THE UTILITY OF ANAEROBIC GUT FUNGI IN THE PRODUCTION OF LIGNOCELLULOSIC BIOFUELS

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Abstract: The overall aim of this dissertation was to determine the prevalence and distribution of anaerobic gut fungi (AF) in nature and to evaluate the potential of AF isolates in producing biofuels from lignocellulosic plant substrates. Three different research projects were undertaken to achieve this aim. I started by investigating the diversity and community structure of anaerobic gut fungi in fecal samples obtained from a large number of mammalian and reptilian herbivores using a culture-independent approach that involved the amplification and sequencing of the internal transcribed spacer (ITS-1) region in the rRNA operon. This work revealed a highly diverse anaerobic fungal community within herbivores, with many novel, previously un-encountered lineages identified. Eight distinct AF groups representing putatively novel genera were detected, several of which have subsequently been independently confirmed by other research groups around the world. In the second project, multiple isolation strategies were employed in an effort to obtain robust anaerobic fungal isolates capable of growing on various lignocellulosic substrates. This effort yielded a novel anaerobic fungal isolate, *Orpinomyces* sp. strain C1A, isolated on media supplemented with cellobiose and switchgrass. Experimental analyses indicated that strain C1A is a remarkable biomass degrader, capable of simultaneous saccharification and fermentation of the cellulosic and hemicellulosic fractions in multiple grasses and crop residues, with and without biomass pretreatment. In my final project, I evaluated the utility of hydrothermal biomass pretreatment in degradation schemes using strain C1A. Hydrothermolysis-pretreated lignocellulosic biomass (corn stover and switchgrass) was more amenable to degradation by strain C1A when compared to untreated biomass. However, when factoring in the proportion of biomass lost during the pretreatment process, hydrothermolysis provided negligible or negative improvements to the extent of corn stover and switchgrass degradation by strain C1A. Collectively, the results of these projects demonstrate the remarkable genus and species level diversity within the anaerobic gut fungal communities in nature, and suggests that these organisms could represent a promising platform for biofuel production from lignocellulosic biomass. However, since anaerobic fungi produce organic acids rather than alcohols as their major fermentation products, efforts towards improving alcohol production and tolerance via physiological and genetic manipulations are still required to achieve efficient and commercially appealing ethanol production.

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Preface

The desire for sustainable alternatives to fossil fuels has lead to considerable improvements in the production of biofuels from agricultural crops. Efficient production of bioethanol using homofermentative microorganisms is a well-established and economical process. However, production of biofuels from crops (e.g. corn and sugar cane) is not desirable since it leads to higher commodity prices as well as the expansion of farming acreage and fertilizer usage. A promising alternative is the production of biofuels from lignocellulosic biomass, defined as the raw, non-edible plant biomass that is mainly composed of sugar (cellulose and hemicellulose) and aromatic (lignin) polymers. Currently, such processes are technically feasible, but extremely expensive due to the structural complexity of plant substrates and the high costs associated with sugar extraction saccharification from the complex cellulose and hemicellulose fraction in lignocellulosic biomass.

One of the most intriguing candidates for microbial deconstruction of lignocellulosic biomass is the anaerobic gut fungi. Anaerobic gut fungi represent a distinct early-branching fungal phylum (*Neocallimastigomycota*), and reside in the rumen, hindgut, and feces of ruminant and non-ruminant herbivores. This dissertation focuses on evaluating this potential role for anaerobic fungi in biofuel production from lignocellulosic substrates. At the start of my graduate research in 2008, the paucity of information regarding their overall prevalence in nature led to the first research project

described in Chapter II, which investigated the composition, size, and distribution of the anaerobic fungal populations within herbivores. In the second project (Chapter III), we applied the knowledge gained through the first study to successfully obtain an anaerobic fungal isolate, *Orpinomyces* sp. strain C1A, and described its degradative capabilities on multiple lignocellulosic substrates with and without biomass pretreatment. The third project (Chapter IV) critically evaluated hydrothermolysis as a pretreatment strategy for biomass degradation by strain C1A.

Chapter I was written to provide a general introduction to the *Neocallimastigomycota*, anaerobic gut fungi, and their emerging recognition as key players in biomass utilization within herbivores and proposed utility in applied systems for lignocellulosic biofuels. The challenging aspects to elucidating the phylogenetic diversity for this group and several limitations to biofuel production that are the addressed targets in Chapters III and IV will also be introduced.

The prevalence and distribution of anaerobic fungi (AF) in herbivores was the focus of the work in Chapter II. Prior to this study, the limited phylogenetic diversity reported for this group had been inferred primarily from culture-based and microscopic studies, with community composition assessed through the use of various fingerprinting approaches. As such, little was known regarding the extent of global phylogenetic diversity within the AF, the presence and prevalence of novel yet-uncultured AF genera, the complexity of the AF community within a single host, and the influence of various

ecological and environmental factors on AF diversity and community composition within various hosts. Using a high-throughput barcoded pyrosequencing approach, a survey of fecal samples from 33 ruminant and non-ruminant herbivores revealed the presence of an extremely diverse AF community that varied widely between different hosts and identified multiple novel AF fungal genera. The study also presented evidence that host phylogeny may be an important factor in determining the AF diversity and community composition within the different samples. The work is published in the journal *International Society for Microbial Ecology* (Liggenstoffer et al., 2010 The ISME J 4:1225-1235).

The work presented in Chapter III describes the isolation and degradative capabilities of an anaerobic fungal isolate, *Orpinomyces* sp. strain C1A. The information gained in the previous study, detailed in Chapter II, allowed for targeted AF isolation efforts from multiple herbivores possessing high diversity and unique AF communities. The resulting strain, C1A, was successfully maintained in a cellobiose medium supplemented with rumen fluid without the loss of culture viability or degradative capacity. It was capable of simultaneous saccharification of the cellulosic and hemicellulosic components of multiple lignocellulosic plants with combined fermentation of the resulting hexose and pentose sugars. The invasive nature and filamentous growth pattern of strain C1A allowed plant biomass degradation to proceed without pretreatment, and was shown to be significantly enhanced using mild pretreatments. Collectively,

strain C1A was shown to be an effective, versatile biomass degrader and a potential role in consolidated bioprocessing for biofuel production was discussed. This work is published as part of a larger study in the journal *Applied and Environmental Microbiology* (Youssef et al., 2013, Appl. Environ. Microbiol. 79:4620-4634).

The necessity of lignocellulosic biomass pretreatment for biofuel production using strain C1A was the focus of the work presented in Chapter IV. Although considered an unavoidable first step in enzyme-based saccharification schemes, its requirement in anaerobic fungal-based schemes was still unclear. Hydrothermal pretreatment uses elevated temperatures and pressure to generate acidic reaction conditions that overcome biomass recalcitrance and render it more amendable to enzyme degradation. This process results in substantial removal of hemicellulose and dislocation of lignin from the pretreated biomass. In addition to substrate losses, pretreatment also comes with increased energy and cost expenditures that must be offset by significant improvements in biomass degradation to justify its use. This study found that the improvements in degradability realized through hydrothermal pretreatment did not justify the losses resulting from the process. This work has been accepted by the *Journal of Microbiological Methods*.

CHAPTER I

EMERGING RECOGNITION OF ANAEROBIC GUT FUNGI IN BIOMASS DEGRADATION AND PROPOSED UTILITY IN APPLIED SYSTEMS FOR LIGNOCELLULOSIC BIOFUELS

Abstract

Anaerobic fungi (phylum *Neocallimastigomycota*) inhabit the gastrointestinal tract of ruminant and non-ruminant herbivores, where they play an important role in the degradation of plant materials. Phylogenetically, the *Neocallimastigomycota* represent a separate basal fungal phylum with very little known regarding their true distribution in nature. They combine mechanisms for biomass deconstruction from anaerobic prokaryotes and aerobic fungi into a single, highly fibrolytic microorganism. Anaerobic fungi produce a wide array of cell-bound and cell-free cellulolytic, hemicellulolytic, glycolytic, and proteolytic enzymes. Biotechnological applications for anaerobic fungi, and their highly active cellulolytic and hemicellulolytic enzymes, have been a rapidly increasing area of research and development in the last decade. This dissertation focuses on evaluating the potential utility of anaerobic fungi towards one of these applications, the production of lignocellulosic biofuels.

