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Growth and Survival of Anaerobic Fungi in Batch and Continuous-Flow Cultures

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The anaerobic fungus, *Neocallimastix hurleyensis*, was grown in 100 mL batch and continuous-flow cultures on wheat straw concentrations ranging from 5–80 g dry matter/L of culture fluid. In batch culture, *N. hurleyensis* could degrade about 45% of the wheat straw, but only if the substrate was provided at 5 g dry matter/L. In cultures containing 10–80 g dry matter/L, progressively less of the straw was degraded as the substrate concentration was increased. At a wheat straw concentration of 80 g dry matter/L, for example, the fungus was able to remove only about 12% of the substrate. Removal of cell wall non-starch polysaccharides was similarly affected in batch cultures with a decline in removal in cultures containing more wheat straw. Likewise, production of fermentation products by the fungus in batch culture did not increase with increasing substrate concentration. These effects in batch culture were attributed to inhibition, probably by fermentation end products or by the development of adverse physiological conditions. Continuous-flow culture is a procedure new to rumen microbiology in which fermentation end products are removed by continuous passage of liquid medium through the culture vessel at a constant (rumen-like) dilution rate. In this system, *N. hurleyensis* was able to degrade about 45% of wheat straw at concentrations ranging from 5–40 g dry matter/L. Removal of non-starch polysaccharides from plant cell walls and production of fermentation products also increased with increasing substrate concentrations. Growth of *N. hurleyensis* in continuous-flow culture enabled production of greater quantities (up to 20 times larger) of cell wall degrading enzymes (CMCase and β-glucosidase) and demonstrated the ability of the anaerobic fungus to grow in laboratory culture on levels of recalcitrant particulate substrate much in excess of those used in batch cultures.

Introduction

Anaerobic fungi have evolved over millions of years as fibrolytic saprophytes of graminaceous plants. They occupy a unique niche in the digestive tract of ruminants and other mammalian herbivores where they participate in primary colonization of plant cell walls [1]. Descriptions of their activity in the rumen have destroyed the long held belief that fungi require oxygen to grow and resulted in a reappraisal of accepted ideas concerning plant biomass digestion in

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herbivorous animals [2]. The rumen is an open ecosystem providing a relatively stable (steady-state) environment in which micro-organisms grow at relatively high concentrations of digesta dry matter (DM), within the region of 120 g / L [2]. For micro-organisms to survive at such high substrate concentrations, it is essential to prevent the build-up of toxic end products in the rumen and reduce the development of adverse physiological conditions, including dramatic pH decline. This is achieved by salivary flow, the absorption of fermentation end products across the rumen epithelium and the flow of digesta to the lower tract. Under these conditions, and in the absence of any dramatic dietary perturbation, anaerobic fungal populations remain relatively constant, with an equilibrium between fungal biomass production in the rumen and the loss of fungi by death and / or passage from the rumen [3,4]. In the laboratory, however, because of the difficulty of establishing rumen-like culture systems, anaerobic fungi are usually grown in closed batch cultures on particulate substrate concentrations of only 5–10 g DM / L, markedly below those found in the rumen. At these concentrations, growth of the fungus is initially rapid and not inhibited by the accumulation of fermentation end products [5]. As growth proceeds, however, anaerobic fungi are subjected to a changing environment and ultimately fail to survive for more than a few days unless they are subcultured into fresh medium [3,5,6].

A number of continuous culture systems have been used to grow rumen micro-organisms. In general, they involve quite complicated pieces of apparatus in which rumen-like conditions are developed by using dialysis, sequestration or filtration techniques to remove culture liquid and preferentially retain plant and microbial biomass [7–11]. Although these systems have been used to grow rumen bacteria and protozoa, they have not been used to grow anaerobic fungi. The rumen simulation apparatus (Rusitec) [12] has been used to grow anaerobic fungi on fragments of wheat straw in the presence of rumen bacteria [13]. A semi-continuous culture system has also been used for anaerobic fungi [14]; in this system, culture fluids were removed continuously while plant and fungal biomass were selectively retained. We have also grown *Caecomyces, Neocallimastix* and *Piromyces* on soluble substrates in fed-batch cultures (Fernando, N., Theodorou, M. K. and Trinci, A. P. J.; unpublished data).

Other than the research presented in this paper (and in [13,14]) little attention has been given to the growth of anaerobic fungi in continuous culture. In this study, we used batch culture and a simple continuous-flow culture system to investigate and compare the growth of the anaerobic fungus, *Neocallimastix hurleyensis*, on increasing concentrations of wheat straw. A unique feature of our continuous-flow system is the ability to operate up to 20 cultures at the same time, thus enabling comparative investigations in a way that was previously not possible. Growth of anaerobic fungi in continuous-flow culture has not been reported before other than in abstract form [15].

