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Properties of anaerobic fungi isolated from several habitats: complexity of phenotypes

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Abstract. Isolates of anaerobic fungi from rumen, animal faeces and compost displayed morphological similarity with known anaerobic fungi. According to their ITS sequences, species were related to *Neocallimastix* and *Piromyces*. Rumen fungi tolerated exposure to an aerobic atmosphere for at least four days. Under anaerobic conditions, they could grow on both, defined or complex substrates. Growth in liquid media was monitored by the continuous measurement of metabolic gases (O_2, CO_2, H_2, CO, H_2S, CH_4). Monitored metabolism was complex, showed that both CO_2 and H_2 were produced and subsequently consumed by yet unknown metabolic pathway(s). CO and H_2S were evolved similarly, but not identically with the generation of CO_2 and H_2 suggesting their connection with energetic metabolism. Anaerobic fungi from snail faeces and compost produced concentrations of H_2S, H_2, CO near the lower limit of detection. The rumen isolates produced cellulases and xylanases with similar pH and temperature optima. Proteolytic enzymes were secreted as well. Activities of some enzymes of the main catabolic pathways were found in cell-free homogenates of mycelia. The results indicate the presence of the pentose cycle, the glyoxylate cycle and an incomplete citrate cycle in these fungi. Differences between isolates indicate phenotypic variability between anaerobic fungi.

Key words: Anaerobic fungi — Molecular taxonomy — Production of CO, CO_2, H_2, H_2S-hydrolases — Catabolic pathways

Introduction

Since their discovery (Orpin 1975, 1977; Bauchop 1979), anaerobic fungi have attracted the attention of mycologists. They may represent an example of adaptation of aerobic organisms to anaerobic environments (Hackstein et al. 2001). They are inhabitants of the gastrointestinal system of higher animals, and can also be found in related habitats, such as faeces (Davies et al. 1993; Nielsen et al. 1995; Leis et al. 2014; Callaghan et al. 2015). They digest the plant fibres in the gut of animals (Gordon and Phillips 1989, 1998), perhaps, in synergy with rumen bacteria (Marvin-Sikkema et al. 1990; Kopečný et al. 1996). Mechanisms underlying their action in rumen may be similar to those known in aerobic fungi, i.e. being mediated by secreted cellulases, hemicellulases, xylanases and proteases (Bauchop 1989). These hydrolases of anaerobic fungi are probably the most intensively studied (Borneman et al. 1989; Asao et al. 1993; Borneman and Akin 1994; Tripathi et al. 2007; Paul et al. 2010; Haitjema et al. 2014; Wang et al. 2014).

The anaerobic way of life obviously has an impact on the bioenergetics of anaerobic fungi. It is accepted that anaerobic fungi convert the energy of substrates to ATP by fermentative pathways and substrate-level phosphorylation. Multiple fermentative products, such as acetate, lactate, ethanol, formate, succinate, CO_2 and H_2 (Bauchop 1989) have been found. These could be formed in the cytoplasm and in specialized organelles – hydrogenosomes. Hydrogenosomes have been documented in protists (Čerkasov et al.

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1978) but their existence in anaerobic fungi has been documented as well (Muller 1993), and their properties have been extensively studied (Marvin-Sikkema et al. 1994a, 1994b; Benchimol et al. 1997). They possess Fe-hydrogenase, an enzyme catalysing the reduction of protons and H_2 production (Davidson et al. 2002), and some metabolic pathways have been characterised (Akhmanova et al. 1999). The complete map of energy metabolism of anaerobic fungi, however, remains obscure. The origin and biogenesis of hydrogenosomes is also a topic of debate. Although hydrogenosomes are surrounded by a membrane (Benchimol 2009), they do not possess their own DNA and their biogenesis is still unclear (Hackstein et al. 2001).

Despite of the lack of information about the metabolism of anaerobic fungi, there are efforts to use them for biotechnological purposes (Wang et al. 2013, 2014; Gruninger et al. 2014; Haitjema et al. 2014).

In this work, we describe our experiments with new isolates of anaerobic fungi which do not belong to known taxonomic groups. We found that some of these isolates transiently produce H_2, CO and H_2S, and secrete hydrolases. The presence of major catabolic pathways was probed by measurements of activities of relevant enzyme activities. Also, the anaerobic utilization of industrial bulk waste, such as rapeseed or sunflower meals or expellers with indigenous isolates of anaerobic fungi was observed.

Materials and Methods

Microorganisms and culture conditions

The aerobic fungi *Trichoderma atroviride* CCM F–534, and *Aspergillus niger* CCM 8189 from the Czech Collection of Microorganisms, Masaryk University, Faculty of Science, Brno, Czech Republic were cultured in Czapek Dox medium (Biomark Laboratories, Pune, India) at room temperature.

Anaerobic fungi were isolated from the rumen fluid of cattle and sheep (Svaman slaughterhouse, Myjava, Slovakia); from the faeces of llamas, giraffes, and camels (provided by The Zoological Garden, Bratislava, Slovakia). Faeces of snails (*Helix pomatia*) were obtained by feeding snails found in a local garden (Modra, Slovakia) in glass container for 24 h. The isolate from compost was isolated from a sample of mature compost excavated from the depth about 60 cm in a local garden (Modra, Slovakia). The samples were immediately transported to the laboratory in anaerobic boxes (Merck, Darmstadt, Germany) under an atmosphere generated by a BBL GasPakPlus anaerobic system (Becton Dickinson and Company, Sparks, MD, USA). The isolation was performed according to the Koch dilution method using the procedure of Gordon and Phillips (1989). Aliquots of these samples were homogenised with pre-warmed (39°C) medium B (Lowe et al. 1985, without straw). Ten-fold serial dilutions of these homogenates were spread on the Petri dishes with medium B (with straw). Isolated strains were grown anaerobically at 39°C (using a Bactron I-2 anaerobic chamber, Sheldon Manufacturing, USA) or in closed anaerobic boxes, in a defined medium containing different sources of fermentable carbon — medium B or in M2 medium (Teunissen et al. 1991). The bacterial growth was inhibited by adding antibiotics (in g/l: streptomycin sulfate 2, penicillin G 8, chloramphenicol 6, oxytetracycline 5 and neomycin sulfate 6 and lysozyme (4 g/l) into media. In indicated experiments, glucose (medium B) and cellobiose (M2) was replaced by other carbon sources (meadow grass hay, wheat straw, molasses, etc.). Solid media were prepared by adding 18 g/l Agar Biospecial LL (Biolife, Italiana S.r.l., Milano, Italy) into the media. The oxygen-free conditions during cultivation were monitored by a 0.01% solution of resazurin (Gordon et al. 1971), which remained colourless during the cultivation.

