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# The Anaerobic Fungus *Neocallimastix* sp. Strain L2: Growth and Production of (Hemi)Cellulolytic Enzymes on a Range of Carbohydrate Substrates

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**Abstract.** The anaerobic fungus *Neocallimastix* sp. strain L2, isolated from the feces of a llama, was tested for growth on a range of soluble and insoluble carbohydrate substrates. The fungus was able to ferment glucose, cellobiose, fructose, lactose, maltose, sucrose, soluble starch, inulin, filter paper cellulose, and Avicel. No growth was observed on arabinose, galactose, mannose, ribose, xylose, sorbitol, pectin, xylan, glycerol, citrate, soya, and wheat bran. The fermentation products after growth were hydrogen, formate, acetate, ethanol, and lactate. The fermentation pattern was dependent on the carbon source. In general, higher hydrogen production resulted in decreased formation of lactate and ethanol. Recovery of the fermented carbon in products at the end of growth ranged from 50% to 80%. (Hemi)cellulolytic enzyme activities were affected by the carbon source. Highest activities were found in filtrates from cultures grown on cellulose. Growing the fungus on inulin and lactose yielded the lowest cellulolytic activities. Highest specific activities for avicelase, endoglucanase, β-glucosidase, and xylanase were obtained with Avicel as the substrate for growth (0.29, 5.9, 0.57, and 13 IU · $mg^{-1}$ protein, respectively). Endoglucanase activity banding patterns after SDS-PAGE were very similar for all substrates. Minor differences indicated that enzyme activities may in part be the result of secretion of different sets of isoenzymes.

Anaerobic fungi isolated from the digestive tract or feces of herbivorous mammals, both ruminant and nonruminant, produce and secrete a broad range of polysaccharide-degrading enzymes during growth on various soluble and insoluble carbon sources [32]. These enzymes provide these fungi with the potential to effectively degrade the major structural compounds in plant cell walls in vivo [1, 8] as well as in vitro [23, 28, 34, 35, 37]. Among the secreted proteins, the cellulolytic and hemicellulolytic enzymes are studied most extensively. The specific activities of the cellulases from anaerobic fungi are high compared with those of aerobic fungal strains of *Trichoderma reesei* and *T. viride* [28]. The latter fungi are known to be the most powerful in cellulose-degrading activity [5, 10] and are the subject of worldwide studies for their possible industrial use in converting cellulose to glucose. From the economic viewpoint regarding production of (hemi)cellulolytic enzymes from anaerobic fungi, it is desirable to use substrates that enhance enzyme yield.

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To date the fungus *Neocallimastix* sp. strain L2, isolated from a llama, has been studied in detail mainly in relation to its (intracellular) metabolic pathways with emphasis on the role of the hydrogenosomes [15, 16]. No information was found in the literature with respect to the production of extracellular (hemi)cellulolytic enzymes by this organism. This study describes the growth characteristics of *Neocallimastix* sp. strain L2 grown on various carbon sources. In addition, their impact on the production of (hemi)cellulolytic enzymes by the fungus was studied. This information is required for possible future commercial utilization of these enzymes.

## Materials and Methods

**Organism and growth conditions.** *Neocallimastix* sp. strain L2 was isolated from the feces of a llama (*Lama glama*) by Marvin-Sikkema and associates

[14], and stock cultures were maintained on medium M2 containing 0.5% wheat straw [28]. Cultures were subcultured every 3–4 days [27].

**Carbohydrate utilization.** Substrates (mono-, oligo- and polysaccharides) were tested in quadruplicate. Cellobiose, fructose, galactose, pectin, and oat spelt xylan were purchased from Sigma (St. Louis, Missouri, USA); arabinose, glucose, inulin, lactose, maltose, mannose, ribose, sucrose, xylose, glycerol, sorbitol, citrate, and soluble starch from Merck (Darmstadt, Germany); filter paper cellulose (FPC) from Whatman (No. 1; Whatman Ltd., Maidstone, UK) and Avicel (type PH 105) from Serva (Heidelberg, Germany). Experiments for carbohydrate utilization with monomeric substrates, sucrose, and cellobiose were conducted with 180 mM fermentable carbon in 20 ml medium M2 [30]. Soluble substrates were sterilized separately before adding to the medium. The growth experiments with wheat bran, soya, pectin, oat spelt xylan, inulin, FPC, and Avicel were carried out at 5 $g \cdot L^{-1}$. Experimental cultures were obtained after subculturing the fungus thrice on each substrate. Lack of growth of the fungus on a particular substrate was based on four individual experiments, with incubation times of up to 10 days.

