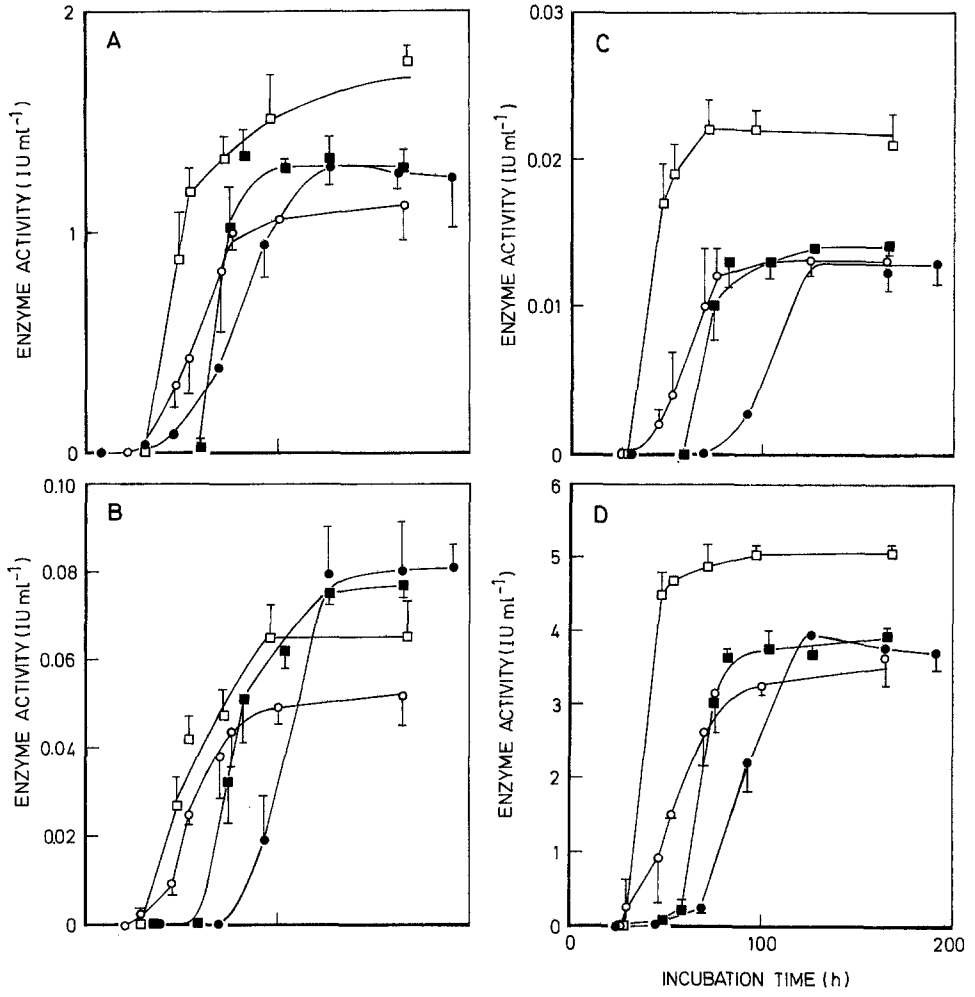

Fig. 2A–D. Time courses for extracellular cellulolytic activities by four anaerobic fungi grown on filter paper cellulose. **A** CMCase activity; **B** β-glucosidase activity, **C** Avicelase activity; **D** Xylanase activity. Each value represents the mean ± SD (n = 4). Symbols as indicated in Fig. 1

3.0 to 8.0. All enzymes had a wide pH optimum at 6.0. At these optimum conditions for temperature and pH enzyme reactions were linear for 40 min with CMC, PNPG and xylan, and for 140 min for Avicel.

Digestion of cellulose by strains E2, N1 and N2 and changes in enzyme activities of culture filtrate appeared to proceed in a similar way during growth. However for strain R1 the major part of the cellulolytic and xylanolytic activities were secreted after the digestion of cellulose was completed. Strain E2 secreted more avicelase, CMCase, and xylanase than the other strains (Fig. 2A, C, D) in a relatively short time. The maximum levels of β-glucosidase for strains N1 and N2 are considerably higher than for strains E2 and R1 (Fig. 2B). Strains N1 and N2 secreted equivalent amounts of each enzyme. The amount of protein secreted at the end of the incubation periods were 0.159 ± 0.011, 0.117 ± 0.007, 0.125 ± 0.005 and 0.160 ± 0.006 mg · ml^{-1} for strains E2, N1, N2, R1, respectively.