Morphological observations

Morphology of isolates was examined by visible light and fluorescence microscopy (Zeiss AxioImager A1, Carl Zeiss, Jena, Germany). The presence of septa was observed using the fluorescent dye thioflavine according to Lakatoš et al. (2010).

Chemical and biochemical assays

Studies on the pH and temperature optima and stability of extracellular enzymes were made with cell-free supernatants from cultures grown in defined media. The activities of hydrolytic enzymes (cellulase, xylanase) were measured by means of determination of reducing sugar released from substrates (1% w/v microcrystalline cellulose or wheat straw) using 3,5-dinitrosalicylic acid (DNS) reagent (Miller 1959). Xylanase activity was measured by incubating 0.2 ml of supernatant with 1% xylan (w/v) in 0.8 ml of 0.1 mol/l acetate buffer (pH 6) at 30°C for 30 min, the supernatant being analysed for reducing sugar with DNS reagent. Cellulase activity was measured by incubating 0.2 ml of supernatant with 1% (w/v) microcrystalline cellulose in 0.8 ml of 0.1 mol/l acetate buffer (pH 5.5) at 40°C for 30 min, the supernatant being analysed for reducing sugar with DNS reagent.

The activities of intracellular enzymes were determined in the intracellular fraction (IF) supernatant. IF was prepared as follows: mycelia were suspended in homogenization solution (50 mmol/l phosphate buffer, 0.7 mol/l KCl, pH 7.5) and mixed with half volume of glass pearls (diameter 0.1–0.2 mm). The suspension was cooled in ice and vor-

texed 10 × 60 s with a 60 s pause in ice. Glass pearls were sedimented and the suspension was spun down for 20 min at 10000 rpm (11000 × *g*) at 4°C in a K24 centrifuge (Zentrifugenbau Engelsdorf, Germany). The supernatant was used for intracellular enzymes activity testing after their protein content was determined. The activities of glyoxylate and citrate cycle (citrate synthase, isocitrate lyase, isocitrate dehydrogenase) enzymes and glutamate dehydrogenase were measured spectrophotometrically (Specord 250, Analytik Jena) after incubation of an enzyme sample (0.02–0.2 ml, according to the enzyme activity) with the corresponding reagent solution (1 ml).

Citrate synthase was measured by the spectrophotometric method using Ellman's reagent (Shepherd and Garland 1969). Activity was calculated using extinction coefficient ε (($\varepsilon_{DTNB\text{-}CoA} = 14.2.10^3$ (mol/l)$^{-1}$·cm^{-1})). Isocitrate dehydrogenase activity was measured spectrophotometrically according to Plaut (1962), using NAD^+ as coenzyme/co-substrate. Isocitrate lyase was measured by the phenylhydrazine method (($\varepsilon_{fenylhydrazon} = 17.10^3$ (mol/l)$^{-1}$·cm^{-1})) described by McFadden (1969). Glutamate dehydrogenase activity was measured by spectrophotometric method of Strecker (1955) using NAD^+ as coenzyme/co-substrate. Enzyme activities involving NADH were calculated using $\varepsilon_{NADH} = 3.3.10^6$ cm^2/mol. Enzyme activities were measured at 37°C.

Proteolytic activity was measured using the chromolytic substrate azocasein (Sigma-Aldrich). 0.1 ml of supernatant was mixed with 0.4 ml of 0.25% azocasein (w/v in 0.1 mol/l phosphate buffer, pH 6.8) at 37°C for 3 h. The reaction was stopped by the addition of 0.4 ml 10% (w/v) trichloroacetic acid (TCA). Non-degraded azocasein was removed by centrifuging samples in a microcentrifuge (MiniSpin Plus Eppendorf, Hamburg, Germany, 10 min, 13.2 × 10^3 rpm). Spectrophotometry (0.85 ml of supernatant with 0.32 ml 1 mol/l NaOH) was performed at 440 nm.

Proteins were analysed by the Bradford method (Bradford 1976).

DNA extraction

Genomic DNA was extracted from fungal biomass using Simax^{TM} Genomic DNA Extraction (Beijing SBS Genetech Co., LTD., China). DNA was resuspended in double distilled water and stored at – 20°C prior to PCR amplification. The quality of the isolated DNA was checked electrophoretically (1% w/v agarose gel run in 1 mmol/l Tris-acetate-EDTA buffer).

Amplification of the ribosomal ITS1 region

The ribosomal ITS1 region defined by primers GM1 (5'-TGTACACACCGCCCGTC-3') and GM2 (5'-CTGCGTTCTTCATCGAT-3') (Brookman et al. 2000) was amplified from genomic DNA by PCR using hot-start HotMaster^{TM} Taq DNA Polymerase (5Prime, Hamburg, Deutschland). The cycling conditions were as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 48°C for 1 min, extension at extension 68°C for 1 min and final extension 68°C for 10 min. The presence of a single product of about 400 bp was verified by agarose gel electrophoresis. The PCR product was purified for subsequent manipulations using a QIA-quick-spin PCR purification column (Metabion, Martinsried, Germany), following the manufacturer's protocol. The PCR products were sequenced using an ABI Prism sequencer at the BITCET (Biotechnology Centre) program facility. The obtained sequences were aligned with published data in gene bank (BLAST http://www.ncbi. nlbi.gov) and the phylogenetic analysis was performed according to [http://phylogeny.lirmm.fr/phylo_cgi/simple_phylogeny.cgi] (Castresana 2000; Edgar 2004; Anisimova and Gascuel 2006; Chevenet et al. 2006; Dereeper et al. 2008; Guindon et al. 2010).

Gas production

The production of gases was monitored using a Micro Oxymax Respirometer (Columbus Instruments, Columbus, USA). Fungal growth was monitored in the 100 ml chambers filled with 40 ml of liquid media and 0.5 g of wheat straw with the continuous measurement of metabolic gases under pyrogallol-treated N_2 at 39°C. The results of these measurements are presented as the readouts from the instrument. The same data are plotted either as% (v/v) in the gaseous atmosphere, or as a cumulative readout, i.e., the sum of all previously read data.

