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Effects of Sequential Sub-Culturing on the Survival and Enzyme Activity of *Neocallimastix hurleyensis*

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Abstract: This study investigated the long-term adaptation of a rumen anaerobic fungus, *Neocallimastix hurleyensis*, grown on each of 3 different substrates as the sole energy source. The rumen anaerobic fungus was cultivated on Avicel, Xylan and glucose for up to 31 subcultures, and its survival and enzymatic activity were monitored. The fungus was able to survive on each of these substrates. Significant increases in the xylanase, avicelase and carboxymethylcellulase (CMCase) activities of *N. hurleyensis* were observed after 31 subcultures on Avicel and Xylan. However, there was a significant decrease in these enzyme activities after 31 consecutive subcultures of the *N. hurleyensis* fungus on the medium containing glucose as the sole energy source.

Key Words: Anaerobic fungi, *Neocallimastix hurleyensis*, xylanase, avicelase, CMCase

Neocallimastix hurleyensis'in Ardışık Olarak Alt Kültüre Alınmasının Yaşamına ve Enzim Aktivitesine Etkileri

Özet: Bu çalışmada rumen fungusunun farklı üç enerji kaynağı üzerinde yetiştirilerek uzun süreli adaptasyonu incelenmiştir. Rumen anaerobik fungusu *Neocallimastix hurleyensis* avisel, ksilan ve glikoz üzerinde 31 alt kültüre kadar yetiştirilmiş ve fungusun hayatta kalabilmesi ve enzimatik aktivitesi gözlemlenmiştir. Anaerobik fungusun bu enerji kaynaklarının her biri üzerinde yaşayabildiği gözlemlenmiştir. *N. hurleyensis*'in ksilan üzerindeki 31'nci alt kültürden sonraki ksilanaz aktivitesinde ve avisel üzerindeki 31'nci alt kültürden sonraki aviselaz ve karboksimetilselulaz aktivitelerinde önemli miktarda artış olduğu gözlemlenmiştir. *N. hurleyensis*'in enerji kaynağı olarak glikoz içeren besi ortamı üzerindeki 31'nci alt kültürden sonraki ksilanaz, aviselaz ve karboksimetilselulaz aktivitelerinde önemli düzeyde düşüşler belirlenmiştir.

Anahtar Sözcükler: Anaerobik fungus, *Neocallimastix hurleyensis*, ksilanaz, aviselaz, karboksimetilselulaz

Introduction

The main component of plant material is lignocellulose, whose biodegradation is vital not only for animal nutrition but also for environmental, industrial and agricultural applications (1). The diet of ruminants consists largely of plant biomass; however, as mammalian herbivores do not themselves produce the necessary fiber degrading enzymes, they are dependent on the fibrolytic microorganisms that inhabit their digestive tract. Many rumen microorganisms are able to convert lignocelluloses to some extent to mono or disaccharides, which can be readily utilized by ruminants (2,3). However, the complete biodegradation of this material in the rumen is still limited due to the physical structures of plants such as lignifications and high crystallinity (4).

Gut anaerobic fungi are uniquely adapted to the pre- and post-gastric organs of the digestive tract of ruminant and monogastric herbivores, where they survive in an open (continuous-culture) ecosystem (5-7). Gut anaerobic fungi have stimulated considerable worldwide interest since their original discovery (8), not only because of their uniqueness among fungi but also because of their important role in plant biomass breakdown in the digestive tract of most herbivores. Studies indicated that these organisms are highly fibrolytic microorganisms that are capable of colonizing and degrading the major polysaccharides of plant materials (such as cellulose and hemicellulose) in the rumen ecosystem (9-11). These fungi produce a wide range of cell-bound and cell-free glycolytic, cellulolytic, and hemicellulolytic enzymes (12).

Fibrolytic enzymes of anaerobic fungi have been found to be associated with the rhizomycelium and many were also secreted into the environment (9,13,14). Furthermore, anaerobic fungi produce a large amount of xylanase constitutively together with cellulolytic enzymes even when cellulose is used as the main carbon source (15,16). Enzyme production and activity of these fungi are thought to be influenced by the stage of the life cycle and growth conditions (13,17,18). The aim of the current study was to investigate the survival of an anaerobic rumen fungus, *Neocallimastix hurleyensis*, in media containing different substrates and to detect any changes in the enzymatic activity of this microorganism.