Product release by enzymes active on Avicel and influence of inhibition of β-glucosidase

Exoglucanases, which can hydrolyse Avicel, produce either glucose or cellobiose. The products formed after incubation of Avicel with culture filtrates were determined (Table 3). The main part of the reducing sugars (RS) formed appeared to be glucose; 88.2 to 99.0%. No cellobiose was found for all strains. Cellobiose formed during hydrolysis of Avicel could have been subsequently hydrolysed by β-glucosidase which is also present in the enzyme sample. Therefore the influence of specific inhibition of β-glucosidase on product formation by enzymes acting on Avicel was studied. β-Glucosidase activity of all strains was inhibited by 0.25 mg · ml^{-1} D-glucurono-1,5-δ-lactone (GL) for more than 99.8% while no inhibition of CMCase or xylanase was found at this concentration of GL. In the presence of GL 20 to 40% less of each product was formed (Table 3). The reducing sugars were accounted for 96.3 to 106.2% by glucose for the individual strains and still no cellobiose was found. The amount of total soluble sugars (TSS) formed was both in the presence and absence of GL 15 to 65% higher than the amount of reducing sugars or glucose and probably consist of oligomers. Possibly endoglucanase has also a small activity on Avicel.

Enzyme activity on p-nitrophenyl-β-D-cellobioside

p-Nitrophenol-β-D-cellobioside (PNPC) is a substrate used to determine the exoglucanase which acts as

Table 3. Influence of β-glucosidase inhibition on product release by enzymes acting on Avicel[a]

Fungal isolate	No inhibition β-glucosidase			Glucose/RS	β-Glucosidase inhibited[b]			Glucose/RS
	TSS (mM glucose)	RS (mM glucose)	glucose (mM glucose)		TSS (mM glucose)	RS (mM glucose)	glucose (mM glucose)	
Neocallimastix								
Strain N1	0.86 ± 0.01	0.58 ± 0.01	0.51 ± 0.00	0.87 ± 0.02	0.69 ± 0.01	0.43 ± 0.00	0.41 ± 0.01	0.96 ± 0.04
Strain N2	0.68 ± 0.02	0.59 ± 0.00	0.59 ± 0.02	0.99 ± 0.04	0.57 ± 0.00	0.49 ± 0.00	0.48 ± 0.01	0.98 ± 0.02
Piromyces								
Strain E2	0.91 ± 0.01	0.83 ± 0.00	0.79 ± 0.03	0.96 ± 0.04	0.63 ± 0.00	0.58 ± 0.01	0.56 ± 0.04	1.06 ± 0.02
Strain R1	0.78 ± 0.02	0.66 ± 0.03	0.59 ± 0.01	0.88 ± 0.06	0.46 ± 0.00	0.40 ± 0.01	0.40 ± 0.00	1.01 ± 0.01

[a] Avicel was incubated during 140 min with 0.2 ml culture filtrate in 0.1 M phosphate-citrate buffer (pH = 6.0)
[b] In the presence of 0.25 mg/ml glucurono-1,5-δ-lactone, inhibitor of β-glucosidase
Each value represents the mean ± SD (n = 4)

cellobiohydrolase. The activities in culture filtrates of the strains were very low and varied from 2.2 nmol · min^{-1} · ml^{-1} for strain R1 to 4.9 nmol · min^{-1} · ml^{-1} for strain N2. Such low activities could have resulted from the presence of β-glucosidase and endoglucanase which have also a small activity on PNPC. In accordance the enzyme activities on PNPC were about 50% lower for all strains in the presence GL. The results indicate that cellobiose is not a product of enzymes with exoglucanase activity.

Discussion

Anaerobic fungi isolated from ruminants have been studied extensively in contrast to strains isolated from non-ruminants. This study compares two fungal strains from a ruminant (*Neocallimastix* spp.) with two strains from non-ruminants (*Piromyces* spp.).