Results

Isolation and cultivation of anaerobic fungi

In our effort to isolate anaerobic fungi suitable for the treatment of lignocellulosic materials we searched in habitats known as possible sources of anaerobic fungi, i.e., rumen, faeces, and compost. We succeeded in obtaining isolates, two from the cattle rumen (Ana1, Ana2), one from sheep rumen (Ana3), one from compost (Comp1), two from faeces of snails (Snl1, Snl2), and one each from llama, camel and giraffe faeces. Not all isolates survived the course of experiments, so that most of the data presented in detail in this paper are obtained from rumen and compost fungi.

The macroscopic morphology of these isolates was uniform, they formed white fluffy and irregular mycelia at 38°C (not shown). At 40°C, the fungi grew faster and formed

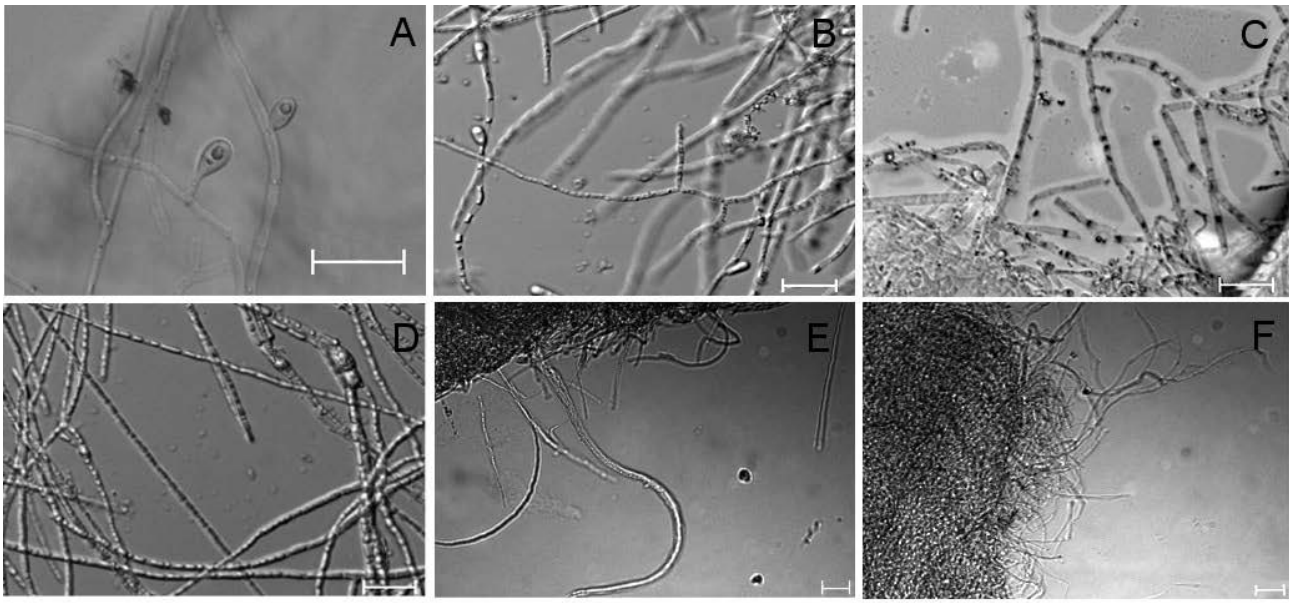


Figure 1. Microscopic features of isolates: Ana1 (**A**), Ana2 (**B**), Ana3 (**C**), Comp1 (**D**), Snl1 (**E**), and Snl2 (**F**). Preparations were made from 1 week cultures. A–D: magnification ×630; E–F: magnification ×400. The scale bar indicates 20 μm.

a copious mycelium. Lower temperatures were not used for growing these fungi. The macroscopic formation of spores was not observed. Septate mycelia were seen on microscopic observation (Fig. 1). Staining with thioflavin (Lakatoš et al. 2010) also indicated the presence of septate mycelia with relatively distant septa (not shown). Spores were observed in Ana1, Ana2, Ana3 and Comp1.

The growth of all isolates was irregular. Unlike aerobic fungi, the daily increment of colony diameter was not proportional to the time of culture and acceleration or deceleration or even temporary cessation of growth was observed (not shown). We attempted to overcome these problems by supplementing the media with various additives (plant fibres, plant extracts, blood, etc.) but we did not succeed in establishing a stable and regular growth which could yield amounts of mycelia sufficient for extensive biochemical studies. Variability in redox potential is probably not the cause of the unstable growth as our isolates tolerated the oxygen atmosphere well. The cultures of Ana1, Ana2 and Ana3 exposed to the ambient atmosphere for 96 h and subsequently transferred to anaerobic did not change their patterns of growth. Ana1 changed its appearance during the air exposure from white to light grey, probably, due to spore formation (not shown).

Taxonomical characterisation of fungal isolates

Isolates were identified by means of their ITS rRNA sequences. PCR amplicons co-migrated with bands of 400–500 bp (not shown). Their sequences (GenBank: Ana1 KR078301, Ana2 KR078302, Ana3 KR078303, Comp1 KR078306, Snl1 KR078304, Snl2 KR078305) were compared with known sequences of anaerobic fungi and a phylogenetic tree was constructed (Fig. 2). The sequence data showed that none of our isolates was identical with known anaerobic fungi. Nevertheless, they are related to known taxa. Ana1 (cattle rumen) was more related to the Ana3 fungus (sheep rumen) and Snl1 (snail faeces), while Ana2 (cattle rumen) was more related to another snail fungus (Snl2) and the fungus found in compost (Comp1). All fungi formed a clade distinct from *Neocallimastix* on the one hand, and from the complex group of *Piromyces,* or *Anaeromyces, Orpinomyces and Caecomyces* on the other hand (Fig. 2).

The taxonomy of the isolates from the faeces of llama, camel, and giraffe was analysed similarly and showed only one taxon. Surprisingly, these isolates were unequivocally identified as *Aspergillus fumigatus*. All three isolates could be passaged several times anaerobically but, finally, lost their capability to grow under anaerobic conditions. These results will be not shown here in detail.