Anaerobic gut fungi (AF). Members of the anaerobic fungi (*Neocallimastigomycota*) were originally discovered in sheep, but have since been shown to exist in the rumen, hindgut, and feces of ruminant and non-ruminant herbivorous mammals, as well as reptilian herbivores (47, 51). Currently, only 6 genera and 20 species have been described, although multiple uncharacterized isolates have also been reported (Table 1). Further, multiple culture-independent diversity surveys have documented the presence of novel, yet-uncultured anaerobic fungal lineages within the gut of various herbivores (Table 2).

AF role in the rumen. Considering the model system of the cow, biomass undergoes relatively mild physical and chemical pretreatment before much of the "work" is done by its digestive microbiota, including the anaerobic gut fungi (2, 3, 8, 17, 22, 25, 33, 42, 48, 60, 73, 74, 78). Anaerobic fungi are unique in being both anaerobic and filamentous, capable of coupling saccharification and fermentation of recently ingested plant materials, and in their capacity to utilize both cellulose and hemicellulose fractions (47). This ability has evolved during their long evolution in the gut from exposure to selective forces including mixed lignocellulosic substrates, short retention times for consumed materials, a consistently anaerobic and warm environment, and co-habitation with anaerobic cellulolytic bacteria (47, 77). Within the rumen, anaerobic fungi are thought to be responsible for initial attack on ingested plant materials due to their physical invasiveness and hyphal penetration of plant cell walls, as well as their capability to produce multiple saccharolytic enzymes (47, 50). Studies showing preferential colonization of motile zoospores, the reproductive phase of AF, to lignin-rich regions of ingested plant materials further supports their primary role in producing an accessible

lignocellulosic substrate within the digestive tract of the host (2, 17, 22, 50). Many of these characteristics enabling anaerobic fungi to degrade lignocellulosic biomass in the cow rumen could conceivably be useful in biofuel production from lignocellulosic biomass. The sole purpose of this dissertation is hence to explore the utility and applicability of utilizing anaerobic fungi for biofuel production from lignocellulosic biomass.

The need for lignocellulosic biofuels. The continued depletion of, and the projected increase in the demand for fossil fuels necessitates the development and production of cost effective fuels from renewable energy sources, including biofuels (29, 39, 56, 70, 72, 75). Advancements towards this goal have been made, with each successive generation of biofuel research endeavoring to remedy the limitations of its predecessor in an effort to replicate the abilities endowed to natural systems (74). However, certain challenges still hinder the widespread use and cost-effective conversion of plant biomass to biofuels. A major drawback in "first generation" biofuels was the use of agricultural biomass sources that alternatively would be used as a direct food source for humans or feed for livestock (29, 39, 71, 76, 79). The production of this biomass often required the redirection of agricultural resources, such as arable lands and fresh water (28). To surmount these issues, second generation biofuels utilized biomass sources not directly applicable for human consumption or livestock feeds (39). However, this decrease in usability results from an increase in structural complexity and recalcitrance of these substrates (39). Chemical, thermal or enzymatic pretreatment is commonly required to loosen or remove lignin from the cellulose and hemicellulose carbohydrates before fermentation (1, 4, 18, 21, 29, 32, 34, 56, 70, 79). Thus, efficient depolymerization of structural carbohydrates

to monomeric sugar residues continues to be a rate-limiting step in the subsequent saccharification and fermentation (32, 69). Focused efforts have advanced the effectiveness in using chemical or thermal pretreatments but the use of enzymes is preferred owing to their selectivity in the reaction chemistry (29, 32, 39, 62, 69). However, the low functional stability and efficiency of industrial enzymes results in high concentration requirements and are considered cost-prohibitive factors in the development of lignocellulosic biofuels (32, 39). These issues have yet to be overcome in a cost-effective manner (32).

Costs associated with biofuel production from lignocellulosic biomass. Currently, the greatest hindrances to wide scale production of lignocellulosic biofuels stems from the costs associated with pretreatment and enzymatic saccharification (4, 32). This process is shown in Figure 1. First, a chemical or thermal pretreatment is used to create an enzymatically receptive substrate (ERS), which reduces the structural complexity and allows for increased saccharification. However, this often removes or degrades potentially fermentable substrates and may produce toxic degradation products. Next, there is an enzymatic saccharification step that often requires, or is at least enhanced by, multiple lignocellulolytic enzymes.

Similar to crop-based biofuels, the production of lignocellulosic biofuels is a biochemical process, in which enzymes are utilized to extract sugar from plant polymers, and the produced sugars are then converted into biofuels using dedicated sugar-fermenting microorganisms (4, 39). However, the sugar extraction process from lignocellulosic biomass is far more complicated than sugar extraction from cereal grains (mainly corn in the US) due to differences in the composition of sugar polymers in both;

starch in case of corn, as opposed to cellulose and hemicellulose in case of lignocellulosic biomass. Since starch is a temporary storage pool for glucose in plants, it is quickly and easily metabolized by few, often one, enzyme(s) (39). However, cellulose and hemicellulose are structural components of plant cell walls that are chemically bound to a variety of complex macromolecules, mainly lignin (39, 59). Therefore, to effectively metabolize cellulose and hemicellulose, a combination of chemical pretreatments and exogenous enzyme cocktail additions are required (4).

Pretreatment processes often involve high temperatures, harsh chemicals, and/or high pressures that cause sugars to be degraded into furfurals and organic acids that inhibit microbial fermentation of remaining sugars to biofuels and chemicals (4, 35). Enzymatic treatment of lignocellulosic biomass is a complex endeavor requiring a mixture of multiple enzymes to depolymerize cellulose and hemicellulose. Cellulose requires at least three distinct enzymes (endogluconases, cellobiohydrolases, and β-glucosidaes) for degradation. While hemicellulose, a term that describes multiple heterogeneous structural polymers with highly substituted xylans, mannans, xyloglucans, glucomannans, or β-(1→3,1→4)-glucan backbones (39, 59), requires an even greater number of enzymes for efficient hydrolysis. For example, efficient utilization of glucoronoarabinoxylan, the most common form of hemicellulose in grasses, requires the concerted action of mobilizing (ferulic and cinnamoyl esterases), debranching (α-arabinofuranosidase, acetylxylan esterase, polysaccharide deacetylase, α-glucuronidase), and depolymerizing (xylanase and xylosidase) enzymes (59). Finally, the dependence on a single type of lignocellulosic biomass as a starting substrate is an inducement for planting bioenergy crops on a large scale on marginal lands, an issue that could lead to

loss in plant biodiversity (67). Due to these difficulties, the National Research Council report explicitly states that "*biofuel production from cellulosic biomass will not reach the mass efficiency or economic viability of ethanol production from grain unless techniques are developed to break down both cellulose and hemicellulose effectively into sugars*" (12).