Anaerobic fungi metabolise carbohydrates via a mixed acid-type fermentation with acetate, formate and lactate as major endproducts (Borneman et al. 1989; Lowe et al. 1987a; Phillips and Gordon 1989). The fermentation pattern of *Neocallimastix* strain N1 resembles that of *Neocallimastix frontalis* (Bauchop and Mountfort 1981) more than that of *Neocallimastix patriciarum* strain N2 (from AFRC culture collection) which was also used in this study. The anaerobic fungi tested in this study appear to produce almost no carbon dioxide which was calculated on the basis of other metabolic productes formed. Moreover, the ratio of oxidized to reduced products confirms this conclusion. For other anaerobic fungal strains carbon dioxide productions were reported ranging from 0.19 to 0.62 mol CO_2 per mol of glucose fermented (Borneman et al. 1989; Lowe et al. 1987a). It is not clear if these differences are caused by different culture conditions or by the use of different strains.

A carbon and redox balance was constructed to determine if the main products of fermentation were detected for all strains. The carbon recoveries varied between 79.8 and 99.6% indicating that all major endproducts were found. The balance may consist of extracellular protein and some additional products like glycerol or pyruvate. Small amounts of ethanol, lactate, malate, and succinate were found as minor endproducts. This is the first time malate production by anaerobic fungi has been shown. Yarlett et al. (1986) suggested that malate is produced in the cytosol and than transported into the hydrogenosome where it is further metabolised. For *Tritrichomonas foetus*, an anaerobic flagellate, it has been found that malate is a product of the hydrogenosomes which is further reduced to succinate (Steinbüchel and Müller 1986).

The anaerobic fungi used in this study digest cellulose at a high rate. *Neocallimastix* species have been shown to digest cellulose with rates (estimated from reported cellulose digestion curves) varying between 0.04 and 0.06 g · l^{-1} · h^{-1} (Bauchop and Mountfort 1981; Lowe et al. 1987b; Phillips and Gordon 1989). The cellulose digestion rates of the two *Neocallimastix* species, strains N1 and N2, were three to five times higher. Both *Piromyces* species, isolated from non-ruminants, used in this study digest cellulose at a rate comparable to *Neocallimastix patriciarum* (strain N2). No detailed information on cellulose digestion by *Piromyces* species is available in literature.

All strains, from ruminant and non-ruminant herbivores, produced an array of enzymes that allowed them to hydrolyse plant cell walls. The pH and temperature optima of the enzymes of both *Piromyces* species from non-ruminants were similar to those found for a ruminal *Piromyces* strain (Borneman et al. 1989) and did not differ from *Neocallimastix* species (Lowe et al. 1987b; Mountfort and Asher 1985). The release of enzymatic activity parallelled the growth of the strains as reported for other fungi isolated from ruminants (Borneman et al. 1989; Lowe et al. 1987b). *Piromyces* strain E2 produced the highest amount of cellulolytic and xylanolytic enzyme activities in a relatively short time. The avicelase activities and β-glucosidase activities for anaerobic fungi used in this study were similar to those reported in literature for other fungal strains (Borneman et al. 1989; Gordon and Phillips 1989; Lowe et al. 1987b). However, for *Neocallimastix* strain N1 a 25 times lower avicelase activity was reported. CMCase activities of strains N1 and N2 were 2 to 6 times higher than those reported for other cellulose grown fungal strains (Borneman et al. 1989; Gordon and Phillips 1989; Lowe et al. 1987b; Mountfort

and Asher 1985). Direct comparison of xylanolytic activities produced by anaerobic fungi as measured in other studies have not been made because of the different composition of the xylan.

Borneman et al. (1989) reported specific enzyme activities for a *Neocallimastix* strain and *Piromyces* strain which are comparable to the specific activities after growth on cellulose for the strains used in this study. The specific enzyme activities found for all anaerobic fungal strains are high compared to those of aerobic fungal strains of *Trichoderma reesei* and *Trichoderma viride* which are known to be most powerful in cellulose hydrolysis (Gosh et al. 1984; Beldman et al. 1985). For example, the specific CMCase activity is three to six times higher for anaerobic fungi than for a hyper-cellulolytic mutant of *Trichoderma reesei* (Gosh et al. 1984) and comparable to purified CMCase of *Trichoderma viride* (Beldman et al. 1985). This indicates that anaerobic fungi may be of importance for the commerical production of cellulases.