Materials and Methods

Micro-organisms and culture maintenance

The anaerobic fungus, *N. hurleyensis*, was isolated from the rumens of sheep and subsequently classified [16,17]. Cultures were maintained at 39°C on wheat straw (10 g DM / L milled to pass through a 1 mm dry mesh screen) in medium C, a complex liquid medium containing rumen fluid [18]. Cultures were incubated without agitation and sub-cultured every 5 days.

Batch culture

Milled wheat straw (5–80 g DM / L) was weighed into 125 mL serum bottles (Phase Separations Ltd., Clwyd, U.K.; nominally of about 160 mL capacity, but retailed as 125 mL bottles) and was gassed for up to 1 h with CO_2 before dispensing 90 mL reduced medium C into each bottle. The bottles were sealed with butyl rubber stoppers and aluminium crimp seals (Bellco Glass Inc., Vineland, NJ, U.S.A.) under an atmosphere of 100% CO_2 and sterilized by autoclaving at 121°C for 15 min. Chloramphenicol (1 mL of a 5 mg / mL solution in 50% aqueous ethanol) was injected into each bottle and the bottles were then placed at 39°C overnight prior to inoculation with 10 mL two-day old culture suspension. Cultures (three replicates) were grown at 39°C without agitation. For determination of substrate losses, acetate production and enzyme activities, bottle contents (solids and liquids) were harvested at daily intervals. Bottles were vented twice daily for the first 5 days and daily thereafter by inserting a disposable hypodermic syringe needle (23 gauge × 1 inch) through the butyl stopper. This procedure ensured that gaseous end products did not accumulate in the head-space of the culture bottles.

Continuous-flow culture

This technique has not been described previously and was as follows. The culture medium was prepared and inoculated as described above. Cultures (three replicates) were incubated without agitation at 39°C for 20–24 h before attachment of the cultures to the continuous-flow culture system.

The continuous-flow apparatus shown in Figure 1

was installed in a large, gas tight, perspex chamber (125 × 45 × 63 cm; constructed in the IGER workshop) in an incubation room at 39°C. The chamber had several medium inlet and gas inlet/outlet ports and was constantly flushed with a stream of CO_2. The front panel of the chamber had two large doors (45 × 32 cm), through which equipment and culture bottles could be passed at the beginning and end of the experiment. Three smaller doors (29 × 15 cm) were installed on the upper surface of the chamber and served for routine maintenance of cultures during experiments, including retrieval and replacement of receptacles into which culture effluents were collected. Two multichannel peristaltic pumps (Watson Marlow Ltd., Falmouth, Cornwall, U.K.), each with 10 channels, were placed in the chamber and each channel was connected via peristaltic pump tubing (Central Lab Supplies, Basingstoke, Hants, U.K.) to a continuous-flow vessel.

To connect culture bottles to the continuous-flow system, crimp seals and butyl rubber stoppers were removed from culture bottles and replaced with similar stoppers through which two stainless steel, luer-lock, blunt-ended, lumbar puncture needles (5 inches long, 16 and 18 gauge, Alfred Cox Surgical Ltd) had been passed. The needles served as medium inflow and liquid and gaseous effluent outflow ports respectively and were adjusted such that each bottle operated at a constant volume of about 100 mL; needles carrying the inflowing medium were positioned near the centre of the vessel. On the medium input side of the pump, each of the 20 peristaltic pump tubes were connected to the medium reservoir by two multiple stream-dividers constructed from glass T-pieces and butyl rubber tubing. The medium reservoir was housed outside of the anaerobic chamber and consisted of a glass aspirator bottle of 20 L capacity containing 16 L of medium C. The medium was autoclaved (121°C for 60 min), cooled to 39°C, gently stirred with a magnetic stirrer and bar and flushed with CO_2 overnight. Sterile L-cysteine hydrochloride (16 g in 100 mL distilled water) and chloramphenicol (160 mL prepared as above) was then added aseptically to the medium reservoir and the completed medium was again flushed with CO_2 overnight before being connected to the continuous-flow cultures. Gassing and gentle stirring of the medium vessel continued until the medium was used up and a new reservoir connected via the stream divider. Effluent from continuous-flow cultures was collected into separate collection vessels and the volumes recorded at regular intervals. The collection vessels had narrow necks and were stoppered with cotton wool to limit evaporation losses. For chemical analysis, effluents were collected daily over a 2 h period and acidified for volatile fatty acid analysis, or kept at –80°C for determination of enzyme activities and reducing sugar concentrations, as described below.