Evaluation of the role of AF in lignocellulosic biomass production schemes. Overall, research progress on AF has been hampered by their anaerobic and eukaryotic nature. Mycologists usually display little interest in working with strict anaerobes, and similarly, bacteriologists display little interest in working with eukaryotes. Left in the proverbial no man's land, very few research laboratories in the world are currently studying aspects of the biology of AF. This is unfortunate, since AF play a prominent role in plant biomass degradation within herbivores, and many of the capabilities acquired during their evolutionary history and adaptation to herbivorous guts represent extremely desirable traits for direct conversion of lignocellulosic biomass to sugars and biofuels. These traits include: 1. Coupling an anaerobic fermentative mode of metabolism and accumulation of acid and alcohol end products, a trait associated with prokaryotes, with the invasive and filamentous growth patterns associated with fungi; 2. The capability to degrade multiple types of plant substrates (e.g. ryegrass, barley, wheat straw, corn stover, energy cane); and 3. The capability to degrade both cellulosic and hemicellulosic (arabinoxylans, glucoxylans, and glucomannans) fractions of lignocellulosic biomass by producing a large array of synergistic catalytic and accessory enzymes for biomass deconstruction (40, 77).

Table 1-1. Anaerobic fungi detected using enrichment and isolation based approaches.

Animal host	**Latin name**	**Family**	**Gut type**	**AF genera detected**	**References**
White antelope	*Addax nasomaculatus*	Bovidae	Foregut Ruminant	*Piromyces*	(68)
African elephant	*Loxodonta africana*	Elephantidae	Hindgut	*Piromyces*	(63)
Alpine ibex	*Capra ibex*	Bovidae	Foregut Ruminant	*Neocallimastix, Caecomyces*	(36)
Arabian oryx	*Oryx leucoryx*	Bovidae	Foregut Ruminant	*Neocallimastix*	(44)
Asian elephant	*Elephas maximus*	Elephantidae	Hindgut	*Neocallimastix*	(37, 44, 45, 65)
Bactrian camel	*Camelus bactrianus*	Camelidae	Foregut Pseudoruminant	*Neocallimastix*	(26, 44)
Banteng cattle	*Bos javanicus*	Bovidae	Foregut Ruminant	*Neocallimastix*	(68)
Blackbuck	*Antilope cervicapra*	Bovidae	Foregut Ruminant	*Anaeromyces, Neocallimastix, Orpinomyces*	(58)
Black rhinoceros	*Diceros bicornis*	Rhinocerotidae	Hindgut	*Neocallimastix, Piromyces*	(44, 63)

Bongo	*Tragelaphus eurycerus*	Bovidae	Foregut Ruminant	*Neocallimastix*	(44)
Domestic sheep	*Ovis aries*	Bovidae	Foregut Ruminant	*Neocallimastix, Orpinomyces, Piromyces, Anaeromyces, Caecomyces*	(6, 7, 10, 27, 41, 44, 45, 48, 51, 52, 54, 58, 63, 66)
Domestic cattle	*Bos taurus, B. indicus, B. gaurus*	Bovidae	Foregut Ruminant	*Neocallimastix, Piromyces, Orpinomyces, Anaeromyces, Cyllamyces*	(5, 6, 8-11, 13, 15, 20, 23, 24, 44, 45, 55, 77)
Common zebra (Plains zebra)	*Equus quagga*	Equidae	Hindgut	*Neocallimastix, Piromyces*	(44, 45, 68)
Deer	unknown species	Cervidae	Foregut Ruminant	*Neocallimastix*	(45)
Goat	*Capra aegagrus hircus*	Bovidae	Foregut Ruminant	*Neocallimastix, Orpinomyces, Piromyces, Anaeromyces*	(10, 26, 45, 58, 66)
Guinea pig	*Cavia porcellus*	Caviidae	Hindgut	*Caecomyces*	(52)
Hog deer	*Hyelaphus porcinus*	Cervidae	Foregut Ruminant	*Anaeromyces*	(58)
Horse	*Equus ferus* ssp. *caballus*	Equidae	Hindgut	*Piromyces, Caecomyces*	(19, 37, 49)
Indian	*Rhinoceros*	Rhinocerotidae	Hindgut	*Piromyces*	(64)

rhinoceros	*unicornis*				
Kangaroo	*Macropus* sp. (unidentified)	Macropodidae	Foregut Nonruminant	*Piromyces*	(10)
Greater kudu	*Tragelaphus strepsiceros*	Bovidae	Foregut Ruminant	*Neocallimastix*	(44)
Kudu	*Tragelaphus* sp.	Bovidae	Foregut Ruminant	*Orpinomyces*	(68)
Llama	*Lama glama, L. pacos, L. guanicoe* (all housed in a single enclosure)	Camelidae	Foregut Pseudoruminant	*Neocallimastix*	(44)
Mara	*Dolichotis patagonum*	Caviidae (order rodenta)	Hindgut	*Piromyces*	(63)
Marine iguana	*Amblyrhynchus cristatus*	Iguanidae	Hindgut	unidentified (microscopic identification of anaerobic fungal spores)	(43)
Mule	*Equus* sp.	Equidae	Hindgut	*Piromyces, Anaeromyces*	(26)
Nilgai (Blue bull)	*Boselaphus tragocamelus*	Bovidae	Foregut Ruminant	*Piromyces*	(45, 57, 58, 68)

Roan antelope	*Hippotragus equinus*	Bovidae	Foregut Ruminant	*Neocallimastix*	(44)
Sable	*Hippotragus niger*	Bovidae	Foregut Ruminant	*Neocallimastix*	(68)
Spotted dear	*Axis axis*	Cervidae	Foregut Ruminant	*Neocallimastix*	(58)
Svalbard reindeer	*Rangifer tarandus*	Cervidae	Foregut Ruminant	*Neocallimastix*	(53)
Vicuna	*Vicugna vicugna*	Camelidae	Foregut Pseudoruminant	*Neocallimastix*	(44)
Water buffalo	*Bubalus bubalis*	Bovidae	Foregut Ruminant	*Neocallimastix*, *Orpinomyces*, *Piromyces*, *Anaeromyces*	(10, 26, 45, 58)

Table 1-2. Culture-independent studies examining AF community.

Study	**Animal host**	**Latin name**	**Family**	**Gut type**	**Anaerobic fungal genera**	**Method**
(14)	Domestic cattle	*Bos taurus*	Bovidae	Foregut Ruminant	*Anaeromyces*, *Orpinomyces*	ARISA, Cloning and Sanger sequencing
(38)	Indian hog deer	*Hyelaphus porcinus*	Cervidae	Foregut Ruminant	*Piromyces*, *Caecomyces*, *Anaeromyces*, AL1, AL2, AL3, AL5	Pyrosequencing
	American bison	*Bison bison*	Bovidae	Foregut Ruminant	*Neocallimastix*, *Piromyces*, *Caecomyces*, *Anaeromyces*, *Orpinomyces*, *Cyllamyces*, AL2, AL4	
	American elk	*Cervus canadensis*	Cervidae	Foregut Ruminant	*Neocallimastix*, *Piromyces*, AL3	
	Black rhinoceros	*Diceros bicornis*	Rhinocerotidae	Hindgut	*Piromyces*, *Neocallimastix*	
	Bontebok	*Damaliscus pygargus*	Bovidae	Foregut Ruminant	*Neocallimastix*, *Piromyces*, *Caecomyces*, *Anaeromyces*, *Orpinomyces*, AL1	
	Domestic	*Bos taurus*	Bovidae	Foregut	*Neocallimastix*, *Piromyces*, *Caecomyces*, *Anaeromyces*,	

	cattle			Ruminant	*Orpinomyces*, AL1, AL3, AL5, AL7
	Gerenuk	*Litocranius walleri*	Bovidae	Foregut Ruminant	*Neocallimastix, Piromyces, Caecomyces, Orpinomyces*, AL3
	Goat	*Capra aegagrus hircus*	Bovidae	Foregut Ruminant	*Neocallimastix*, *Piromyces*, *Anaeromyces*, AL1, AL5
	Goral	*Nemorhaedus* sp.	Bovidae	Foregut Ruminant	*Neocallimastix*, *Piromyces*, *Caecomyces*, *Anaeromyces*, AL1, AL2, AL3
	Grant's gazelle	*Nanger granti*	Bovidae	Foregut Ruminant	*Piromyce*s, AL1, AL3
	Grant's zebra	*Equus quagga boehmi*	Equidae	Hindgut	*Anaeromyces*, AL1
	Greater kudu	*Tragelaphus strepsiceros*	Bovidae	Foregut Ruminant	*Anaeromyces*, AL6
	Green iguana	*Iguana iguana*	Iguanidae	Reptilian Hindgut	*Neocallimastix, Piromyces, Anaeromyces*, AL1, AL3, AL5
	Grevy's zebra	*Equus grevyi*	Equidae	Hindgut	*Piromyces*, AL1, AL3