**Sample collection.** Fungal growth was monitored by hydrogen production. After growth ceased, four parallel cultures were harvested by filtration through glass-fiber filters (Whatman, grade C). Samples of the culture filtrates were stored at −20°C until used for determination of enzyme activities and analysis of water-soluble fermentation products and reducing sugar content. The remaining filtrates were combined for SDS-PAGE analysis. Concentration and collection of the protein was performed as described [30].

**Analytical procedures.** Enzyme assays were performed in duplicate at optimum pH and temperature as described previously [28]. All enzyme reactions were linear over the period of the assays. Enzyme and substrate controls were included in all assays.

The fermentation products—lactate, ethanol, and acetate—were determined as described previously [26]. Hydrogen in head space samples was quantified by gas chromatography [27]. Formic acid was determined by the colorimetric method of Sleat and Mah [25]. For Fig. 1, calculations were made as follows: for each growth substrate the total molar amount of fermentation products was defined as the sum of the hydrogen, acetate, formate, ethanol, and lactate produced. Relative amounts were expressed as the molar ratio (%) of specific products and the total amount of fermentation products.

Protein concentrations in the enzyme preparations were determined with Bio-Rad protein reagent (Bio-Rad Laboratories, Richmond, Virginia, USA) with bovine γ-globulin as a standard. Reducing sugar contents were determined according to the method of Miller [17]. Residual polysaccharide was determined as described by Updegraff [33].

SDS-PAGE was performed in 10% polyacrylamide slab gels in the presence of SDS (0.1%, wt/vol) as described by Laemmli [12]. Enzyme samples were pretreated with sample buffer for 18 h at 20°C [30]. SDS-PAGE molecular weight standards (High Molecular Weight Calibration Kits) were obtained from Bio-Rad Laboratories. Electrophoresis was conducted at 15°C with a constant current of 40 mA until the tracking dye reached the anode end of the slab gels. Gels were stained for protein with Coomassie Brilliant Blue G-250 (Serva, Heidelberg, Germany). Zymogram analysis for the detection of endoglucanase was performed as described [30], with the substrate carboxymethylcellulose (CMC) added before polymerization of the gel.

## Results

**Growth of *Neocallimastix* sp. strain L2 on various substrates.** *Neocallimastix* sp. strain L2 was tested for its ability to grow on various carbon sources. The fungus exhibited growth on glucose, fructose, cellobiose, maltose, lactose, sucrose, inulin, soluble starch, Avicel, and FPC, but was not able to grow on arabinose, galactose, mannose, ribose, xylose, sorbitol, pectin, xylan, glycerol, citrate, soya, and wheat bran. The time period within which growth occurred, as measured by $H_2$ production, was dependent on the substrate tested and varied from 60 h for fructose to 228 h for maltose. Reducing sugar measurements revealed nearly complete utilization of mono- and disaccharides by the fungus, except for lactose. The high amount of sugar present in culture filtrates after growth on this substrate is due to galactose accumulation, since no growth was obtained on this carbon source. Avicel, FPC, and soluble starch were completely digested, as almost no sugars (less than 2%)