Metabolic characterization of isolates

Due to the irregular growth of the isolates, we were unable to construct typical growth curves. In order to monitor their growth we monitored the production of CO_2 as an indicator of metabolic activity and/or growth. These measurements were performed in the liquid culture with

Table 1. Maximal specific activities of hydrolytic enzymes of anaerobic isolates and aerobic fungi attained during cultivation

Microorganism (culture medium)		Specific activity ($U.mg^{-1}$/($nkat.mg^{-1}$))		Extracellular proteins ($\mu g.ml^{-1}$)
		Cellulase	Xylanase	
T. atroviride (CzD)		231.00 (3850.77)	64.00 (106.88)	6
A. niger (CzD)		77.60 (1293.59)	33.00 (550.11)	16
Ana1	M2 + cellulose	0.65 (10.84)	1.09 (18.17)	56
	M2 + straw	0.65 (10.84)	0.23 (3.83)	129
	H_2O + straw	0.09 (1.50)	0.90 (15.00)	59
Ana2	M2 + cellulose	0.38 (6.33)	0.21 (3.50)	300
	M2 + straw	3.38 (56.34)	3.05 (50.84)	29
	H_2O + straw	0.59 (9.84)	0.19 (3.17)	10
Ana3	M2 + cellulose	2.52 (42.01)	3.80 (63.35)	N.D.
	M2 + straw	4.30 (71.68)	4.38 (73.01)	N.D.
	H_2O + straw	22.12(368.74)	10.14 (169.03)	N.D.

CzD, Czapek Dox medium; M2, medium M2; N.D., not determined.

added wheat straw as an additional carbon and/or nutrient source mimicking the "natural" conditions of a fungal habitat. The time course of the CO_2 generation for Ana1 is shown in Figure 3A. Ana1 started to produce CO_2 after 2–3 h of cultivation and reached a steady state after about 200 h attaining 16 mmol/l CO_2. Most of the CO_2 was produced during first 100 h of cultivation. Measurements of other metabolic gases, H_2, H_2S, and CO showed that they are produced almost exclusively during the early phase of growth, with a short delay compared with CO_2, and with some differences in the kinetics and in the concentrations attained in the steady state. It is noticeable that their concentrations are two orders of magnitude lower than those of CO_2 (Fig. 3A).

Similar patterns of generation of CO_2 were displayed by the Ana2 isolate with a slightly lower maximum concentration (12 mmol/l). However, the production of H_2 was restricted to two bursts during the phase of rapid growth only. Patterns of changes in H_2S and CO followed those of CO_2 but were produced at a proportionally lower level compared with those of Ana1 (Fig. 3B).

The Ana3 isolate produced CO_2 in a different manner compared with the other isolates (Fig. 3C). The onset of production was sluggish and its level oscillated rather periodically instead of changing continuously. The maximal production reached only 3 mmol/l. The production of H_2 occurred in two bursts and its yield was almost ten times lower than in the Ana1 or Ana2 isolates. The production of CO was similar to that of CO_2, but the oscillatory pattern of CO concentrations did not precisely match those of CO_2 (Fig. 3C). This fungus produced H_2S in amounts close to the lower limit of detection of our instrument.

The Comp1 fungus was the poorest producer of metabolic gases. The generation of CO_2 was low (less than 0.5 mmol/l at the end of experiment) and its time course did not have the normal characteristics of a growth curve. The production of H_2 was marginal, if present at all. This fungus did not produce H_2S and the production of CO was negligible, occurring in two bursts (Fig. 4).

It should be noted that the level of O_2 was also measured during the above experiments. Its levels confirmed that the anaerobiosis was maintained during the experiments

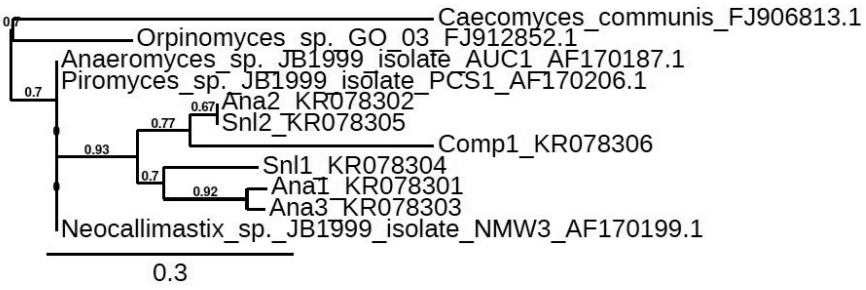


Figure 2. Phylogenetic tree presenting relationships of isolates to known anaerobic fungi – *Anaeromyces, Piromyces, Orpinomyces, Caecomyces* and *Neocallimastix sp*. The phylogenetic tree was constructed using ITS1 sequences of novel isolates deposited in the GenBank according to http://phylogeny.lirmm.fr/phylo_cgi/simple_phylogeny.cgi.

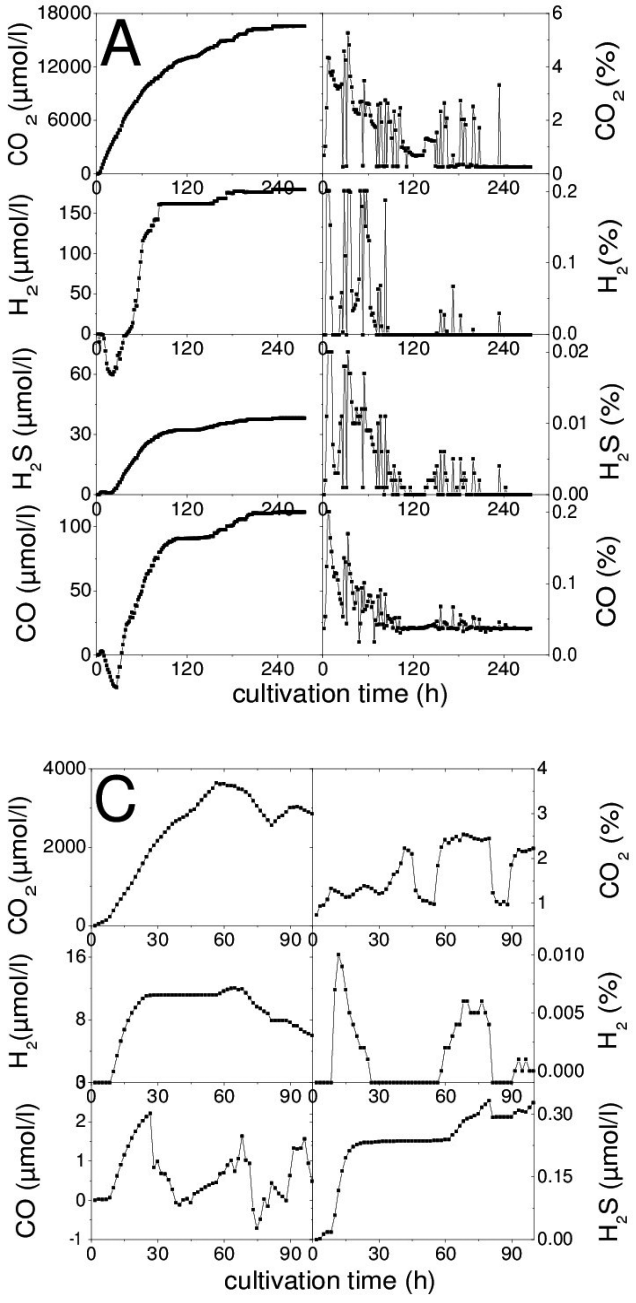
(not shown). It should be noted also that the decrease of cumulative values seen in some readouts could be ascribed to the measurements of values close to zero. In this case the instrument reads noise which could be positive or negative and distorts the cumulative curve.