	Horse	*Equus ferus* ssp. *caballus*	Equidae	Hindgut	*Neocallimastix*, *Piromyces*, *Caecomyces*, *Anaeromyces*, AL1, AL2, AL3, AL5
	Indo-Chinese sika deer	*Cervus nippon*	Cervidae	Foregut Ruminant	*Piromyces*, *Anaeromyces*, AL1, AL3, AL5
	Llama	*Llama* sp.	Camelidae	Foregut Pseudoruminant	*Piromyces*, *Neocallimastix*, *Caecomyces*, *Anaeromyces*, *Orpinomyces*, AL6
	Miniature donkey	*Equus africanus asinus*	Equidae	Hindgut	*Neocallimastix*, *Piromyces*, *Anaeromyces*, NG3
	Nile lechwe	*Kobus megaceros*	Bovidae	Foregut Ruminant	*Neocallimastix*, *Piromyces*, *Caecomyces*, *Anaeromyces*, AL1, AL2, AL3, AL5
	Okapi	*Okapia johnstoni*	Giraffidae	Foregut Ruminant	*Neocallimastix*, *Piromyces*, *Anaeromyces*, AL1, AL6
	Pere David's deer	*Elaphurus davidianus*	Cervidae	Foregut Ruminant	*Piromyces*, *Caecomyces*, *Anaeromyces*, AL1, AL2, AL3
	Pronghorn	*Antilocapra americana*	Antilocapridae	Foregut Ruminant	*Piromyces*, *Anaeromyces*, AL3, AL5

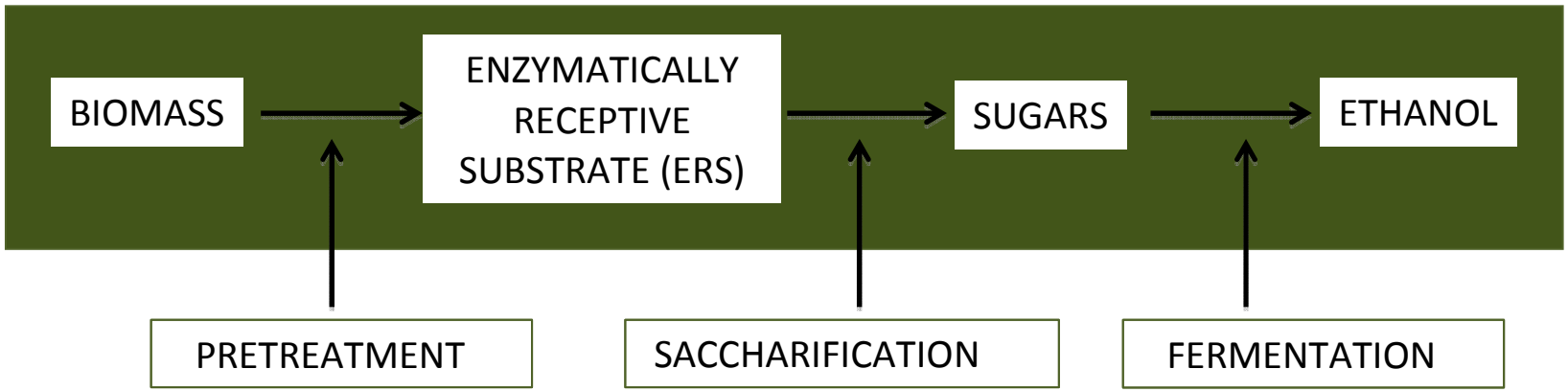
	Pygmy hippopotamus	*Choeropsis liberiensis*	Hippopotamidae	Foregut Pseudoruminant	*Piromyces*, *Anaeromyces*, AL1, AL3, AL5	
	Red kangaroo	*Macropus rufus*	Macropodidae	Foregut NonRuminant	*Piromyces*, *Anaeromyces*, AL1, AL3, AL8	
	Rothschild's giraffe	*Giraffa camelopardalis rothschildi*	Giraffidae	Foregut Ruminant	*Anaeromyces*, *Orpinomyces*, AL1, AL3, AL5, AL6	
	Sable antelope	*Hippotragus niger*	Bovidae	Foregut Ruminant	*Neocallimastix*, *Piromyces*, *Caecomyces*, *Anaeromyces*, *Orpinomyces*, *Cyllamyces*, AL1, AL3, AL4, AL5	
	Domestic sheep	*Ovis aries*	Bovidae	Foregut Ruminant	*Neocallimastix*, *Piromyces*, *Caecomyces*, *Anaeromyces*, *Orpinomyces*, AL5	
	Somali wild ass	*Equus africanus somaliensis*	Equidae	Hindgut	*Neocallimastix*, *Piromyces*, *Caecomyces*, AL1, AL3, AL7	
	Western tufted deer	*Elaphodus cephalophus*	Cervidae	Foregut Ruminant	*Neocallimastix*, *Piromyces*, *Anaeromyces*, AL1, AL2, AL3, AL5	
	White fronted	*Macropus*	Macropodidae	Foregut	*Piromyces*, *Anaeromyces*,	

	wallaby	*parma*		NonRuminant	AL1, AL2, AL3, AL5	
(16)	Domestic cattle	*Bos taurus*	Bovidae	Foregut Ruminant	*Cyllamyces, Piromyces, Anaeromyces, Neocallimastix, Caecomyces,* Nov KF1, SK1, SK3, AL6	Cloning and sequencing
(46)	African buffalo	*Syncerus caffer*	Bovidae	Foregut Ruminant	Collectively for African buffalo, Impala, Eland, African elephant, African hippopotamus, and Zebra: *Anaeromyces, Orpinomyces*, MN1 and MN2	DGGE, followed by classification according to banding pattern in DGGE, excising bands and sequencing. Sequencing was done for water buffalo only. Size fractionation was done, but not clear if it was used to select clones for sequencing
	African elephant	*Loxodonta africana*	Elephantidae	Hindgut		
	African hippopotamus	*Hippopotamus amphibius*	Hippopotamidae	Foregut Pseudoruminant		
	Eland	*Taurotragus derbianus*	Bovidae	Foregut Ruminant		
	Impala	*Aepyceros melampus*	Bovidae	Foregut Ruminant		
	Zebra	*Equus quagga*	Equidae	Hindgut		
	Domestic cattle	*Bos taurus*	Bovidae	Foregut Ruminant	Collectively for Domestic cattle and sheep:	DGGE, followed by classification

	Domestic sheep	*Ovis aries*	Bovidae	Foregut Ruminant	*Neocallimastix*, *Piromyces*, *Orpinomyces*, *Cyllamyces*, MN3, MN4	according to banding pattern in DGGE, excising bands and sequencing. Size fractionation was done, but not clear if it was used to select clones for sequencing
(30)	Domestic cattle	*Bos taurus*	Bovidae	Foregut Ruminant	*Caecomyces*, *Neocallimastix*, *Orpinomyces*, *Piromyces*, SK1, SK3, Black Rhino group	Cloning and Sanger sequencing
	Red deer	*Cervus elaphus*	Cervidae	Foregut Ruminant	*Neocallimastix*, *Orpinomyces*, *Caecomyces*, *Piromyces*, AL6, SK1, SK2, SK3, Black Rhino group	
	Domestic sheep	*Ovis aries*	Bovidae	Foregut Ruminant	*Neocallimastix*, *Orpinomyces*, *Piromyces*, SK1, SK3, SK4, Black Rhino group	