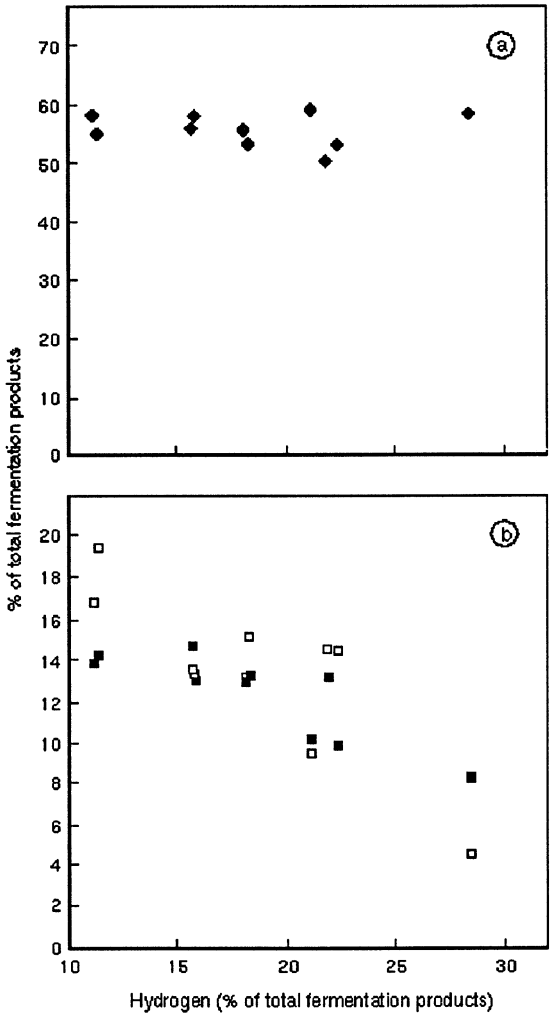


Fig. 1. Relative formation of fermentation products by *Neocallimastix* sp. strain L2 grown on various carbohydrate substrates as a function of the relative hydrogen production. (a) acetate + formate; (b) ethanol (■) and lactate (□). Calculations were performed as described in Materials and Methods.

Table 1. Fermentation products after growth of *Neocallimastix* sp. strain L2 on various carbohydrate substrates

| Carbon source | Fermentation product (mM) | | | | |
|---|---|---|---|---|---|
| | Hydrogen | Formate | Acetate | Ethanol | Lactate |
| Glucose | 8.8 ± 1.3 | 23.8 ± 2.0 | 22.5 ± 1.7 | 11.1 ± 0.2 | 13.4 ± 1.4 |
| Cellobiose | 14.0 ± 0.5 | 18.0 ± 5.0 | 15.4 ± 2.5 | 6.2 ± 1.3 | 9.1 ± 1.7 |
| Fructose | 12.3 ± 0.4 | 24.2 ± 1.5 | 21.0 ± 0.7 | 10.2 ± 0.6 | 10.5 ± 0.6 |
| Lactose | 13.9 ± 1.7 | 14.3 ± 0.7 | 14.3 ± 1.4 | 4.1 ± 0.3 | 2.3 ± 0.6 |
| Maltose | 16.1 ± 1.7 | 23.5 ± 1.9 | 21.4 ± 2.6 | 7.8 ± 1.0 | 7.3 ± 0.5 |
| Sucrose | 12.8 ± 0.8 | 18.4 ± 2.4 | 21.0 ± 1.7 | 9.2 ± 0.9 | 9.4 ± 1.7 |
| Inulin | 13.9 ± 1.9 | 18.6 ± 4.7 | 21.9 ± 1.7 | 10.1 ± 1.3 | 11.6 ± 3.1 |
| Starch | 13.7 ± 1.2 | 10.0 ± 1.2 | 21.5 ± 0.8 | 8.3 ± 2.3 | 9.1 ± 1.1 |
| Avicel | 10.7 ± 1.1 | 26.7 ± 3.9 | 22.0 ± 1.2 | 12.6 ± 0.6 | 17.2 ± 1.6 |
| Filter paper | 12.3 ± 0.7 | 22.2 ± 2.5 | 21.9 ± 1.5 | 11.6 ± 1.4 | 10.7 ± 3.4 |

Each value represents the mean ± SD (n = 4).