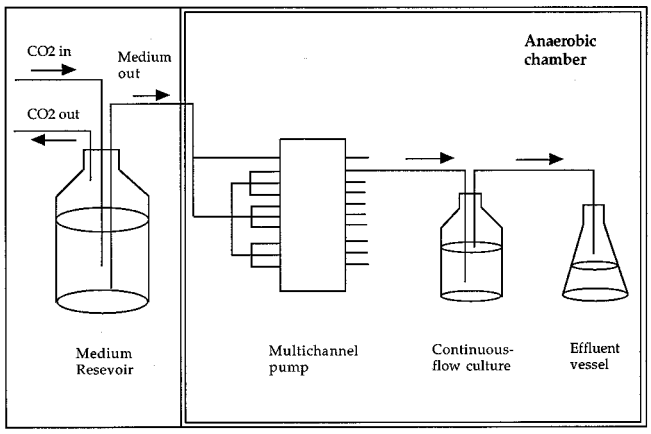

Figure 1. A schematic diagram of the continuous-flow culture system. The 20 L capacity medium reservoir was positioned external to the anaerobic chamber and constantly flushed with a stream of CO_2. Reduced medium from the reservoir was pumped by a multichannel 10 lane peristaltic pump to the continuous-flow cultures. These were operated at a constant volume of 100 mL and effluent from the cultures collected individually in effluent collection vessels kept in the anaerobic chamber. The volume (mL) of liquid flowing through each continuous-flow culture was recorded and the dilution rates (per hour) determined for use in data calculation. Two continuous-flow systems were operated in one anaerobic chamber providing 20 cultures for experimentation. The mean dilution rate across all bottles was 0.038/h with SEM = 0.0004.

Volatile fatty acid analysis

Volatile fatty acids were determined using a Chrompack model CP9000 gas chromatograph with flame ionisation detector (FID) detection, split injection and an automatic (model 911) sampler (Chrompack U.K. Ltd., London, U.K.). The column used was a wall coated open tubular (WCOT) fused silica capillary column (25 mm × 0.32 mm internal diameter) coated with free fatty acid phase-chemically bonded (FFAP-CB). The machine was linked to an IBM PC with MOSAIC (Chrompack) integration software. Aliquots (1.5 mL) of culture fluid from batch culture or culture effluent from continuous-flow culture were added to Eppendorff tubes and acidified to pH 2 with 20 mL of orthophosphoric acid. This mixture was stored at 4°C for 2 days to allow for precipitation and then centrifuged at 3500 × *g* for 3 min using a microfuge (Sorvell Ltd., U.K.). Clear supernatant (1 mL) was removed and mixed with 0.2 mL of internal standard (15.0 mM 2-methylvaleric acid) in a 2 mL glass crimp vial (Vials Direct., Macclesfield, U.K.). The crimped vials were loaded on the machine and 0.5 μL samples were injected onto the column.

Enzyme assays

Carboxymethylcellulase (CMCase) activity was determined as described [19]. Reducing sugars were determined by the Nelson-Somogyi method [20]. The CMCase activity was expressed as the difference between the total amount of reducing sugar (glucose) released from the enzyme reaction and that of the reducing sugar present in the effluents. β-glucosidase activity was determined as described previously [21] by quantifying the release of *p*-nitrophenol (*p*NP) from *p*-nitrophenol β-*D*-glucopyranoside (*p*NPG). Assays were conducted in microtitre plates and values determined using a microtitre plate reader (EL 340 Microplate, BIO-TEK Instruments, Luminar Technology Ltd., Southampton, U.K.). Units (IU) of enzyme activity are defined as μM of product released / min under assay conditions.

Harvest of cultures and determination of cell-wall sugars

Residual wheat straw and adherent fungal biomass was harvested by filtering through pre-weighed filter crucibles (Sintaglass porosity 1 crucibles, 70 mL capacity; Gallenkamp, U.K.) using vacuum filtration. Part of the residue was used immediately for viability assay as described below. The rest of the residue was washed with distilled water through crucibles and freeze dried to constant weight. The original wheat straw and residues remaining after fermentation were analysed for their cell wall polysaccharide (or non-starch polysaccharide: NSP) content and composition [22]. Alditol acetate derivatives of carbohydrate monomers derived from acid hydrolysates of de-starched samples were quantified by gas chromatography. Gas chromotography was performed on a Varian 3400 chromatograph (Varian U.K. Ltd, Walton on Thames, Surrey, U.K.) fitted with a flame-ionisation detector and an automatic sampler (Varian 8000) and linked to a Dell PC with Dionex Al-450 integration software (Dionex U.K. Ltd, Camberley, Surrey, U.K.). The uronic acid contents of the hydrolysates were determined by a colorimetric method [23].