Materials and Methods

Microorganisms and culture maintenance

The monocentric anaerobic fungus *Neocallimastix hurleyensis* was isolated from the rumen of sheep and characterized (19), and was obtained from the culture collection of the Institute of Grassland and Environmental Research, Aberystwyth, UK. Cultures were maintained at 39 °C in modified medium (M2) (20), a complex liquid medium containing rumen fluid (21), and wheat straw (*Triticum aestivum* (10 gDM/l) hammer milled and fractionated by dry-sieving through a 2 mm sieve). The medium was prepared by adding 10 ml of basal medium to Hungate tubes (16 X 125 mm, Bellco Glass Inc., Vineland, NJ, USA) containing 5 mg ml^{-1} (final concentration) energy source (e.g., glucose (M2G), microcrystalline cellulose (Avicel) (M2A), Xylan (M2X) or wheat straw (M2WS)). To inhibit bacterial growth 25-50 mg ml^{-1} final concentrations of streptomycin sulfate and/or choloramphenicol were used.

Samples were removed by sterilized loop from liquid cultures that had been vigorously shaken to disperse the contents. The fungal inoculum was then immediately inoculated into a pre-warmed and sterilized culture tube (22). Cultures were incubated without agitation and subcultured twice weekly. All fungi were maintained in Hungate tubes or serum bottles (Bellco Inc.) and incubated at 39 °C. The stock cultures were kept in liquid nitrogen (-196 °C) under the cryopreservation of 10% glycerol and thawed at room temperature just before transfer into the medium. All chemicals, unless otherwise stated, were supplied by Sigma (Sigma Chemical Co., Poole, Dorset, UK) and BDH, (British Drug House, Poole, Dorset, UK).

Enzyme assays

Xylanase, avicelase and carboxymethylcellulase (CMCase) activities were determined by measuring the reducing sugar released from Xylan, Avicel and carboxymethylcellulose (CMC) as described by Lever (23) at pH 6.0 and 50 °C. The protein content of samples was determined by the method described by Lowry et al. (24). A 10 mg ml^{-1} (in final concentration) soluble Xylan, obtained from oat spelt Xylan according to Ghangas et al. (25), or 10 mg ml^{-1} Avicel or 10 mg ml^{-1} CMC suspended in 0.05 M sodium phosphate buffer (pH 6.0) was used as substrate. Substrate (0.9 ml) was mixed with 0.1 ml of extract and incubated at 50 °C for 30 min, and 0.1 ml from this mixture was withdrawn and added to test tubes containing 5 ml of PAHBAH (parahydroxybenzoic acid hydrozide) solution as described by Lever (23). This mixture was heated at 70 °C for 10 min and after cooling the absorbance was read at 405 nm wavelength in a FP-901 Coagulation Chemistry Analyzer, LABSYSTEMS. A solution of 0.1 mg ml^{-1} glucose in 0.05 M sodium phosphate buffer (pH 6.0) was used as standard. Units (IU) of enzyme activity are defined as mM of product released per minute under assay conditions.

Results

Survival of *Neocallimastix hurleyensis*

N. hurleyensis was grown on 3 different carbon sources, (Xylan, glucose and Avicel) and was subcultured twice a week for up to 23 generations (Xylan and Avicel) or 32 generations (glucose). After every 10 subcultures (the 11th, 21st and 31st subcultures) the viability of *N. hurleyensis* was recorded (by eye, and with the aid of an inverted microscope), making observations for the presence/absence of fungal zoospores, sporangia and colonizing rhizoids (Figure 1). These substrates did not inhibit the growth or viability of *N. hurleyensis.*

Enzyme activities

To determine the optimum extracellular enzyme producing life stage of *N. hurleyensis*, the time-course of enzymatic activity was performed. For this purpose the fungus was incubated for 3, 5 and 7 days on M2X and M2A, or for 1, 3 and 5 days on M2G medium. After growth on the particular media (M2G, M2X or M2A) the culture supernatants were separated from fungal biomass and residual substrate centrifugation and xylanase,

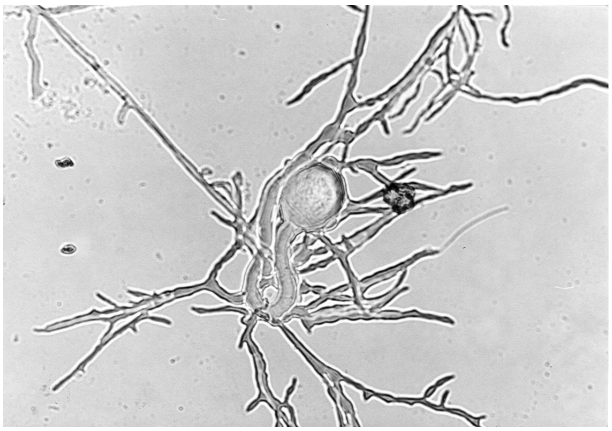

Figure 1. Observation of *N. hurleyensis* after 31 subcultures grown on M2G medium.

avicelase and CMCase activities of culture supernatants were determined.