Table 2. Enzyme production by *Neocallimastix* sp. strain L2 after growth on various substrates

| Substrate | Enzyme activity (IU · ml$^{-1}$) | | | | Protein[a] |
|---|---|---|---|---|---|
| | Avicelase | β-Glucosidase | Endoglucanase | Xylanase | |
| Glucose | 0.008 ± 0.001 | 0.034 ± 0.002 | 0.27 ± 0.06 | 1.13 ± 0.13 | 0.235 ± 0.023 |
| Cellobiose | 0.008 ± 0.001 | 0.038 ± 0.008 | 0.24 ± 0.02 | 0.75 ± 0.10 | 0.156 ± 0.019 |
| Fructose | 0.007 ± 0.002 | 0.036 ± 0.009 | 0.26 ± 0.08 | 1.49 ± 0.51 | 0.180 ± 0.043 |
| Lactose | 0.004 ± 0.001 | 0.017 ± 0.006 | 0.15 ± 0.04 | 0.92 ± 0.07 | 0.079 ± 0.007 |
| Maltose | 0.010 ± 0.003 | 0.068 ± 0.002 | 0.37 ± 0.19 | 1.98 ± 0.21 | 0.149 ± 0.038 |
| Sucrose | 0.008 ± 0.002 | 0.052 ± 0.001 | 0.21 ± 0.05 | 1.71 ± 0.05 | 0.197 ± 0.033 |
| Inulin | 0.005 ± 0.003 | 0.021 ± 0.007 | 0.016 ± 0.03 | 1.31 ± 0.22 | 0.123 ± 0.019 |
| Starch | 0.011 ± 0.001 | 0.058 ± 0.009 | 0.39 ± 0.07 | 1.79 ± 0.12 | 0.160 ± 0.039 |
| Avicel | 0.055 ± 0.006 | 0.109 ± 0.027 | 1.13 ± 0.13 | 2.49 ± 0.18 | 0.192 ± 0.059 |
| Filter paper | 0.051 ± 0.005 | 0.150 ± 0.010 | 1.29 ± 0.21 | 2.10 ± 0.17 | 0.175 ± 0.026 |

Each value represents the mean ± SD (n = 4).
[a] Protein secreted is expressed as mg · ml$^{-1}$.

could be detected upon hydrolysis with sulfuric acid. Moreover, reducing sugars were hardly detectable in culture filtrates after growth on these substrates.

**Fermentation products.** End products from the fermentation of the tested substrates were hydrogen, formate, lactate, ethanol, and acetate (Table 1). Malate and succinate were not determined because of the small amounts produced by anaerobic fungi [6, 28]. The major fermentation products—formate and acetate—were formed in nearly equal amounts with the exception of cultures grown on starch. Lactate and ethanol formation showed strong variation by the cultures on different substrates. Hydrogen production varied from 8.8 mM to 16.1 mM for glucose- and maltose-grown cultures, respectively. The amount of carbon recovered in fermentation products varied from 50% to 80%. When the relative production of acetate plus formate was plotted against the relative hydrogen production (Fig. 1a), no interdependence was observed. However, increased hydrogen production resulted in lower relative levels of both ethanol and lactate (Fig. 1b).

**Production of (hemi)cellulolytic enzymes.** During growth of the fungus, cellulolytic and xylanolytic enzymes were secreted into the surrounding medium (Table 2). Enzyme activities were observed to be dependent on the substrates tested. In general, highest activities were found in filtrates from cultures grown on the insoluble substrates Avicel and FPC. Growing the fungus on inulin and lactose yielded the lowest cellulolytic activity. Avicelase activities varied from 0.004 to 0.055 IU · ml$^{-1}$. Very high production was observed when Avicel was the substrate for growth. On the other hand, inulin and lactose were found to be rather poor inducers of avicelase activity. Activities towards CMC, used to estimate endoglucanase activity, ranged from 0.15 to 1.29 IU · ml$^{-1}$ for lactose- and FPC-grown cultures, respectively. A similar profile was obtained for β-glucosidase activities (0.017–0.150 IU · ml$^{-1}$). In contrast to the findings for cellulo-