Viability assays

For fungal viability assays, 0.5 g of the fresh residue from harvested cultures was transferred immediately to medium C in triplicate anaerobic culture tubes (Bellco Glass Inc.) containing approximately 5 g DM / L of milled wheat straw. The tubes were incubated at 39°C for up to 10 days and examined at intervals by eye and with the aid of an inverted microscope (Olympus CK; Olympus Optical Co (U.K.) Ltd, London, U.K.) for the presence of anaerobic fungi; culture were scored positive on identification of fungal zoospores, sporangia and colonising rhizoids.

Mathematical treatment

Cumulative production (F_p) of acetate and enzymes in continuous-flow culture was determined as follows. The outflow rate (f_t) of acetate (mM / h) and enzymes (IU / h) at any time is given by:

$$f_t = k X_t$$

where t(h) is the time since the start of outflow, k(/ h) is the fractional outflow rate, and X_t is the amount of acetate (mM) or enzyme (IU) in the bottle. The outflow rate (f_t) is equal to the rate of change of the cumulative outflow (dF_t / dt). As the cumulative outflow (F_t) at the start of flow ($t = 0$) is 0, the cumulative outflow of acetate (mM) or enzymes (IU) is given by:

$$F_t = k A$$

where $A = ({}_0\int^t X_t dt)$ denotes the area under the acetate time or the enzyme activity time curve. The value of this area is approximated by a piece wise linear integration between each measurement of acetate and enzyme concentration in the effluent, multiplied by the liquid volume in the bottle. At time t ($t > 0$), the cumulative production of each bottle is given by:

$$P_t = F_t + X_t - X_{(t-1)}$$

where P_t is the cumulative production of enzyme (IU) or acetate (mM) at time t, assuming there is no disappearance of enzyme or acetate other than by outflow from the bottle, and X_t and $X_{(t-l)}$ are the amounts of acetate or enzyme measured at the end and the start of the time interval of interest.

Results

Wheat straw degradation and non-starch polysaccharide losses

Wheat straw at 5, 10, 20, 40 and 80 g DM / L (three replicates) was fermented in batch culture and in continuous-flow culture at a dilution rate of 0.038 / h (SEM = 0.0004). At the end of the incubation period, wheat straw residues and adherent fungal biomass were harvested and analysed for DM loss and NSP content and composition. Prior to fermentation, the wheat straw contained 616 g NSP / kg DM which comprised glucose (369 g), xylose (196 g), arabinose (24 g), galactose (7 g), mannose (4 g) and uronic acids (16 g). Glucose and xylose were the major NSP components, accounting for 60 and 32% of the total

NSP content, respectively. Arabinose, uronic acids, galactose and mannose were all minor components, together accounting for the remaining 8% of the NSP fraction. In batch culture, DM losses declined from 38 to 12% as substrate concentrations increased from 5 to 80 g DM / L [Figure 2(a)]. Except for cultures grown on 5 g DM / L of wheat straw, all continuous-flow cultures exhibited significantly higher ($p < 0.05$) DM losses than the comparable batch cultures. The pattern of NSP loss in batch and continuous-flow cultures closely mirrored that of DM loss [Figure 2(b)] as was also observed for the major NSP constituent monomers, glucose and xylose [Figure 2(c)]. Similar data was obtained for the minor NSP components but this has not been presented. There was a logarithmic decline in NSP and constituent losses from batch cultures with increasing substrate concentrations [Figure 2(b and c)]. In contrast, in continuous-flow cultures, the percentage losses of NSP and constituent monomers were similar across the range of substrate concentrations up to and including 40 g DM / L. Thereafter, from continuous-flow cultures containing 80 g DM / L, there was a marked decrease in percentage NSP loss. Except for cultures grown on 5 g DM / L wheat straw, all continuous-flow cultures exhibited significantly higher NSP and constituent monomer losses than the comparable batch cultures ($p < 0.05$).