(31)	Domestic cattle	*Bos taurus*	Bovidae	Foregut Ruminant	*Caecomyces, Neocallimastix, Piromyces, Orpinomyces*, SK3, SK1, Al6, KF1	Pyrosequencing (Same samples from Kittelmann 2012)
	Red deer	*Cervus elaphus*	Cervidae	Foregut Ruminant	*Piromyces, Neocallimastix, Anaeromyces, Orpinomyces, Cyllamyces*, SK3, SK4, Black Rhino group	
	Domestic sheep	*Ovis aries*	Bovidae	Foregut Ruminant	*Piromyces, Neocallimastix, Caecomyces*, SK1, SK3, KF1, Al6	
(61)	Domestic cattle	*Bos indicus*	Bovidae	Foregut Ruminant	*Orpinomyces, Cyllamyces, Anaeromyces*	

Figure 1-1. Process flow diagram for the production of lignocellulosic biofuels.



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CHAPTER II

PHYLOGENETIC DIVERSITY AND COMMUNITY STRUCTURE OF ANAEROBIC GUT FUNGI (PHYLUM NEOCALLIMASTIGOMYCOTA) IN RUMINANT AND NON-RUMINANT HERBIVORES

Abstract

The phylogenetic diversity and community structure of members of the gut anaerobic fungi (Phylum *Neocallimastigomycota*) were investigated in thirty different herbivore species that belong to 10 different mammalian and reptilian families using the internal transcribed spacer region-1 (ITS-1) rRNA region as a phylogenetic marker. A total of 267, 287 sequences representing all known anaerobic fungal genera were obtained in this study. Sequences affiliated with the genus *Piromyces* were the most abundant, being encountered in 28 different samples, and representing 36% of the sequences obtained. On the other hand, sequences affiliated with the genera *Cyllamyces* and *Orpinomyces* were the least abundant, being encountered in 2, and 8 samples, and representing 0.7, and 1.1% of the total sequences obtained, respectively. Further, 38.3% of the sequences obtained did not cluster with previously identified genera and formed eight phylogenetically distinct novel anaerobic fungal lineages. Some of these novel lineages were widely distributed (e.g. NG1, NG3), while others were animal specific, being encountered in only one or two animals (e.g. NG4, NG6, NG7, and NG8). The impact of various physiological and environmental factors on the diversity and community structure of anaerobic fungi was examined. The results suggest that animal host phylogeny exerts the most significant role on shaping anaerobic fungal diversity and community composition. These results greatly expand the documented global phylogenetic diversity of members of this poorly studied group of fungi that plays a key role in initiating plant fiber degradation during fermentative digestion in ruminant and non-ruminant herbivores.

Introduction

Although flagellated zoospores of anaerobic fungi (AF) were observed as early as 1910, definitive proof that AF are an important constituent of the cow rumen came relatively late (15, 25-27). The accidental discovery and proof that such flagellates were actually spores of a new fungal lineage rather than ciliated protozoa came when vegetative fungal growth was consistently observed while attempting to isolate anaerobic ciliated protozoa from sheep rumen (27). Anaerobic fungi are now classified in a single order (*Neocallimastigales*) within the recently erected phylum *Neocallimastigomycota* (14). Originally described in sheep, members of the AF have since been shown to exist in the rumen, hindgut, and feces of ruminant and non-ruminant herbivorous mammals, as well as reptilian herbivores (3, 22, 36). Currently, only 6 genera and 20 species have been described (13), although multiple uncharacterized isolates have also been reported (15, 29).

The presence of anaerobic fungi in multiple (at least 50) ruminant and non-ruminant herbivorous mammals (20), as well as reptilian herbivores (22) has been well documented. However, the presence of AF in such habitats has mainly been assessed through isolation of a single or few AF strains (7, 15, 28, 37) or through microscopic observation of the characteristic zoospores of AF in rumen content (22). Collectively, these culture-based and microscopic studies have provided valuable insights on the prevalence and association of specific genera with certain animals. Recently, PCR primers that selectively amplify the internal transcribed spacer region 1 (ITS-1) within the ribosomal RNA (rRNA) of members of the *Neocallimastigomycota* has been described and validated (5, 10). These primers have mainly been used either to identify

AF isolates (5, 11, 40) or to identify AF community composition using various finger printing approaches, e.g. DGGE, T-RFLP, ARISA, and size based selection (sephadex) (10, 23). To our knowledge, an examination of the phylogenetic diversity of AF community using a high throughput sequencing approach (either by cloning and sequencing a large number of clones or by pyrosequencing) has not yet been attempted, and only 236 ITS-1 AF sequences from pure cultures and environmental isolates are available in GenBank (as of October 2009). As such, little is currently known regarding the extent of global phylogenetic diversity within the AF, the presence and prevalence of novel yet-uncultured anaerobic fungal genera, the complexity of AF community within a single host, and the influence of various ecological and environmental factors on AF diversity and community composition within various hosts.

As part of a broader effort on exploring the utility of AF in direct fermentation schemes and biofuel production from lignocellulolytic biomass, we sought to explore the diversity of AF in multiple herbivores using a culture independent sequencing approach. We present the results of a pyrosequencing-based survey of the Neocallimastigomycota from the fecal samples of a wide range of herbivores that belong to ten different animal families. We document the presence of an extremely diverse AF community that varies widely between different hosts, identify multiple novel AF fungal genera, and present evidence that host phylogeny is an important factor in determining the AF diversity and community composition in different samples.

Materials and Methods

Sampling. Fecal samples were obtained from domesticated animals from farms surrounding the cities of Stillwater and Cushing in Payne county, OK USA, from non-domesticated animals housed at the Oklahoma City Zoo (Oklahoma City, OK, USA), and from a reptile (Green iguana) housed within the Learning Resource Center, Department of Zoology at Oklahoma State University (Stillwater, OK, USA) in November and December 2008. Fresh fecal samples were collected from animals in 50ml sterile falcon tubes immediately after deposition, stored on ice on-site, promptly transferred and stored in a -200C freezer, usually within no more than 30 minutes of collection. Care was taken in order to avoid cross contamination between different samples. A detailed description of the animals, locations, feed, and gut type is presented in Table 2-3.

DNA extraction, PCR amplification, Pyrosequencing, and sequence quality control. DNA was extracted from 0.5 grams of fecal material from each sample using the FastDNA SPIN kit for soil (MP Biomedicals, Santa Ana, CA). The extraction was conducted according to the manufacturer's instructions, with the exception that the lysis step was conducted for thirty seconds thrice, to allow for disruption of fungal tissues (zoospores and vegetative growth) (8). PCR was conducted using forward primers GCCTCCCTCGCGCCATCAG-(barcode)-TCCTACCCTTTGTGAATTTG and reverse primer GCCTTGCCAGCCCGCTCAG-CTGCGTTCTTCATCGTTGCG. These primers are a modification of the previously described ITS-1 primers MN100 and MNGM2, (10, 23) with the universal forward pyrosequencing adaptor, and one of twelve barcode sequences (Multiplex Identifiers (MIDs), 454 Life Sciences, Roche Diagnostics Corp.) attached to the 5' end of the forward primer, and the universal reverse pyrosequencing

adaptor added to the 3' end of reverse primers. The utilization of 12 different barcode decamers allows for sequencing of up to twelve different samples in a single plate quadrant, and a total of 48 different samples in a single pyrosequencing run. PCR amplification was conducted in 50 μl reaction mixtures containing: 2 μl of extracted DNA, 2.5 mM MgSO4, 0.2 mM dNTPs, 1.5 U of GoTaq Flexi DNA polymerase (Promega), and 10 μM of each of the forward and reverse primers. PCR amplification was carried out as follows: initial denaturation for 5 min at 95^{o}C, followed by 40 cycles of denaturation at 95^{o}C for 30 s, annealing at 48^{o}C for 30 s, and elongation at 72^{o}C for 1.5 min. PCR products from different animal samples with different barcodes were pooled and purified using an Invitrogen PureLink PCR Purification kit (Invitrogen Corporation Carlsbad, CA). 54 FLX LR70 sequencing of pooled, purified, and barcoded PCR products was carried out at the University of South Carolina EnGenCore facility.