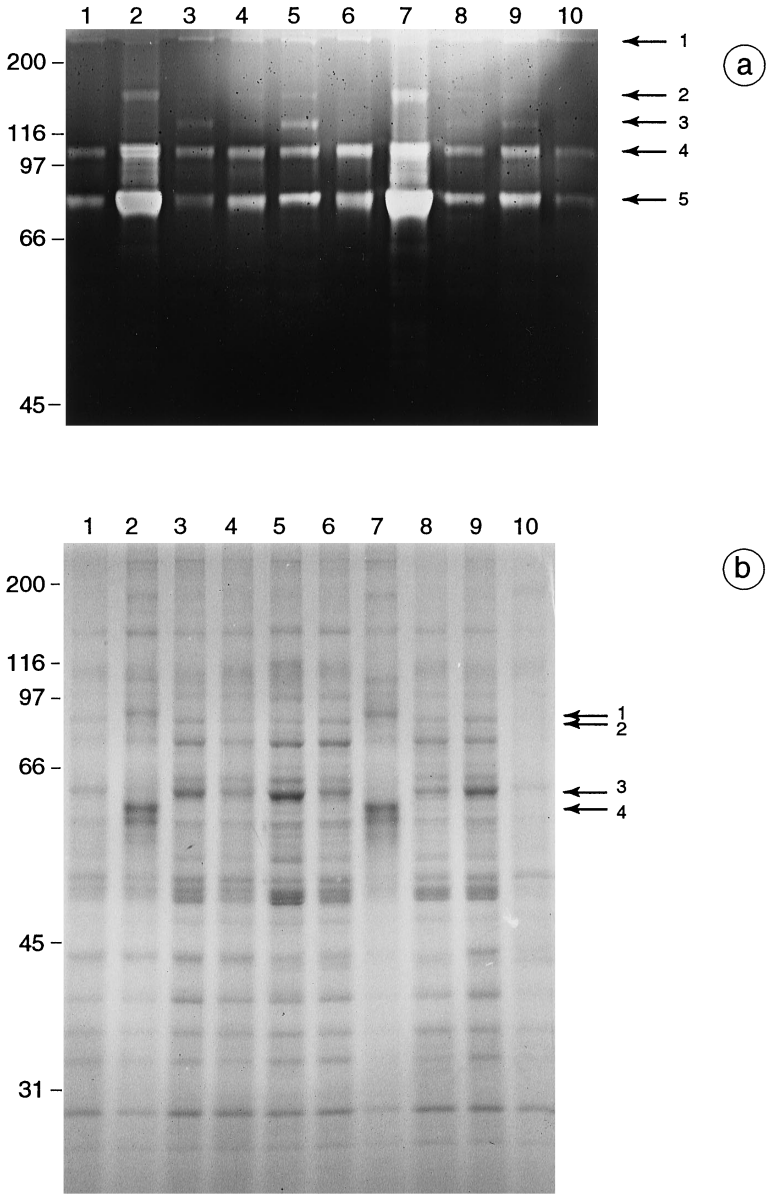


Fig. 2. Enzyme activity and protein banding patterns of extracellular protein from *Neocallimastix* sp. strain L2 grown on several carbon sources. After SDS-PAGE, gels were stained for endoglucanase activity (a) or protein (b). Lanes from left to right: (1) inulin, (2) Avicel, (3) sucrose, (4) fructose, (5) soluble starch, (6) glucose, (7) filter paper cellulose, (8) cellobiose, (9) maltose, and (10) lactose. The amounts of protein applied to the gels are (lanes 1, 3, 4, 5, 6, 8, 9, 10) 20 μg for endoglucanase and protein staining; (lanes 2 and 7) 5 μg and 15 μg for endoglucanase and protein staining, respectively. Activity and protein bands discussed in the text are marked with arrows.

lytic activities, the differences between xylanase activities for FPC- or Avicel-grown cultures and cultures on soluble substrates were not dramatic.

The amount of protein secreted was relatively constant (Table 2). However, relatively low values were found for cultures on lactose. The highest specific activities of avicelase, endoglucanase, and β-glucosidase were obtained with Avicel as growth substrate and were 0.29, 5.9, and 0.57 IU · $mg^{-1}$ protein respectively. Maltose- and starch-grown cultures also showed high values. Specific xylanase activities were highest for maltose- and Avicel-grown cultures (13 IU · $mg^{-1}$ protein). Glucose and cellobiose gave rise to relatively low specific xylanase activities.