Occasionally, the production of methane was observed in cultures grown in medium without added antibiotics (not shown). We ascribed the production of methane to the close association of methanogenic bacteria with anaerobic fungi (Jin et al. 2011; Leis et al. 2014), which was probably only temporary under our experimental conditions.

Biochemical characterisation of fungal isolates

As the production of hydrolytic enzymes is a prerequisite for the utilization of lignocellulosic substrates, the activities of hydrolytic enzymes were measured in supernatants from mycelia-free media. First, extracellular proteins were determined and it was found that their concentrations were very different (Table 1). Xylanase and cellulase activities were measured using specific substrates and their specific activities were calculated. These are shown in Fig. 5.

Cellulase and xylanase activities appeared in the media after several days of cultivation of Ana1–Ana3 isolates with wheat straw or with added cellulose. The specific activities of both enzymes were two to three orders of magnitude lower than those of prototypal cellulase- and xylanase producing strains *Trichoderma atroviride*, or *Aspergillus niger* (cultivated under aerobic conditions). Also, they were not stable throughout the culture period (Fig. 5). The maximal activities found in individual isolates are shown in Table 1.



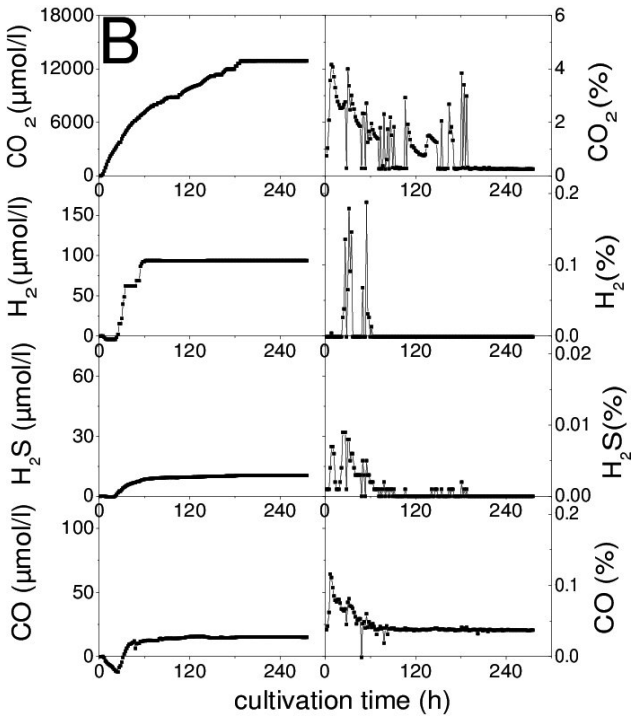


Figure 3. The production of metabolic gases by isolates: Ana1 (**A**), Ana2 (**B**), Ana3 (**C**). The isolates were cultured under nitrogen at 39°C in liquid medium B, with wheat straw as a carbon source, for the time indicated in the figure. Presented are characteristic results from one of three (Ana1, Ana2) or two (Ana3) experiments. The graphs show gas accumulation (µmol/l) and immediate gas concentration (%).

The dependence of cellulase and xylanase activities of the anaerobic fungi Ana1–Ana3 on both temperature and pH were remarkably similar. Cellulase activities had a pH optimum at pH 5.5, while xylanase activities had the optimum at pH 6. Temperature optima of cellulases were close to 40°C, while xylanase optima were at 30°C with the conspicuously flat curve (Fig. 6).

The unstable pattern of xylanase and cellulose production could be caused by the concomitant secretion of proteases. We found that protease activities appeared in the media to various extents and with various times of production onset (Fig. 7). Surprisingly, similar activities were found whether wheat straw or cellulose was used as a substrate. In general, cellulose was a better inducer of protease activity than wheat straw. The onset of protease secretion was earlier in Ana2 than in the Ana1 or Ana3 isolates. The activity and specific activity of secreted proteases in Ana1 was lower than in the Ana3 isolates. The secretion of proteinase could account for the loss of cellulase activity during the storage of the medium after cultivation and separation of mycelia. In an experiment not detailed here we stored this medium for 7 days at 4°C (35% activity remained), while more than 90% remained

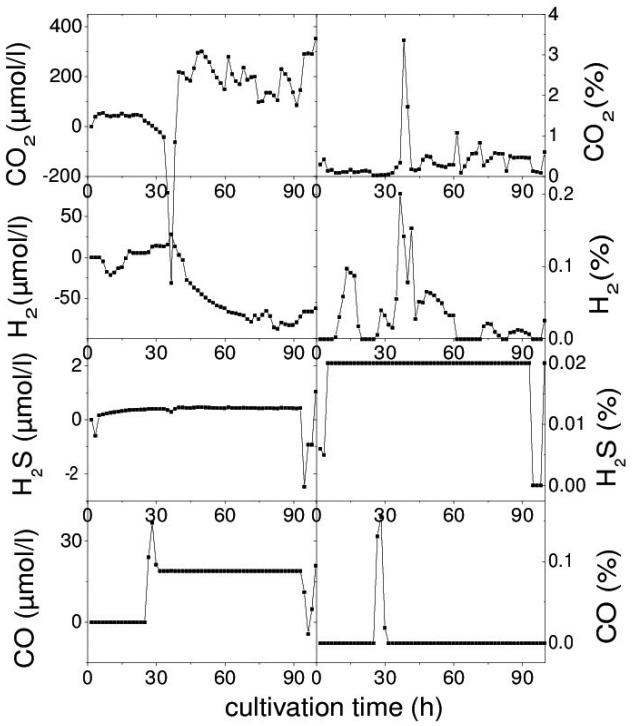


Figure 4. The production of metabolic gases by Comp1 isolate. The isolate was cultured under nitrogen at 39°C in liquid medium B, with wheat straw as a carbon source, for the time indicated in the figure. Presented are characteristic results from one of two experiments. The graphs show gas accumulation (µmol/l) and immediate gas concentration (%).