Anaerobic fungi can degrade crystalline cellulose (Wood et al. 1986). For all fungi, except brown rot fungi, this characteristic is associated with the production of cellobiohydrolases (Wood 1989). There is no direct evidence that the cellulase complex from anaerobic fungi contains an exoglucanase that acts as a cellobiohydrolase. However, Wood et al. (1986) showed by thermal inactivation studies that an extracellular enzyme is present that acts synergistically with endoglucanase and β-glucosidase. The thermal inactivated enzyme can be replaced by the cellobiohydrolase of *Trichoderma koningii* and this author suggested that the thermal inactivated enzyme was an exoglucanase that acts as a cellobiohydrolase. Avicel, a microcrystalline cellulose, is widely used as a substrate to determine exoglucanase activity. In this study all anaerobic fungi produced significant enzyme activity towards avicel. However, glucose, appeared to be the main product on this substrate, both in the presence and absence of glucurono-1,5-δ-lactone (GL) which is a specific inhibitor of β-glucosidase (Deshpande et al. 1984). In all cases no cellobiose could be detected. The very low activities towards p-nitrophenol-β-D-cellobioside (PNPC), a substrate to determine cellobiohydrolase activity, can be caused by cross-reactivity of β-glucosidase and endoglucanase on this substrate (Deshpande et al. 1984). In the presence of GL activity on PNPC was 50% lower. These findings indicate that anaerobic fungi secrete a glucohydrolase instead of a cellobiohydrolase. To elucidate this further study will concentrate on the purification of cellulolytic enzymes of the four anaerobic fungi.

Acknowledgements. This research was supported by a grant from the Ministry of Economic Affairs of the Netherlands.

References

Barr DJS, Kudo H, Jakober KD, Cheng K-J (1989) Morphology and development of rumen fungi: *Neocallimastix* sp., *Piromyces communis*, and *Orpinomyces bovis* gen. nov., sp. nov. Can J Bot 67:2815–2824

Bauchop T, Mountfort DO (1981) Cellulose fermentation by a rumen anaerobic fungus in both the absence and presence of rumen methanogens. Appl Environ Microbiol 42:1103–1110

Beldman G, Searle-Van Leeuwen MF, Rombouts FM, Voragen FGJ (1985) The cellulase of *Trichoderma viride*. Purification, characterization and comparison of all detectable endoglucanases, exoglucanases and β-glucosidases. Eur J Biochem 146:303–308

Bergmeyer HU, Bernt E (1974) D-glucose. Bestimmung mit Glucose-Oxydase und Peroxydase. In: Bergmeyer HU, Gawehn K (eds) Methoden der enzymatischen Analyse, Band 2. Verlag Chemie, Weinheim, pp 1250–1257

Borneman WS, Akin DE, Ljungdahl LG (1989) Fermentation products and plant cell wall-degrading enzymes produced by monocentric and polycentric anaerobic ruminal fungi. Appl Environ Microbiol 55:1066–1073

Deshpande MV, Erikson KE, Peterson LG (1984) Assay for selective determination of exo-1,4-β-glucanases in a mixture of cellulolytic enzymes. Anal Biochem 138:481–487

Gold JJ, Heath IB, Bauchop T (1988) Ultrastructural description of a new chytrid genus of caecum anaerobe, *Caecomyes equi* gen. nov., sp. nov., assigned to the Neocallimasticaceae. Biosystems 21:403–415

Gordon GLR, Phillips MW (1989) Degradation and utilization of cellulose and straw by three different anaerobic fungi from the ovine rumen. Appl Environ Microbiol 55:1703–1710

Gosh A, Gosh BK, Trimino-Vazquez H, Eveleigh DE, Montenecourt BS (1984) Cellulase secretion from hyper-cellulolytic mutant of *Trichoderma reesei* Rut-C30. Arch Microbiol 140:126–133