The highest avicelase, CMCase and xylanase activities were obtained from 7-day-old cultures of *N. hurleyensis* grown on both M2X and M2A media, whereas the highest enzymes activities were obtained from 3-day-old cultures of *N. hurleyensis* grown on M2G medium (Table). Therefore 7-day-old cultures grown on M2X and M2A media and 3-day-old cultures grown on M2G medium were used for further enzyme assays.

Changes in the enzymatic activity of *N. hurleyensis* were investigated by subculturing on anaerobic medium containing different energy sources including Avicel, glucose or Xylan. Therefore *N. hurleyensis* was subcultured twice a week up to 31 times. During this period, 8 tubes were taken out for enzyme assays after every 10 subcultures (11, 21, and 31). Subculturing of *N. hurleyensis* on M2G medium resulted in decreases in avicelase, CMCase and xylanase activities (Figure 2). On the 11th subculture on M2G medium, avicelase, CMCase and xylanase lost about 65%, 70% and 90% of their activities, respectively. At the end of the 31st subculturing on M2G medium avicelase and CMCase activities were decreased remarkably (Figure 2) (lost activity approximately 15%).

Subculture of *N. hurleyensis* on M2A medium resulted in increases in avicelase and CMCase activities but a decrease in xylanase activity (Figure 2). On the 11th subculture on M2A medium, avicelase and CMCase activities increased approximately 25% and 20%, respectively. By the 31st subculture on M2A medium, avicelase and CMCase activities increased significantly by 83% and 162%, respectively (Figure 2). However, xylanase activity decreased gradually from 5% to 20% and to 30% (Figure 2).

Subculture of *N. hurleyensis* on M2X medium resulted in an increase in xylanase activity but decreases in avicelase and CMCase activities (Figure 2). Although on the 11th subculture on M2X medium, xylanase activity increased by about 35%, the 31st subculturing on M2X medium resulted in a remarkable increase in xylanase activity (approximately 178%, Figure 2). However, avicelase activity decreased gradually from 18% to 36% and to 40%, whilst CMCase activity decreased from 15%

Table. Determination of extracellular enzyme activities (IU) of *N. hurleyensis* grown on different substrates at different time intervals.

Energy sources-culture age	Avicelase	CMCase	Xylanase
Avicel-3 days	1.2 ±0.4	4.8 ± 0.6	20.5 ± 4.3
Avicel-5 days	4.7 ±0.5	15.5 ±0.9	39.2 ± 5.4
Avicel-7 days	9.1 ± 0.9	15.6 ± 1.2	49.6 ± 6.2
Xylan-3 days	11.4 ± 1.7	7.2 ± 0.7	51.3 ± 6.8
Xylan-5 days	14.2 ± 2.1	12.6 ± 1.4	98.6 ± 8.5
Xylan-7 days	12 ± 0.8	14.8 ± 1.3	107.3 ± 9.7
Glucose-1 day	3.8 ± 0.4	3.4 ± 0.8	5.6 ± 1.1
Glucose-3 days	7.3 ± 1.6	10.9 ± 3.4	47.8 ± 7.4
Glucose-5 days	3.2 ± 0.5	6.1 ± 1.3	30.9 ± 6.1