**Enzyme activity and protein patterns after SDS-PAGE.** The carbon sources on which *Neocallimastix* sp. strain L2 was grown had an effect on the type and amount of isozymes with endoglucanase activity (Fig. 2). The most distinct endoglucanase banding patterns were observed for cultures grown on cellulose (Fig. 2a, lanes 2 and 7). In all cases an activity band was observed at a molecular mass of over 300 kDa (arrow 1). Growth on all substrates yielded two major endoglucanase activity bands at molecular weights of 105 and 77 kDa (arrows 4 and 5). An activity band at a molecular mass of 151 kDa (arrow 2) was present in all cases, except when inulin or lactose was used as the substrate. The presence of this activity band was most obvious for Avicel- and FPC-

grown cultures. An activity band at a molecular weight of 126 kDa (arrow 3) was found for cultures on sucrose, fructose, starch, cellobiose, and maltose.

The influence of the carbon source on the synthesis of individual proteins is visualized in Fig. 2b. The banding pattern for the lactose-grown culture was very faint, despite the fact that the amount of protein applied to the gel was almost the same. The most obvious difference in the banding patterns is the fact that two proteins of respectively 75 and 61 kDa (arrows 1 and 4) were found only for Avicel- and FPC-grown cultures. Furthermore, a protein of 72 kDa (arrow 2) was observed only for cultures grown on sucrose, fructose, starch, glucose, cellobiose, and maltose. A protein of 63 kDa (arrow 3) was present in all cases, except when Avicel or FPC were the growth substrate. In general, banding patterns were quite similar and differed mainly in intensity.

## Discussion

*Neocallimastix* sp. strain L2 was able to utilize a range of carbon sources for growth. No growth occurred on xylose or xylan. This is contradictory to previous observations [15] and also in conflict with the finding that this fungus, grown on various carbon sources, secreted enzymes with xylanolytic activity. Furthermore, all *Neocallimastix* species studied thus far are able to utilize these carbon sources [2, 24, 31, 34, 36], with the exception of *N. patriciarum* strain N2 [31]. A possible explanation could be the poor adaptational capacity of this organism to changes in the medium. After transfer from one carbon source to another, we observed a long lag phase. In contrast to previous findings [15], we observed growth of *Neocallimastix* sp. strain L2 on inulin, and culture filtrates from inulin-grown fungus contained inulinase activity (1.1 IU $\cdot$ $mg^{-1}$ protein at 50°C). The only exception for utilization of inulin by *Neocallimastix* species to date has been reported for *N. frontalis* strain N1 [31]. Lactose could serve as a source of carbon for *Neocallimastix* sp. strain L2, but only the glucose part of this disaccharide is fermented by the fungus. Galactose accumulates in the medium, since it is not a growth substrate. The same results were reported for other anaerobic fungi [24, 31]. Only a few examples exist of utilization of galactose [2, 22].

The fermentation pattern of *Neocallimastix* sp. strain L2 was influenced by the growth substrate. Increased hydrogen production correlated with decreased formation of other reduced products (e.g., lactate and ethanol), whereas the relative amounts of acetate and formate were constant. Comparable results were obtained from calculations with data reported for *Piromyces* sp. strain E2 [30]. Apparently, growth substrates had an impact on the carbon flow through the hydrogenosome. Similar shifts were observed when anaerobic fungi were co-cultivated with hydrogen-consuming microorganisms [4, 11, 14, 18, 29].

As for other anaerobic fungi [31, 34, 35], the production of cellulolytic enzymes by *Neocallimastix* sp. strain L2 was substrate dependent. Specific enzyme activities fell in the range reported in the literature for anaerobic fungi [20]. Highest production was observed when the fungus was grown on cellulose (Avicel or FPC). Soluble substrates generally yielded lower enzyme production. Similar results were obtained with *N. frontalis* [19, 22] and *N. patriciarum* [21, 23, 31]. The effect of cellulose was most pronounced for avicelase activity, which was 5- to 10-fold higher compared with soluble substrates. In this study, cellobiose was shown to be not so effective an inducer of cellulases as reported for *N. frontalis* [19].