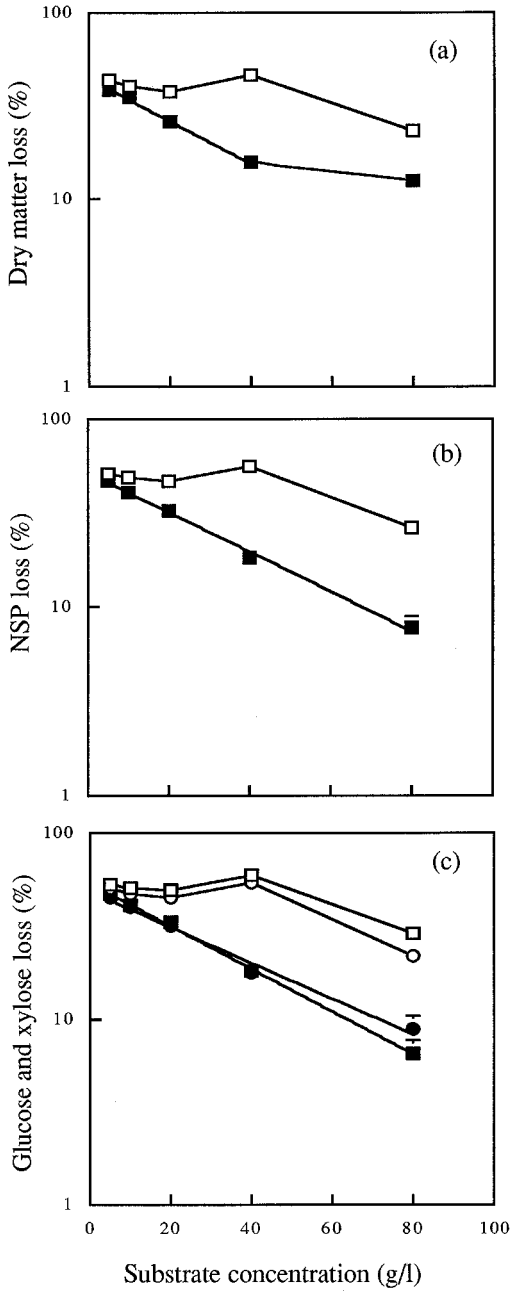

Figure 2. (a) Degradation of wheat straw, (b) removal of total non-starch polysaccarides and (c) removal of glucose (●, ○) and xylose (■, □) residues by *N. hurleyensis* in batch (closed symbols) and continuous-flow (open symbols) cultures. The fungus was grown at 39°C in both culture systems on wheat straw concentrations ranging from 5–80 g DM / L. Error bars represent SEM where n = 3. Where error bars are not shown the values were less than the size of the symbols used to represent the data.

Fungal viability

Viable *N. hurleyensis* was recovered at the end of the incubation period from batch cultures containing 5, 10 and 20 g DM / L wheat straw, but not from those containing 40 and 80 g DM / L wheat straw (Table 1). At the end of the incubation period in continuous-flow cultures, however, *N. hurleyensis* was recovered from cultures containing 20 and 40 g DM / L wheat straw, but not from those containing 5, 10 or 80 g DM / L wheat straw (Table 1). Although viable zoospores were removed in culture effluents during incubation of continuous-flow cultures, they were not quantified. In addition to determining the viability of *N. hurleyensis* in freshly harvested residues, viability assays were performed on both washed and unwashed residues from batch and continuous-flow cultures after the residues had been dried and kept in air at ambient temperature for 7 days. Viable *N. hurleyensis* was not obtained from these dried residues in any investigation.

Table 1. Viability of *Neocallimastix hurleyensis* in residues harvested from batch and continuous-flow cultures

Wheat straw (g / L)	Batch cultures	Continuous-flow cultures
5	+	–
10	+	–
20	+/-	+
40	–	+
80	–	–

Wheat straw residues plus adherent fungal biomass were harvested after 15 days of incubation and suspended in fresh medium (plus straw) in anaerobic culture tubes (three replicates). These were incubated at 39°C for up to 10 days and scored for the presence or absence of *N. hurleyensis*. +, fungal growth; +/–, growth in two tubes but not in the third tube, –, no fungal growth.

Production of fermentation end-products

In the current work, only acetate was measured in batch and continuous-flow cultures as a representative fermentation end product of the anaerobic fungus. Other fermentation products, including formate, lactate and ethanol are produced by *N. hurleyensis* [5], but these are not detected using the gas chromatographic procedure described here and are usually quantified using enzymic procedures. A description of all fermentation products and how their production is affected by dilution rate in continuous-flow culture is the subject of a forthcoming publication. In the current paper we present acetate profiles merely as a comparison of the mode of production of fermentation end-products by an anaerobic fungus in batch and continuous-flow cultures.