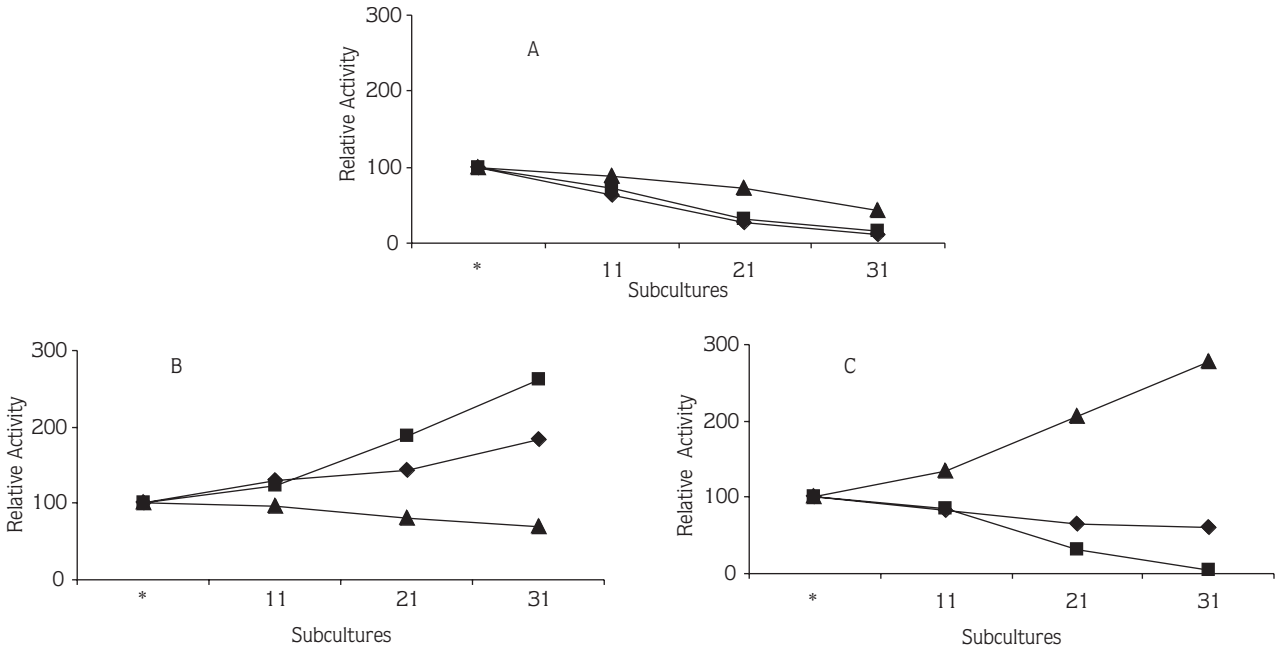

Figure 2. Determination of relative (%) avicelase (■), CMCase (◆) and xylanase (▲) enzyme activities of *N. hurleyensis* grown on glucose (A), Avicel (B) and Xylan (C) up to 31 subcultures. * control culture, grown on M2WS before subculturing.

to 69% and to 96% for the 11th, 21st and 31st subcultures, respectively (Figure 2).

Discussion

Rumen fungi play an important role in the degradation of plant structural and storage polysaccharides (9,12). *N. hurleyensis* was able to utilize a range of carbon sources including Avicel, Xylan and glucose for growth. Survival of *N. hurleyensis* on these substrates was demonstrated until about the 23rd generation. All *Neocallimastix* species studied so far are able to utilize Xylan or xylose (11,26,27) except for *Neocallimastix* sp. strain L2 (27). Pearce and Bauchop (28) reported that the enzymatic activity of *N. frontalis* was highest in the extracellular fraction, reaching maximal levels after 6-7 days although the intracellular activity peaked more rapidly. The degradation of complex polysaccharides can only occur extracellularly, and the invasion of plant tissue by the fungi will require an array of active degradative enzymes. However, the penetrative mode of growth may ensure that the secreted enzymes are in the immediate proximity of the plant cell wall and able to interact with substrate.

As with other anaerobic fungi, the production of polysaccharide degrading enzymes by *N. hurleyensis* was substrate dependent. The highest production of cellulolytic enzymes was observed when the fungus was grown on highly crystalline cellulose (Avicel). Soluble substrate (e.g., glucose) and Xylan yield lower enzyme production. Similar results were obtained with *N. frontalis* (27) and *N. patriciarum* (18,26). The effects of Xylan and cellulose were most pronounced for xylanase and cellulase (avicelase, CMCase) activities (Figure 2). Studies with *N. frontalis* (11,29) indicated that enzyme formation by this fungus was also substrate dependent, and it has been demonstrated that the production of cellulases was repressed by glucose and production of xylanases repressed by xylose and arabinose (29), and that the soluble sugars were less effective inducers of cellulase. Although there is evidence that some or all of the enzymes are partly constitutive, they can also be induced in the presence of cellulose (27,29,30) or other cellulose containing fiber (26,31). Indeed relatively little work has been carried out on the regulation of genes encoding xylanolytic enzymes produced by rumen microorganisms. It has been reported that these enzymes are synthesized under separate regulatory control from

cellulases in aerobic fungi (4). It would thus appear that a similar control mechanism modulates the production of other polysaccharide depolymerases in *Neocallimastix* spp. and other rumen fungal genera. The mode of the regulatory mechanism was not established but it was apparent in this survey that although the xylanase, avicelase and CMCase were formed constitutively the enzyme activities were lower following growth on the medium containing glucose as the sole energy source.

Although it cannot be concluded for all energy sources, studies suggest that the production of cellulases and xylanases is likely to be under different regulatory control (1). This study also suggests that production of cellulases and xylanases of *N. hurleyensis* might be under different regulatory control. Enzyme production and activity by microorganisms can be affected both adversely and positively by the presence of soluble or insoluble energy sources in the growth medium (30). Therefore, 2 basic regulatory mechanisms are suggested in cellulase synthesis: I) cellulase systems are repressed by the presence of low molecular mass carbon sources that are degraded relatively readily, and II) cellulose biosynthesis is induced by the presence of cellulose and its degradation products in the culture medium (32). However, we still know little about the enzyme regulatory mechanism of anaerobic gut fungi and more investigations are needed.

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