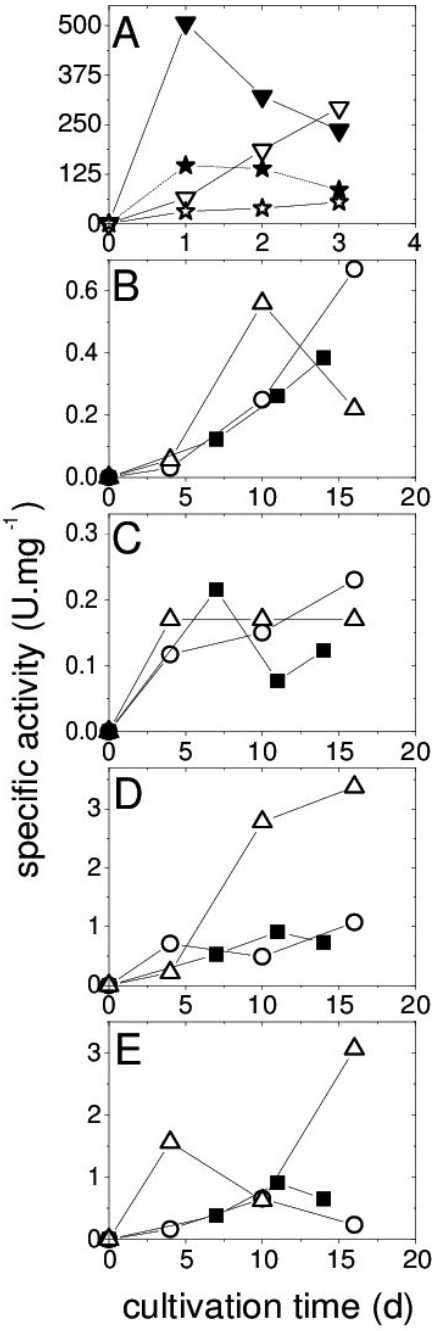


Figure 5. Appearance of hydrolase activities during cultivation of anaerobic fungi. Comparison with typical aerobic fungi: **A.** *T. atroviride* (down-pointing triangle), *A. niger* (star) cellulase (black) and xylanase (white) activities, cultivation in CzD medium, **B.** Ana1, Ana2, Ana3 cellulase activities, cultivation in M2 medium with wheat straw 1% (w/v), **C.** Ana1, Ana2, Ana3 xylanase activity, cultivation in M2 medium with wheat straw 1% (w/v), **D.** Ana1, Ana2, Ana3 cellulase activity, cultivation in M2 medium with cellulose 1% (w/v), **E.** Ana1, Ana2, Ana3 xylanase activity, cultivation in M2 medium with cellulose 1% (w/v). Measurements were performed as described in the section Material and Methods. -○-Ana1, -▪- Ana2, -Δ-Ana3.

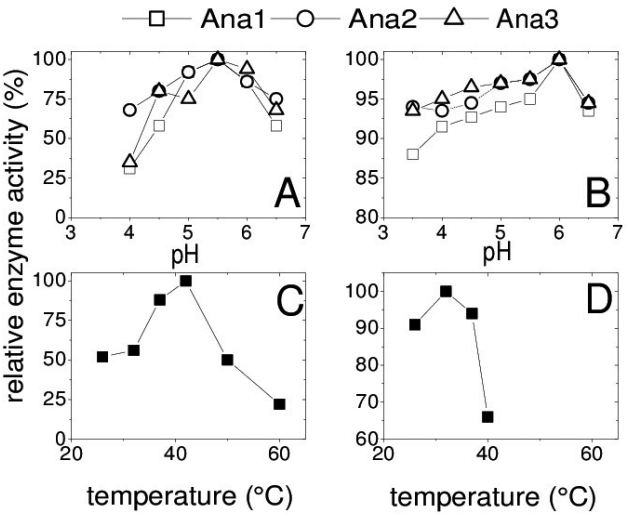


Figure 6. Temperature- and pH-dependence of secreted cellulases and xylanases. Relative enzyme activities of cellulases **(A, C)** and xylanases **(B, D)** of Ana1, Ana2, Ana3 depending on pH and temperature. pH dependence of enzyme activities was measured in media buffered with 50 mmol/l Good´s buffers. Results are expressed as % of maximal measured activities. The results of temperature dependencies from all three fungi were identical so that only one representative curve is shown. Note different scales in individual parts of the figure.

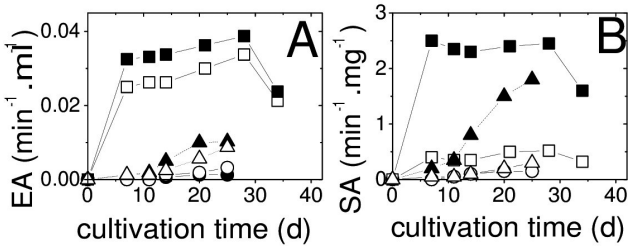


Figure 7. Proteolytic enzymes and specific enzymes activities of anaerobic isolates. **A.** Liquid M2 medium with 1% (w/v) wheat straw. **B.** Liquid M2 medium with 1% (w/v) cellulose. Mycelia were cultivated at 39–40°C without shaking for the time indicated in the figure. Analyses were performed as described in Materials and Methods. EA, enzyme activity; SA, specific activity; -●-Ana1 cellulose, -○-Ana1 straw, -▪- Ana2 cellulose, -□-Ana2 straw, -▲-Ana3 cellulose, -Δ-Ana3 straw.

when the sample of medium was frozen, in accord with the possible attack of protease.