Figure 3(a) shows the acetate concentrations in culture effluents from continuous-flow cultures grown on 5–80 g DM/L of wheat straw over 350 h incubation period at a culture fluid dilution rate of 0.038/h. In general, there was a rapid increase in acetate concentration in culture effluents within the initial 100 h of incubation and concentration declined thereafter. Except for the 80 g DM/L wheat straw cultures, effluent acetate concentrations increased with increasing substrate concentrations. Growth of *N. hurleyensis* on the highest level of wheat straw (80 g DM/L) produced an acetate concentration profile somewhat similar to that for growth in the 40 g DM/L wheat straw cultures [Figure 3(a)]. By expressing the acetate concentration profiles in Figure 3(a) as cumulative production, cultures grown on 40 and 80 g DM/L of wheat straw were shown to accumulate similar amount of acetate over the experimental period, while progressively lesser amounts were produced from cultures grown on lesser quantities of wheat straw [Figure 3(b)]. When grown in batch culture, however, *N. hurleyensis* produced similar amount of acetate irrespective of substrate concentration, with < 3.0 mM of total acetate being produced within the same period of incubation (data not shown). A linear relationship between total acetate production and initial wheat straw concentration was observed for data from continuous-flow cultures within the range of 5–40 g DM/L of wheat straw, reaching a plateau thereafter (Figure 4); a similar relationship was not observed in batch culture.

CMCase and β-gluocosidase activities

Anaerobic fungi produce a wide range of cell wall degrading enzymes and here we describe the accumulation of CMCase and β-glucosidase activities in batch and continuous-flow cultures grown on 40 g DM/L wheat straw over 8 and 15 day incubation periods, respectively. The CMCase and β-glucosidase activity profiles from continuous-flow cultures are shown in Figure 5(a). CMCase activity increased rapidly, reaching a maximum of 160 IU/L at 80 h of incubation, and remained relatively constant for next 100 h before a rapid decline. A similar profile was observed for β-glucosidase activity, with a maximal value of 40 IU/L. In batch culture, the maximal values of CMCase and β-glucosidase activities were 100 and 15 IU/L, respectively (data not shown); these values were significantly ($p < 0.05$) lower than their corresponding maxima in continuous-flow cultures. By expressing the enzyme activity profiles in Figure 5(a) as cumulative production, it was found that both accumulated in a similar (linear) fashion during the initial 200 h of

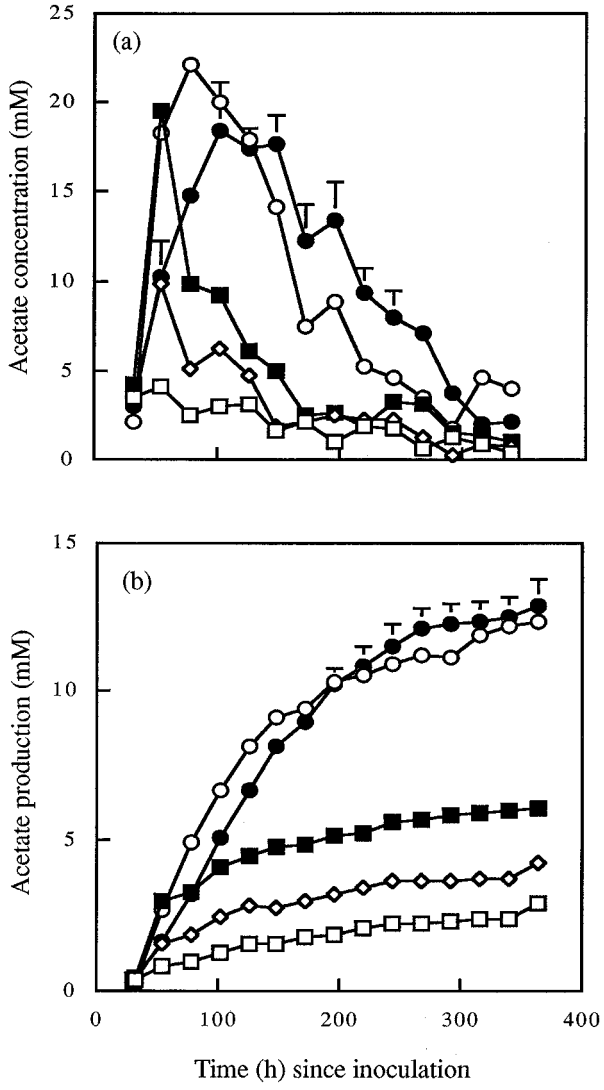

Figure 3. Production of acetate by *N. hurleyensis* grown in continuous-flow cultures. (a): acetate concentration, and (b): cumulative production. The fungus was grown at 39°C on wheat straw concentrations of 5 (□), 10 (◇), 20 (■), 40 (○) and 80 (●) g DM/L. Error bars represent SEM where n = 3. Where error bars are not shown the values were less than the size of the symbols used to represent the data.