Sequences obtained were binned into different host animal groups using a perl script that identifies unique decamer barcodes (available upon request). Sequences with <130 bases and with quality scores of <25 were removed. Additionally, sequences with ambiguous bases or homopolymers strings of >10 (the maximum length of homopolymer strings in Sanger sequenced ITS-1 anaerobic fungal sequences present in GenBank database) were also removed from the datasets. The remaining sequences were examined against a database of all available ITS-1 rRNA sequences belonging to anaerobic fungi using BLAST search (1) conducted on a local server. Sequences with no similarity, or partial similarity (i.e. a segment of the amplicon has similarity to an ITS-1 sequence in the database, while the remainder of the amplicon does not have any similarity to ITS-1 sequence) were further removed from the dataset.

Phylogenetic analysis.

Operational taxonomic unit (OTU) assignments. ITS rRNA regions within Bacteria, Archaea, and Fungi are known to be more variable than SSU regions, and hence the established putative species (3%) and genus (6%) sequence divergence values that cater to 16S rRNA gene based diversity surveys of Bacteria and Archaea are unsuitable as universal thresholds for operational taxonomic assignments in ITS-1 diversity surveys (24). Therefore, to group sequences obtained into OTUs representing relevant AF species and genera, we used the publicly available ITS-1 AF sequences to empirically determine a putative species and genus cutoff for the ITS-1 sequenced fragment in AF. Sequences derived from AF pure cultures were used to confirm phylogenetic affiliations or to assign uncultured clones or uncharacterized isolates to specific species and genera. Using this approach, the percentage of sequence-level difference between morphologically identified AF genera and species was directly calculated, and therefore provided a better means of resolving the diversity detected in each sample. This approach is limited by several factors including the unknown variation between phenotypic and phylogenetic characteristics, and dependence upon the quality of the cultured isolates with deposited ITS1 sequences. In all, 83 sequences (with the full portion of the ITS-1 region theoretically amplified by this primers pair) were assignable to known genera. These sequences were aligned using ClustalX (39), and a distance matrix was created using PAUP (Version 4.01b10; Sinauer associates, Sunderland, MA, USA). Sequence divergence values between all possible pairs belonging to the same genus were averaged to compute a species level sequence divergence cutoff. Also, sequence divergence between all possible pairs belonging to different genera was averaged to compute a genus

level sequence divergence cutoff. Using this approach, a species cutoff of 4.80% and a genus cutoff of 16.95% were obtained. Species cutoff value of 0.05 was thus used for estimation of the number of putative species within each sample, and for computing the various diversity estimates and rankings described below. Genus level cutoff of 0.17 was used in conjunction with phylogenetic analysis (see below) to identify novel genus-level diversity within datasets.

In addition to OTU identification in individual datasets, we identified OTUs shared between different datasets by constructing a single alignment for all sequences obtained in this study, followed by distance matrix generation and OTU assignment using mothur (33). Shared OTU information gained through analysis of the entire dataset (267,287 sequences) were used for various comparative diversity approaches between the different datasets. The Petascale Data Analysis Facility (PDAF), a data-intensive computing cluster part of the Triton Resource located at the San Diego Supercomputing Center, University of California San Diego (http://tritonresource.sdsc.edu/pdaf.php), was used for generating all mothur outputs for the entire dataset.

Phylogenetic placement. Classification and identification of AF lineages have mainly been based on pattern of thallus / rhizoid morphology (monocentric or polycentric), and zoospore flagellation (uniflagellate or polyflagellate) (15, 23, 28). The use of molecular phylogenetic approaches in AF taxonomy has recently been examined (5), and ITS-1 based phylogeny have shown that while multiple AF genera are monophyletic (*Cyllamyces*, *Anaeromyces*, and *Orpinomyces*), members of the genus *Piromyces* appear to be polyphyletic (5), and members of the genus *Caecomyces* cluster as a distinct subgroup within the genus *Neocallimastix* (11). In spite of such discrepancies

between microscopic-based and phylogenetic-based classification of AF, no revisions (e.g. species reassignment, proposition of new genera) based on molecular taxonomic data have been proposed, and microscopic-based taxonomical schemes are still currently in use.

To determine the phylogenetic affiliation of $OTU_{0.05}$ obtained, representative sequences were searched against all ITS-1 sequences available in public databases. Sequences with high (>94%) sequence similarity to multiple isolates belonging to a single genus were assigned to that genus. On the other hand, OTUs with lower sequence similarity or similarity to multiple sequences from different genera were further probed by examining their phylogenetic position relative to other AF ITS-1 sequences in a PAUP-generated tree using various distance-based and character-based phylogenetic placements. OTUs with more than 17% sequence divergence that formed distinct phylogenetic lineages with high bootstrap support were judged to constitute a novel AF lineage at the genus level. The effect of filtering hypervariable regions on tree topologies was analyzed using GBlocks (35) under multiple stringency conditions. No significant differences in tree topology or bootstrap support were identified between the most relaxed conditions and the most stringent conditions that still maintained the primer regions, therefore relaxed parameters were used in Figure 2-5. The program JModelTest (31) was used to determine the optimum nucleotide substitution model to be used in constructing phylogenetic trees.

Diversity estimates, rankings, and evaluation of various factors affecting AF diversity and community structure within individual datasets. Basic diversity estimates, as well as rarefaction curves were computed on $OTU_{0.05}$ outputs using mothur

(33). Good's coverage was computed for each sample as described previously (12). Three different approaches were used to rank all datasets obtained according to diversity: number of genera per sample, rarefaction curve analysis, and diversity rankings approaches. Diversity ranking-based approaches have been widely utilized in macro ecology (19), and only recently introduced to microbial ecology (43). We used both an information-related diversity ordering method (Renyi generalized entropy), and an expected number of species-related diversity ordering method (Hulbert family of diversity indices) to reach a consensus ranking of fungal diversity for all the 33 animals studied (19, 43).

To identify the factors that most affect fungal diversity, we examined the correlation between AF diversity and various multiple measurable factors (Table 2-3) that might influence the AF communities diversity estimates calculated for the 33 datasets. Since these factors are nominal, χ^2 Contingency tables was the method of choice for correlation (9, 30). However, the dependent variable (ordinal in cases of diversity rankings, and rarefaction curve rankings, and quantitative in case of identified number of fungal genera) had to be converted to nominal variables first. Ordinal variables were grouped into: low-medium-high diversity categories such that; ranks 1-11 were classified as low diversity, ranks 12-22 were classified as medium diversity, and ranks 23-33 were classified as high diversity. As for the quantitative variables, we first ranked these from the least to the most (1-33) then the ordinal ranks were converted to nominal variables as discussed above. With the 2 variables (dependent and independent) being nominal, χ^2 Contingency correlation was carried out. To measure the degree of association between the 2 variables, the obtained χ^2 value was used to calculate Cramer's V statistics;

$V = \sqrt{\frac{\chi^2}{n \times \min(M-1, N-1)}}$, where χ^2 is the calculated χ^2 value, n is the number of species (33), M is the number of rows (or dependent variables), and N is the number of columns (or independent variables).