Pilot experiments were performed in order to identify the presence of several enzymes relevant to the energetic metabolism under anaerobic conditions. These included glucose-6-phosphate dehydrogenase (G6PDH), the first enzyme of pentose cycle, pyruvate dehydrogenase (PDH), the crucial enzyme for the metabolic activation of pyruvate, and glutamate dehydrogenase (GlDH), an enzyme important for ammonia utilisation. Further, we measured three enzymes of the citrate cycle, citrate synthase (CS), isocitrate dehydrogenase (ICDH), α-ketoglutarate dehydrogenase (KGDH), and isocitrate lyase (ICL), the key enzyme of the glyoxylate cycle, important for the catabolism of acetate (acetyl-CoA). The results of these measurements are shown in the Table 2.

All isolates except Ana1 possessed the comparable activities of G6PDH suggesting that the pentose cycle or Entner-Douroff-pathway of glucose-6-phosphate catabolism could be active. PDH activity was detected in all isolates except in the rumen fungi Ana1–Ana3. Enzymes of the citrate cycle were present in isolates with different specific activities. CS was present in all isolates attaining its highest value in *T. atroviride* followed by Comp1. The rumen fungus Ana1 had only 30% of the activity of *T. atroviride* but other rumen fungi had CS specific activity an order of magnitude lower than *T. atroviride*. The specific activities of ICDH were, conversely, one order of magnitude higher in all anaerobic isolates than in *T. atroviride*. The third enzyme, α-KGDH was absent in all rumen fungi but Comp1 had a similar activity to *T. atroviride*. ICL specific activity was higher in rumen fungi

Table 2. Specific activities of enzymes involved in main catabolic pathways in homogenates of isolates of anaerobic fungi and of aerobic fungus *Trichoderma atroviride*

Microorganism	Specific activity (nkat·mg^{-1})						
	G6PDH	PDH	CS	ICDH	KGDH	ICL	GlDH
T. atroviride	3.50	0.40	6.16	3.00	0.25	0.50	80.18
Ana1	N.D.	N.D.	2.16	20.33	N.D.	1.33	5.66
Ana2	1.66	N.D.	0.66	68.18	N.D.	0.83	3.16
Ana3	1.16	N.D.	0.33	76.18	N.D.	1.83	3.33
Comp1	1.50	0.15	4.00	23.33	0.53	0.83	102.02

Cultivation conditions: *T. atroviride* CCM F-534, CzD medium, 18 h, Ana1–Ana3, medium M2 with straw (1% w/v), 34 days (CS, citrate synthase; ICL, isocitrate lyase; GlDH, glutamate dehydrogenase; ICDH, isocitrate dehydrogenase; PDH, pyruvate dehydrogenase; KGDH, α-ketoglutarate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; N.D., not detected.

than in Comp1 from compost and *T. atroviride*. Dramatic differences were observed in the GlDH specific activities. The highest specific activity was found in Comp1 and in *T. atroviride* (102.02 and 80.18 nkat/mg, respectively), while the highest value in rumen fungi was 5.67 nkat/mg. It should be noted at this point that the activities of several enzymes could be increased by the presence of additional nutrients, e.g. molasses or xylan etc. (not shown). Therefore the data shown in Table 2, obtained for mycelia grown in M2 medium cannot be generalised for different conditions.

In other experiments we tested several waste materials derived from sunflower and rape seeds as substrates for anaerobic fungi for the production of the hydrogen gas. These materials were less efficient substrates for H_2 production than wheat straw, which we used as a reference material (Table 3). For the comparison, isolates cultivated in glucose medium (with final concentration of glucose 0.1 g/l and trace elements solution 1.10^{-3} l by Lowe et al. 1985). Ana1 cultivated in glucose medium produced 2210 μmol/l CO_2 and 349 μmol/l H_2, Ana2 produced 1330 μmol/l CO_2 and 0.94 μmol/l H_2 and Ana3 produced 761 μmol/l CO_2 and 91 μmol/l H_2 (data after 150 h of cultivation). Note that data shown in Figures 3–4 were for cultures at 100 or 280 h.

Discussion

The morphological characteristics of the isolates were similar to those observed earlier by other authors (Bauchop 1979; Benchimol 2009). According to the rRNA sequences they were different from each other and did not belong to any of the known genera (Fig. 2). Rather, they are akin to two genera – *Neocallimastix* and *Piromyces* but they may originate from a unique clade. It is interesting that not only isolates from rumen but also those from compost or snail faeces belonged to this putative clade. This may reflect some geographical aspects (there are no data about other isolates from this part of Europe). They were in part aerotolerant, similar to isolates of *Orpinomyces* (Davies et al. 1993; Leis et al. 2014; Struchtemeyer et al. 2014). A more interesting aspect is that some isolates seem to have substantially different physiology when their production of gases, or enzyme activities (Figures 3, 4, 5, Table 2) are compared.

The measurement of the production of multiple gaseous metabolites enabled us to get some insight into the physiological processes, including the energetic metabolism, of the isolates. The generation of CO_2, a product of the catabolism of glucose, could reflect the "general" metabolic activity of the isolates. The isolates displayed different rates of CO_2 production (Ana1 > Ana2 > Ana3 > Comp1). The production of H_2, which should be closely related to the catabolism of glucose, occurred concomitantly with CO_2 only in Ana1. In Ana2 it lagged CO_2 production and in other isolates was only just discernible. This indicates that H_2 production is not an integral part of glucose metabolism and is activated only optionally. It is feasible that the activation occurs at the peak of metabolic activity of fungus (possibly as overflow metabolism). If this is true, we may conclude

Table 3. Production of metabolic gases by Ana1-Ana3 grown up on complex substrates

Accum. gas concentration (μmol/l)	Substrate					
	sunflower meals	sunflower peels	sunflower expeller	rapeseed meals	rapeseed expeller	straw
isolate Ana1						
CO_2	3612.80	2096.81	4589.22	2302.95	2810.64	14145.15
CO	0.36	0.30	0.47	0.10	0.00	92.56
H_2	0.35	0.38	0.27	0.24	0.02	162.08
H_2S	0.39	0.25	0.56	1.03	0.53	33.84
isolate Ana2						
CO_2	3209.52	1766.10	13823.26	2682.18	3445.70	11364.64
CO	0.39	0.37	0.55	0.29	0.00	14.48
H_2	0.34	0.37	0.48	0.25	0.18	93.70
H_2S	0.07	0.00	0.21	1.19	1.25	10.01
isolate Ana3						
CO_2	2727.72	1590.75	11268.72	2169.23	1573.17	3562.96
CO	0.42	0.46	0.51	0.67	0.00	0.74
H_2	0.38	0.37	0.44	0.25	0.22	12.07
H_2S	0.000	0.000	0.000	0.98	0.000	0.26