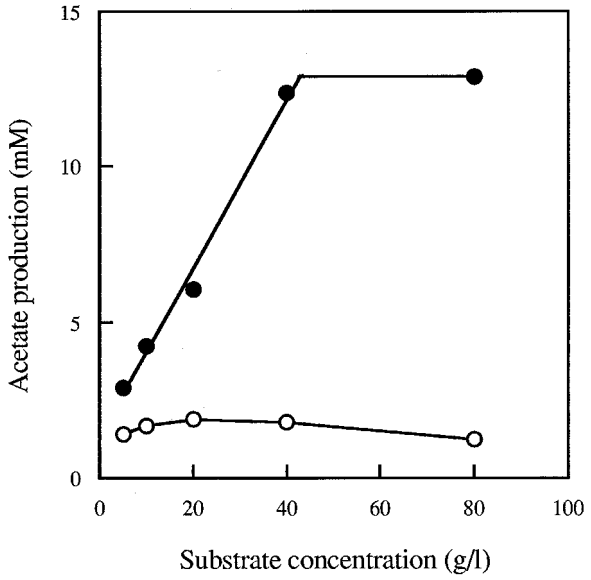

Figure 4. Relationship between substrate concentrations and total acetate production in batch (○) and continuous flow (●) cultures. The fungus was grown at 39°C on wheat straw concentrations ranging from 5–80 g DM/L. Linear regression line of best fit for the first four points of the continuous-flow culture data is $y = 0.269x + 1.342$ ($r^2 = 0.989$).

incubation and the increase declined thereafter. The maximal values for CMCase and β-glucosidase activities in continuous-flow cultures were approximately 100 and 28 IU, respectively [Figure 5(b)]. This compared favourably with the 10 and 1.5 IU of CMCase and β-glucosidase activity, respectively, produced in batch cultures containing the same initial substrate concentration of 40 g DM/L wheat straw.

Discussion

The continuous-flow system described in this paper, although not representative of conventional continuous culture, provided a simple and effective means of growing anaerobic fungi on high concentrations of plant biomass approximating those found in the rumen. An advantage of the system, given the use of relatively simple culture apparatus, was the ability to operate many continuous-flow cultures simultaneously, thus permitting comparative studies. Whilst not strictly a rumen analogue, the continuous-flow culture system described could be attractive to ruminant scientists studying aspects of the rumen ecosystem. Given that almost all studies concerning anaerobic fungi have been conducted in batch cultures containing low concentrations of substrate in comparison to the rumen, our results with continuous-flow cultures containing high substrate concentrations suggest that the potential contribution made by anaerobic fungi to lignocellulose degradation in the rumen may have been grossly underestimated.

In the current study, we have demonstrated that *N. hurleyensis* was able to degrade considerably more wheat straw and produce larger quantities of plant biomass degrading enzymes in continuous-flow compared with batch cultures. Given that the anaerobic fungi have evolved in continuous culture, they might have exploitable characteristics, for example, in the continuous production of plant biomass degrading enzymes or other bio-products. Although it would have been desirable to measure protein concentrations and determine specific enzyme activity, we were reluctant to do this with a monocentric fungus because of its finite life span and the likely contamination of enzyme protein with substantial quantities of autocatylitic breakdown products. Considerably more detailed experimental protocol and procedures are required to obtain meaningful information on the specific activities of extracellular enzymes from monocentric anaerobic fungi.