β-diversity estimates. Network-based analysis (34) and non-metric multidimensional scaling plots were used to visualize differences in community structure between various AF datasets (β-diversity). Network graphs were created with Cytoscape 2.6.3 using a spring-embedded algorithm allowing for visualization of species-level OTUs within and between animal hosts (16, 34). An example of the input file used is shown in Table 2-4. Cytoscape depicts datasets as nodes (animals and OTUs) connected by lines that denote the presence of a specific OTU within or between animal hosts. Animal hosts with more similar AF communities, and therefore, more OTUs that are shared between them, appear spatially closer on the graph. Animal hosts are depicted as circular nodes, whereas, OTU nodes are represented as squares. Generally, datasets with more shared OTUs are pulled towards each other and towards the center of the graph, whereas, datasets with fewer shared OTUs and/or a higher proportion of unique OTUs remain on the periphery. Non-metric multidimensional scaling plots were generated using Bray-Curtis similarity index matrices (4) between the 33 different animals studied. Bray Curtis similarity indices were calculated in mothur program (33), and the function metaMDS in the Vegan library of R statistical program (http://www.r-project.org/)

Nucleotide sequences accession numbers. Sequences generated were deposited under accession numbers GQ576478-GQ843764.

Results

A total of 350, 363 sequences were obtained from thirty-three different samples. 76.3% of the total sequences generated were kept after implementing quality control measures, yielding 267, 287 sequences that were used for further phylogenetic analyses. The range of sequence lengths of amplicons included in the analysis was 130 to 304 bp (average 236). A histogram of sequence read length is provided as Figure 2-3. The average number of sequences per animal sampled was 8,100. Coverage estimates (Table 2-1), as well as rarefaction curve analysis (Figure 2-4) indicates that the sequencing effort was successful in capturing the majority of AF taxa in all samples.

Genus-level taxonomic placement.

Monocentric genera. Sequences affiliated with the genus *Piromyces* were the most abundant in the entire dataset, being encountered in 28 different samples, and representing 36% of the total number of sequences obtained. Although it is currently assumed that *Piromyces* spp. represent the most abundant sequences in hindgut fermenters (25), the distribution of *Piromyces* varied greatly within hindgut fermenters depending on the host animal family. Within the family Equidae, *Piromyces* affiliated sequences were identified in low numbers and were even absent in some horse and Grevy's zebra replicates (Table 2-1). One the other hand, within the hindgut fermenter Black Rhinoceros (family Rhinocerotidae), *Piromyces* affiliated sequences constituted 100% of the AF community. *Piromyces*-affiliated sequences were also encountered in all but two of the ruminants sampled (Rothschild's Giraffe and Greater Kudu).

A total of 22,950 (8.6%) sequences affiliated with the genus *Neocallimastix* were encountered in this study, and *Neocallimastix*-affiliated sequences were identified in 18 different datasets belonging to seven different animal families (Table 2-1). This reinforces the notion that *Neocallimastix* spp. are prevalent in foregut fermenters. However, the results also demonstrate that members of the genus *Neocallimastix* are more widely distributed than previously implied (25), since they also appear to constitute a minor component of AF community identified in multiple hindgut fermenters (0.72% of the sequences from hindgut fermenters). Sequences affiliated with the genus *Caecomyces* were present in both foregut and hindgut fermenters, but were encountered in fewer datasets (fourteen) than *Neocallimastix* and *Piromyces*-affiliated sequences. *Caecomyces* was the most abundant genus only in two datasets (Llama and domestic cattle).

Polycentric genera. Although *Anaeromyces* spp. are generally assumed to be present mainly in foregut ruminants (cattle and water buffalo) as well as non-described species from sheep and goat (15, 25), they were widely distributed in our dataset and were encountered in 26 different samples. However, *Anaeromyces* affiliated sequences typically represented a minor component (average 11% in samples where they were detected) of a specific population, rarely exceeding 30% and never exceeding 50% within any dataset studied (Table 2-1).

Orpinomyces affiliated sequences were identified in only 8 animal species (llama, giraffe and six ruminants). In general, *Orpinomyces* affiliated sequences were present in very low abundance (average of only 3% community composition), and made only 1.1% of the total sequences in this study. *Orpinomyces* affiliated sequences were not identified in any of the hindgut samples analyzed.

Cyllamyces is the most recently described genus of anaerobic fungi and has so far been isolated only from domestic Cattle (28). This study suggests that members of the genus *Cyllamyces* are the least widely distributed in nature, being detected only in two datasets (American bison and Sable antelope). *Cyllamyces*-affiliated sequences comprised less than 10% of the sequences within each of these two datasets, and made up only 0.7% of all the sequences generated in this study. Interestingly, we did not detect any *Cyllamyces* affiliated sequences in cattle, although this is where it was originally identified (28), implying that other factors (e.g. feed type, location) could play an important role in establishing *Cyllamyces* populations in herbivores.

Novel AF groups. In addition to members of previously described genera, a significant fraction (38.3% of total sequences) could not be assigned to any of these six genera. Phylogenetic analysis suggested these groups belonged to eight different novel lineages that were designated novel groups NG1- NG8 (Figure 2-1). These lineages remained monophyletic regardless of the tree-building algorithm used (Parsimony, Maximum likelihood, distance) or the exclusion of hypervariable base pairs from the analysis (Figure 2-5). Some of these groups, e.g. NG 1 and NG3 were present in high abundance in multiple hindgut and foregut samples. NGI and NG3 were the second and third most abundant lineages (with 19.8 and 12.0% of the total number of sequences, respectively). These two groups, either individually or together, constituted the majority of sequences in all hindgut Equidae samples and were also co-identified in multiple foregut fermenters. NG2 and NG5 were present in multiple animals (eight and fourteen, respectively), but typically were present in low abundance in datasets where they were encountered.

Other groups had an extremely limited distribution and abundance. NG4 was a minor constituent within American bison and Sable antelope. NG6 comprised all of the sequences within the Greater Kudu data set, and constituted 15 and 34% of AF sequences in Okapi and Rothschild's giraffe, the two animals belonging to the family Giraffidae in our dataset. Finally NG7 and NG8 were each found in only a single animal, Somali wild ass and Red kangaroo, respectively.

Diversity estimates and factors influencing AF community diversity. Diversity estimates for various datasets were elucidated and compared. Diversity estimates utilized were the number of genera encountered in each datasets, rarefaction curve-based ranking, and diversity ordering-based approaches. The results (Table 2-5) were used as a starting point for diversity correlation using Chi square methods. While gut type, ruminant ability, and feed showed low correlation ($r = 0.20$-0.37) with all three diversity ranking schemes, a higher correlation ($r = 0.56$-0.63) was observed when correlating animal family to various diversity schemes (Table 2-2).

Community relatedness and factors influencing community composition. A network graph based on OTUs that were shared between various dataset was constructed using Cytoscape 2.6.3 (34), and the graphs were color- coded based on different factors potentially affecting AF community relatedness. The results (Figure 2-2A-C) indicate that, similar to diversity studies, feed type appears to be the least relevant factor in shaping community structure, as evident by the scattered color distribution in Figure 2-2A. Gut types (hindgut, foregut-nonruminant, foregut-pseudoruminant, foregut-ruminant, and Iguana), Figure 2-2B, provided slightly better explanation of community relatedness, but members of the same gut type belonging to different animal host families had clearly

different community structures. For example, although both are hindgut fermenters, members of the Equidae appear to have little shared OTUs with Black Rhinoceros. Similarly, the two foregut pseudoruminants belonging to different families had very different community structures, while both foregut nonruminants, both belonging to the family Macropodidae have more similar community structures.