Xylanase production was observed to be more or less constitutive. For other anaerobic fungi, xylanase production was reported to be lower on soluble sugars than on insoluble substrates [9, 13, 31, 34].

SDS-PAGE analysis revealed that growth on different substrates gave similar patterns for endoglucanase activity. However, as for *N. frontalis* [3] and *Neocallimastix* strains N1 and N2 [31], some influence by the substrate was observed. The main differences were due to the nature of the substrate, i.e., soluble or insoluble. Similar observations have been described for *N. frontalis* [3]. Differences in enzyme production on various carbon sources might in part be caused by secretion of different mixtures of isoenzymes. As for activity patterns, protein banding was substrate dependent to a limited extent.

In conclusion, *Neocallimastix* sp. strain L2, like other anaerobic fungi, is able to grow on various carbon sources. The yields of cellulolytic and xylanolytic enzymes were shown to be dependent on the growth substrate. Production was constitutive, but Avicel and FPC gave rise to higher enzyme yields. To explore potential application, the effectiveness of solubilization of (hemi)cellulose polymers by the enzymes produced by the fungus has to be assessed. For several anaerobic fungi, (hemi)cellulolytic enzymes were in part found to be associated in a multiprotein complex [7, 20]. Whether *Neocallimastix* sp. strain L2 also produces complex bound cellulases remains to be elucidated.

### Literature Cited

1. Akin DE, Gordon GLR, Hogan JP (1983) Rumen bacterial and fungal degradation of *Digitaria pentzii* grown with or without sulfur. Appl Environ Microbiol 46:738–748
2. Barichievich EM, Calza R (1990) Media carbon induction of extracellular cellulase activities in *Neocallimastix frontalis* isolate EB188. Curr Microbiol 20:265–271
3. Barichievich EM, Calza R (1990) Supernatant protein and cellulase