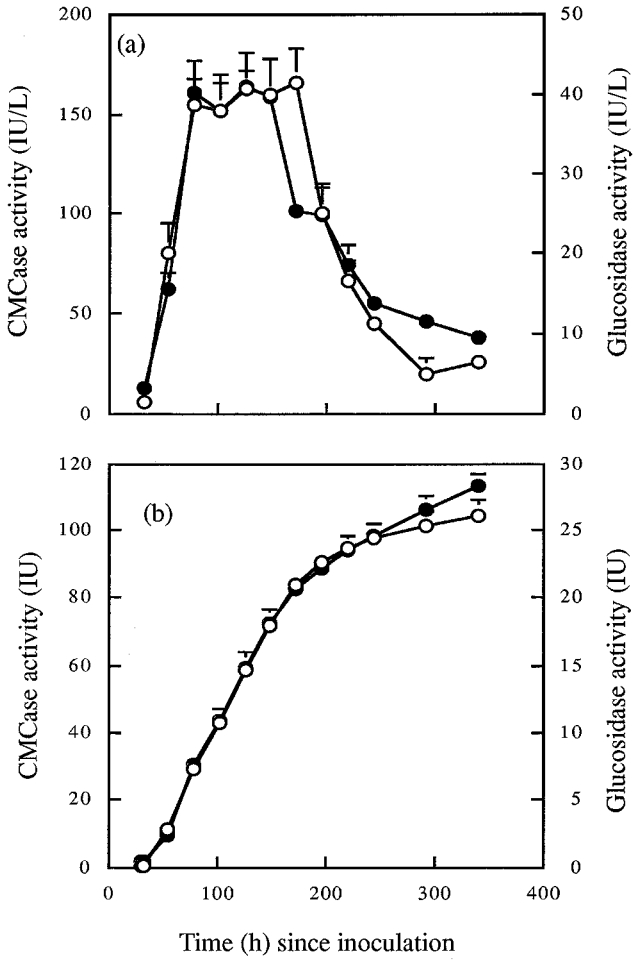

Figure 5. CMCase (○) and β-glucosidase (●) activities (a) and their cumulative activities (b) in continuous-flow cultures grown at 39°C on 40 g DM/L of wheat straw. Error bars represent SEM where n = 3. Where error bars are not shown the values were less than the size of the symbols used to represent the data.

Wheat straw NSP is largely derived from secondary cell walls and this is reflected in the high xylose and cellulose derived glucose content of the samples. The proportions of these constituents in the current study were similar to the 31 and 58% of total NSP accounted for by xylose and glucose, respectively, quoted for secondary cell walls of ryegrass, another member of the *Gramineae* [24]. After fermentation in batch or continuous-flow culture, ratios of the wheat straw NSP constituents remained similar to those of the original sample, suggesting no preferential fermentation of any NSP constituents, but removal of all NSP constituents at similar rates. This phenomenon has also been reported for rumen bacteria [24] and for anaerobic fungi [25].

Losses of wheat straw (DM and NSP) and production of acetate and cellulolytic enzymes were substantially lower in batch cultures than in corresponding continuous-flow cultures, particularly at the higher substrate concentrations. This effect, even at comparatively low substrate concentrations, was indicative of end-product inhibition of the fungus. Removal of end products in the continuous-flow cultures however enabled the fermentation (in cultures containing up to 40 g DM / L) to proceed unhindered. Values for wheat straw losses and acetate production in continuous-flow cultures containing 80 g DM / L wheat straw were similar to those obtained in the 40 g DM / L cultures suggesting that at the dilution rate employed cultures containing 80 g DM / L were inhibited. Conversely, although the percentage loss of NSP from continuous-flow cultures containing 80 g DM / L was only 46% of that from the corresponding 40 g DM / L cultures, the absolute amounts of NSP, glucose and xylose lost from fermentation of the two concentrations of substrate were very similar, suggesting that at the dilution rate used, optimal NSP loss was obtained at approximately 40 g DM / L.

Little information concerning fungal survival in batch and continuous culture is available. In studying the inhibitory effects of mixed acid fermentation intermediates and end products on anaerobic fungi, it was concluded that the most inhibitory compounds were ethanol, formate and lactate [26,27]. Given that substantially higher concentrations of acetate are present in the rumen then, in batch or continuous-flow cultures, this end product is an unlikely candidate for the inhibitor. Adverse physiological conditions, such as a decline in medium pH or limitation of essential nutrients or minerals, may also develop during growth on high substrate concentrations, leading to a loss of viability of the fungus and limited degradation of the substrate. In laboratory cultures, changing pH can have a significant influence on microbial activity, particularly in bicarbonate buffered anaerobic fermentations. Given that the continuous-flow culture system is 'open' and because of the way the system operates within a CO_2 filled chamber, it was not possible to measure pH easily in each of the 20 reaction vessels. Nevertheless, where maximal degradation was observed in continuous-flow cultures, results were consistent with the fact that the pH was optimal for substrate degradation. Where substrate degradation was less than optimal, however, in continuous-flow cultures initially containing 80 g DM / L, a decline in culture pH could have affected fungal activity. Certain inhibitors of cell wall glycosidase activity have been found in the *Gramineae*, the degree of inhibition being dependent on the time of harvesting [28]. Some such inhibitor may have been operating in the current study.

In conclusion, *N. hurleyensis* was able to degrade considerably more wheat straw, produce larger quantities of plant biomass degrading enzymes and survive for longer periods of time in continuous-flow as opposed to batch cultures. Results from continuous-flow cultures were obtained using one dilution rate and a range of substrate concentrations some of which approximating those found in the rumen. In subsequent studies, we have investigated growth, enzyme production and end-product formation using several dilution rates. We also intend to look at the effects of including methanogenic and non-methanogenic rumen bacteria in the culture flow.

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