Fungi were cultivated in M2 medium with addition of 0.5 g of individual substrates at 39°C for 150 h, all measurements were done 2 times except Ana1, Ana2 cultivation in RFM medium with straw (3 times). When straw was used as substrate, RFM medium was used.

that the main catabolic pathway(s) have fermentative character, and hydrogenosomal pathways are activated in order to reoxidise a surplus of reducing equivalents. We did not analyse whether this pathway is ethanolic fermentation as suggested by the results of Bauchop (1989), Marounek and Vovk (1992), Cheng et al. (2013), Teunissen et al. (1991), or another one. It is in accordance with the above explanation that the two isolates with minimal or zero production of H_2 had also the lowest production of CO_2. It is probable that some intrinsic or extrinsic factor limited their metabolism and/or growth rate. Otherwise we would have to conclude that hydrogenosomes do not operate in these isolates, although they belong to a clade similar to canonical anaerobic fungi.

Production of both CO_2 and H_2 was only transient (Fig. 3A,B) or periodical (Fig. 3C) and shows that the production of these gases proceeds concomitantly with their utilisation with a time lag. This observation has a parallel in the study of the metabolism of the bacterium *Succinivibrio dextrinosolvens* (O´Herrin and Kenealy 1993). It is probable that CO_2 generated by the catabolic pathway is also re-utilized in a CO_2-fixation pathway in our fungal isolates (see below).

The generation of other metabolic gases, namely CO and H_2S, which has not been observed in anaerobic fungi so far, is enigmatic. Again, there is a correlation between the production of CO_2 and the production of both CO and H_2S among the tested isolates, suggesting their connection to energetic metabolism, although the limited quantity of produced gases could suggest that they should be regarded as a part of some signalling processes (Kimura 2015). From the mechanistic point of view, we should consider CO and H_2S separately.

CO occurs as metabolite in only one pathway, i.e. the Wood-Ljungdahl CO_2 fixation pathway observed, e.g. in acetogenic bacteria (Ljungdahl 1986; for review, see Ragsdale 2004). It originates from CO_2 reduction and is condensed with bound methyl to form an acetyl residue by the enzyme CO dehydrogenase/acetyl-CoA synthase (CODH/ACS). This enzyme has not been detected in anaerobic fungi. Moreover, evidence has been obtained which shows that CO is channelled through the enzyme (Ragsdale 2004). The production of CO in the CODH/ACS reaction in our experiments is understandable only if we suppose that CO leaks from the CODH/ACS by some unknown mechanism. Nevertheless, the presence of the Wood-Ljungdahl pathway could explain the consumption of CO_2 and H_2 during the late phases of culture shown in the Figures 3 and 4. It should be added that aerobic fungi, such as *T. atroviride*, or *A. niger* did not produce either CO or H_2S (not shown).

The production of H_2S under anaerobic conditions, especially in the bioenergetic context strongly evokes sulphate reduction, although there are also novel mechanisms underlying H_2S production as a signaling molecule also in Eukarya (Kimura et al. 2015). The intensity of H_2S production and its time course (Fig. 3, 4) enable us to consider both possibilities. Thus, the energetic metabolism of our isolates needs further analyses. The data presented in Table 2 show that pyruvate dehydrogenase and α-ketoglutarate dehydrogenase activities are absent in Ana1–Ana3 isolates but were present in the Comp1 isolate and, as expected, in *T. atroviride*. This is in accord with the known metabolic scheme which includes hydrogenosomal metabolism (Hackstein et al. 2001). The presence of both citrate synthase and isocitrate lyase activities enables the utilisation of acetyl-CoA, and thereby acetate, which is one of the products of fermentation metabolism (Brul and Stumm 1994; Hackstein et al. 2001; Paul et al. 2010; Cheng et al. 2013). Isocitrate dehydrogenase is indispensable for the generation of α-ketoglutarate — a precursor of glutamate. These results indicate that the citrate cycle is not complete in isolates Ana1–Ana3. The isolate Comp1 is probably different and may operate the complete citrate cycle. The presence of citrate in the *Piromyces* metabolome was reported recently (Cheng et al. 2013).

The secretion of hydrolytic enzymes (cellulases and xylanases) was lower than expected if compared with two prototypal aerobic fungi, *T. atroviride* and *A. niger*. The difference in secreted activities was at least one order of magnitude in favour of aerobic fungi (Table 1). This difference indicates that the contribution of fungal hydrolase secretion to overall hydrolytic activities may be of less importance compared to the contribution of other microorganisms, such as anaerobic bacteria. It may be of interest to emphasise, that the properties of these enzymes (pH and temperature dependencies) (Fig. 6) indicate that all the isolates we studied probably produce identical hydrolytic enzyme(s), although their pH optima were different from those studied by Barichiewicz and Calza (1990). If M2 medium was replaced with water and wheat straw, the secretion of cellulases and xylanases was higher than in M2 medium with cellulose and wheat straw (Table 1). Thus, the complete medium interferes with the induction of proteases.

The secretion of proteolytic activities was also different from our expectations. Proteolytic activity was better induced by cellulose than by the addition of wheat straw (Fig. 7). Also, the total induced proteolytic activity did not correlate with the induction of xylanolytic or cellulolytic activities in the presence of M2 medium (Fig. 5, 7). Nevertheless, all tested isolates exhibited secreted proteolytic activities, unlike the isolates studied by Michel et al. (1993).

Experiments performed with plant-derived materials as nutrition sources showed that our isolates Ana1–Ana3 were able to utilise them with the production of metabolic gases (Table 3). Although the growth was the best with sunflower expellers, the production of H_2 was highest with wheat straw as substrate. We cannot estimate whether such results could have some application potential, e.g. for H_2 production, if

we consider that the production of gases is only transient in our experimental design. Possibly, growth in an open system, or a system with H_2 and CO_2 traps could change the characteristics of the gas generation.

In summary, the novel isolates of anaerobic fungi described here are different from those described previously by other authors. They may represent a novel clade related to *Piromyces* and *Neocallimastix* and show variable biochemical properties. The metabolic pathways that led to the production of CO are worthy of study in the future in order to understand the energetic metabolism of anaerobic fungi.

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