activities of the anaeobic ruminal fungus *Neocallimastix frontalis* EB188. Appl Environ Microbiol 56:43–48
4. Bauchop T, Mountfort DO (1981) Cellulose fermentation by a rumen anaerobic fungus in both the absence and the presence of rumen methanogens. Appl Environ Microbiol 42:1103–1110
5. 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 146:301–308
6. 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
7. Dijkerman R, Vervuren MBF, Op den Camp HJM, van der Drift C (1996) Adsorption characteristics of cellulolytic enzymes from the anaerobic fungus *Piromyces* sp. strain E2 on microcrystalline cellulose. Appl Environ Microbiol 62:20–25
8. Gordon GLR (1985) The potential of manipulation of ruminal fungi. Rev Rural Sci 6:124–128
9. Gordon GLR, Phillips MW (1989) Comparative fermentation properties of anaerobic fungi from the rumen. In: Nolan JV, Leng RA, Demeyer DI (eds) The role of protozoa and fungi in ruminant digestion. Armidale: Penambul Books, pp 127–131
10. Ghosh A, Ghosh 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
11. Joblin KN, Naylor GE, Williams AG (1990) Effect of *Methanobrevibacter smithii* on xylanolytic activity of anaerobic ruminal fungi. Appl Environ Microbiol 56:2287–2295
12. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
13. Lowe SE, Theodorou MK, Trinci APJ (1987) Cellulases and xylanase of an anaerobic rumen fungus grown on wheat straw, wheat straw holocellulose, cellulose, and xylan. Appl Environ Microbiol 53:1216–1223
14. Marvin-Sikkema FD, Richardson AJ, Stewart CS, Gottschal JC, Prins RA (1990) Influence of hydrogen-consuming bacteria on cellulose degradation by anaerobic fungi. Appl Environ Microbiol 56:3793–3797
15. Marvin-Sikkema FD, Laphor GA, Kraak MN, Gottschal JC, Prins RA (1992) Characterization of an anaerobic fungus from llama faeces. J Gen Microbiol 138:2235–2241
16. Marvin-Sikkema FD, Pedro Gomes TM, Grivet JP, Gottschal JC, Prins RA (1993) Characterization of hydrogenosomes and their role in glucose metabolism of *Neocallimastix* sp. L2. Arch Microbiol 160:388–396
17. Miller GL (1959) Use of dinitrosalicylic acid as reagent for the determination of reducing sugars. Anal Chem 31:426–428
18. Mountfort DO, Asher RA, Bauchop T (1982) Fermentation of cellulose to methane and carbon dioxide by a rumen anaerobic fungus in a triculture with *Methanobrevibacter* sp. strain RA-1 and *Methanosarcina barkeri*. Appl Environ Microbiol 44:128–134
19. 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
20. Op den Camp HJM, Dijkerman R, Teunissen MJ, van der Drift C (1994) Production of depolymerizing enzymes by anaerobic fungi. In: Prins RA, Stewart CS (eds) Microorganisms in ruminant nutrition. Nottingham: University Press, pp 113–126
21. Orpin CG (1983) The role of ciliate protozoa in the rumen digestion of plant cell walls. Anim Feed Sci Technol 10:121–143
22. Orpin CG, Bountiff L (1978) Zoospore chemotaxis in the rumen phycomycete *Neocallimastix frontalis*. J Gen Microbiol 104:113–122
23. Orpin CG, Letcher AJ (1979) Utilization of cellulose, starch, xylan, and other hemicelluloses for growth of the phycomycete *Neocallimastix frontalis*. Curr Microbiol 3:121–124
24. Phillips MW, Gordon GLR (1988) Sugar and polysaccharide fermentation by rumen anaerobic fungi from Australia, Britain and New Zealand. BioSystems 21:377–388
25. Sleat R, Mah RA (1984) Quantitative method for colorimetric determination of formate in fermentation media. Appl Environ Microbiol 47:884–885
26. 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
27. 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
28. Teunissen MJ, Smits TAM, Op den Camp HJM, Huis in 't Veld JHJ, Vogels GD (1991) Fermentation of cellulose and production of cellulolytic and xylanolytic enzymes by anaerobic fungi from ruminant and non-ruminant herbivores. Arch Microbiol 156:290–296
29. Teunissen MJ, Kets EPW, Op den Camp HJM, Huis in 't Veld JHJ, Vogels GD (1992) Effect of coculture of anaerobic fungi isolated from ruminants and non-ruminants with methanogenic bacteria on cellulolytic and xylanolytic enzyme activities. Arch Microbiol 157:176–182
30. Teunissen MJ, de Kort GVM, Op den Camp HJM, Huis in 't Veld JHJ (1992) Production of cellulolytic and xylanolytic enzymes during growth of the anaerobic fungus *Piromyces* sp. on different substrates. J Gen Microbiol 138:1657–1664
31. Teunissen MJ, de Kort GVM, Op den Camp HJM, Vogels GD (1993) Production of cellulolytic and xylanolytic enzymes during growth of anaerobic fungi from ruminant and nonruminant herbivores on different substrates. Appl Biochem Biotechnol 39/40:177–189
32. Trinci APJ, Davies DR, Gull K, Lawrence MI, Nielsen BB, Rickers A, Theodorou MK (1994) Anaerobic fungi in herbivorous animals. Mycol Res 98:129–152
33. Updegraff DM (1969) Semimicro determination of cellulose in biological materials. Anal Chem 32:420–424
34. 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
35. Williams AG, Orpin CG (1987) Glycoside hydrolase enzymes present in zoospore and vegetative growth stages of the rumen fungi *Neocallimastix patriciarum*, *Piromyces communis*, and an unidentified isolate, grown on a range of carbohydrates. Can J Microbiol 33:427–434
36. Williams AG, Withers SE, Orpin CG (1994) Effect of the carbohydrate growth substrate on polysaccharolytic enzyme formation by anaerobic fungi isolated from the foregut and hindgut of nonruminant herbivores and the forestomach of ruminants. Lett Appl Microbiol 18:147–151
37. Zhu WY, Theodorou MK, Longland AC, Nielsen BB, Dijkstra J, Trinci APJ (1996) Growth and survival of anaerobic fungi in batch and continuous-flow cultures. Anaerobe 2:29–37