Ho YW, Abdullah N, Jalaludin S (1990) *Ruminomyces elegans* gen. et spec. nov., a polycentric rumen fungus from cattle. Mycotaxon 38:397–405

Lowe SE, Theodorou MK, Trinci APJ (1987a) Growth and fermentation of an anaerobic rumen fungus on various carbon sources and effect of temperature on development. Appl Environ Microbiol 53:1210–1215

Lowe SE, Theodorou MK, Trinci APJ (1987b) Cellulases and xylanase of an anaerobic rumen fungus grown on wheat straw, wheat straw holocellulose, cellulose, and xylan. Appl Environ Microbiol 53:1216–1223

Miller GL (1959) Use of dinitrosalicylic as reagent for the determination of reducing sugars. Anal Chem 31:426–428

Milne A, Theodorou MK, Jordan MGC, King-Spooner C, Trinci APJ (1989) Survival of anaerobic fungi in feces, in saliva, and in pure culture. Exp Mycol 13:27–37

Möllering H (1974) L-malat. Bestimmung mit Malat-Dehydrogenase and Glutamat-Oxalacetat-Transaminase. In: Bergmeyer HU, Gawehn K (eds) Methoden der enzymatischen Analyse, Band 2. Verlag Chemie, Weinheim, pp 1636–1639

Mountfort DO, Asher RA (1985) Production and regulation of cellulase by two strains of the rumen anaerobic fungus *Neocallimastix frontalis*. Appl Environ Microbiol 49:1314–1322

Müller M (1988) Energy metabolism of protozoa without mitochondria. Annu Rev Microbiol 42:465–488

Orpin CG (1981) Isolation of cellulolytic phycomycete fungi from the caecum of the horse. J Gen Microbiol 123:287–296

Orpin CG (1988) Nutrition and biochemistry of anaerobic Chytridiomycetes. Biosystems 21:365–370

Phillips MW, Gordon GLR (1989) Growth characteristics on cellobiose of three different anaerobic fungi isolated from the ovine rumen. Appl Environ Microbiol 55:1695–1702

Russell JB, Baldwin RL (1978) Substrate preferences in rumen bacteria: evidence of catabolite regulatory mechanisms. Appl Environ Microbiol 36:319–329

Sleat R, Mah RA (1984) Quantitative method for colorimetric determination of formate in fermentation media. Appl Environ Microbiol 47:884–885

Steinbüchel A, Müller M (1986) Glycerol, a metabolic end product of *Trichomonas vaginalis* and *Tritrichomonas foetus*. Mol Biochem Parasitol 20:45–55

Teunissen MJ, Marras SAE, Op den Camp HJM, Vogels GD (1989) An improved method for the quantification of alcohols, volatile fatty acids, and lactate or 2,3-butanediol in biological samples. J Microbiol Methods 10:247–254

Teunissen MJ, Op den Camp HJM, Orpin CG, Huis in 't Veld JHJ, Vogels GD (1991) Comparison of growth characteristics of anaerobic fungi from ruminant and non-ruminant herbivores during cultivation in a defined medium. J Gen Microbiol 137:1401–1408

Updegraff DM (1969) Semimicro determination of cellulose in biological materials. Anal Chem 32:420–424

Williams AG, Orpin CG (1987) Polysaccharide-degrading enzymes formed by anaerobic rumen fungi grown on a range of carbohydrate substrates. Can J Microbiol 33:418–426

Williamson JR (1974) Succinat. In: Bergmeyer HU, Gawehn K (eds) Methoden der enzymatischen Analyse, Band 2. Verlag Chemie, Weinheim, pp 1661–1666

Wood TM (1989) Mechanisms of cellulose degradation by enzymes from aerobic and anaerobic fungi. In: Coughlan MO (ed) Enzyme systems for lignocellulosic degradation. Elsevier Applied Science, London, pp 17–35

Wood TM, Wilson CA, McCrae SI, Joblin KN (1986) A highly active extracellular cellulase from the anaerobic fungus *Neocallimastix frontalis*. FEMS Microbiol Lett 34:37–40

Yarlett N, Orpin CG, Yarlett NC, Greenwood CA (1986) Hydrogenosomes in the rumen fungus *Neocallimastix patriciarum*. Biochem J 236:729–739