Compared to feed type and gut type, animal host phylogeny appears to provide better explanation of community relatedness of AF. Members of the family Equidae clustered at the top of the graph (Figure 2-2C), with replicates of the same animal having highly shared AF community. Both zebras and two of the horse replicates (individuals 1 and 2) had a peripheral position at the top of the graph because such samples, mainly composed of NG1 and NG3, had a very low percentage of shared OTUs with non-Equidae samples (7.82%). On the other hand, Somali wild ass and Miniature donkey had more shared OTUs with non-Equidae samples (22.43%) and on average had fewer unique OTUs (10.28% vs. 30.06% in zebra and two horse replicates), and are therefore more centrally located than the other samples from the Equidae family.

Similar to the Equidae, members of the Cervidae clustered together (Figure 2-2C), as well as the two samples belonging to the family Macropodidae. However, although both families have a high percentage of shared OTUs (86.78%), many of these OTUs are not family specific (i.e. encountered only in these families), and have been encountered in other samples. Therefore members of both the Cervidae and the Macropodidae clustered towards the center of the graph.

Within the large number of samples belonging to the family Bovidae, multiple trends were observed. Some of these samples had a high proportion of shared OTUs with other members of the Bovidae and non-Bovidae (e.g. Nile lechwe, domestic goat, Grant's gazelle, Goral) and as such are centrally located. Others had a high proportion of OTUs that appear to be only shared within certain members of the Bovidae. As such, these animals are collectively located in the periphery of the graph in close proximity to each other (e.g. American bison, domestic cattle and sheep). Southern gerenuk had a strikingly similar community to bontebok, sharing 67.77% of its OTUs. Finally, Greater Kudu had a unique peripheral location in the graph, since its community was mainly composed of OTUs belonging to a lineage of limited distribution (NG6), and had a low proportion of shared OTUs with other animals (only with Okapi and Rothschild's giraffe).

Members of the family Giraffidae analyzed in this study (Rothschild's Giraffe and Okapi) did not have any shared OTUs and thus, are not located in proximity to one another. This represents a deviation from the observed importance of animal host phylogeny on community structure. Although Green iguana, represented the only non-mammalian, cold-blooded animal included in the study, and although it had a unique diet, the AF community in iguana had the lowest proportion of unique OTUs and was centrally located in the network graphs.

In addition to network analysis, a non–metric multidimensional scaling plot was generated to visualize the similarities in AF community structure between the various animal hosts. This non-metric multidimensional scaling plot, Figure 2-2D, shows a striking similarity to the network graph plots and further reinforces the importance of animal host phylogeny in shaping AF community.

Table 2-1. Composition of anaerobic fungal communities in sampled herbivores.

Common name	Rumen Type[1]	Family	No. of seqs	$OTU_{0.05}$	Monocentric			Polycentric			Novel								Coverage
					Neocallimastix	Piromyces	Caecomyces	Anaeromyces	Orpinomyces	Cyllamyces	NG1	NG2	NG3	NG4	NG5	NG6	NG7	NG8	
Horse individual 1	H	Equidae	12772	41		0.3		0.2			56.7		42.8						99.9
Horse individual 2	H	Equidae	8305	33		0.3		0.01			68.3		19.0		0.06				99.9
Horse individual 3	H	Equidae	3650	22	1.9		12.3	0.03			1.1	1.3	92.1						99.8
Miniature donkey	H	Equidae	3827	15	0.03	0.7	3.5	0.4					98.8						99.9
Somali wild ass	H	Equidae	1609	10	44.9	0.1					4.7		34.7				0.6		99.8
Grants zebra	H	Equidae	7591	26			15.0	0.01			99.9								99.9
Grevy's zebra individual 1	H	Equidae	14190	31		0.02					99.9								99.9
Grevy's zebra individual 2	H	Equidae	8789	27							99.9		0.2						99.9
Black rhinoceros	H	Rhinocerotidae	49215	49	0.002	99.9													99.9
White-fronted wallaby	F (N)	Macropodidae	13346	53		49.5		16.9			9.0	0.01	18.8		5.8				99.9
Red kangaroo	F (N)	Macropodidae	5782	28		30.8		12.4			17.8		25.3					13.7	99.9
Pygmy hippopotamus	F (P)	Hippopotamidae	7642	48		39.3		38.6			0.03		11.7		10.3				99.9
Llama	F (P)	Camelidae	11575	58	14.4	5.3		2.4	14.9							0.01			99.9
Rothschild's giraffe	F (R)	Giraffidae	6583	29			63.0	27.1	0.4		0.02		34.4		4.0	34.1			99.9
Okapi	F (R)	Giraffidae	2046	16	8.6	27.9		8.6			39.8					15.2			99.8
Indo-Chinese sika deer	F (R)	Cervidae	5680	31		52.7		18.3			0.1		15.5		13.4				99.9
Indian hog deer	F (R)	Cervidae	5727	27		12.2		20.3			13.9	0.07	39.9		7.3				99.9
American elk	F (R)	Cervidae	48	4	25.0	72.9	6.3						2.1						97.9
Pere David's deer	F (R)	Cervidae	4212	29		33.6		0.3			49.5	0.05	6.8						99.8
Western tufted deer	F (R)	Cervidae	3172	20	0.03	59.6	9.7	0.03			10.1	0.3	2.6		27.3				99.8

Pronghorn	F (R)	Antilocapridae	12950	24		96.7		1.2					0.01		2.0			99.9
Bontebok	F (R)	Bovidae	12431	53	47.3	41.5		0.01	4.4		0.02							99.9
Grant's gazelle	F (R)	Bovidae	4144	19		21.1	6.7				29.6		49.3					99.9
Southern gerenuk	F (R)	Bovidae	4215	23	88.6	10.3			0.02				0.02					99.7
American bison	F (R)	Bovidae	9180	55	37.5	8.4	1.0	31.2	1.3	9.2		0.02		3.0				99.9
Greater kudu	F (R)	Bovidae	4966	31			9.5	0.9								99.1		99.9
Goral	F (R)	Bovidae	3274	23	0.4	12.2		0.03			32.1	0.03	47.5					99.8
Sable antelope	F (R)	Bovidae	11395	46	22.1	9.0	7.6	24.8	0.7	8.4	0.2		5.2	0.4	11.1			99.9
Nile lechwe	F (R)	Bovidae	8768	37	7.4	10.2	18.2	17.3			8.5	0.1	33.7		5.3			99.9
Domestic cattle	F (R)	Bovidae	5448	60	5.3	25.2	17.5	4.3	1.9				2.2		0.09	0.02		99.7
Domestic sheep	F (R)	Bovidae	8554	37	44.0	18.3	61.0	4.0	3.6						8.0		0.01	99.8
Domestic goat	F (R)	Bovidae	5291	22	0.02	32.8	22.1	47.6			0.02				19.6			99.8
Green iguana	n/a	Iguanidae	910	10	0.11	49.8		6.2			1.1		34.8		8.0			99.8

[1] H: Hindgut fermenters; F (N): Non-ruminant foregut fermenters; F (P): Pseudo-ruminant foregut fermenters; F (R): Ruminant foregut fermenters

Figure 2-1. Distance dendrogram highlighting the phylogenetic affiliation of anaerobic gut fungi sequences encountered in this study. Sequences utilized in tree construction include reference sequences of anaerobic fungal isolates, representative OTUs affiliated with known anaerobic fungal genera encountered in this study, and representatives of novel anaerobic fungal lineages. The tree was constructed using neighbor-joining algorithm with the Hasegawa-Kishino-Yano (HKY) substitution model and a gamma shaped distribution of 0.6190. Bootstrap values are based on 1,000 replicates, and are shown for branches with more than 50% bootstrap support. The corresponding ITS-1 region of the ascomycetous yeast *Issatchenikia orientalis* was used as an outgroup. Genbank accession numbers of reference sequences are given in parentheses.

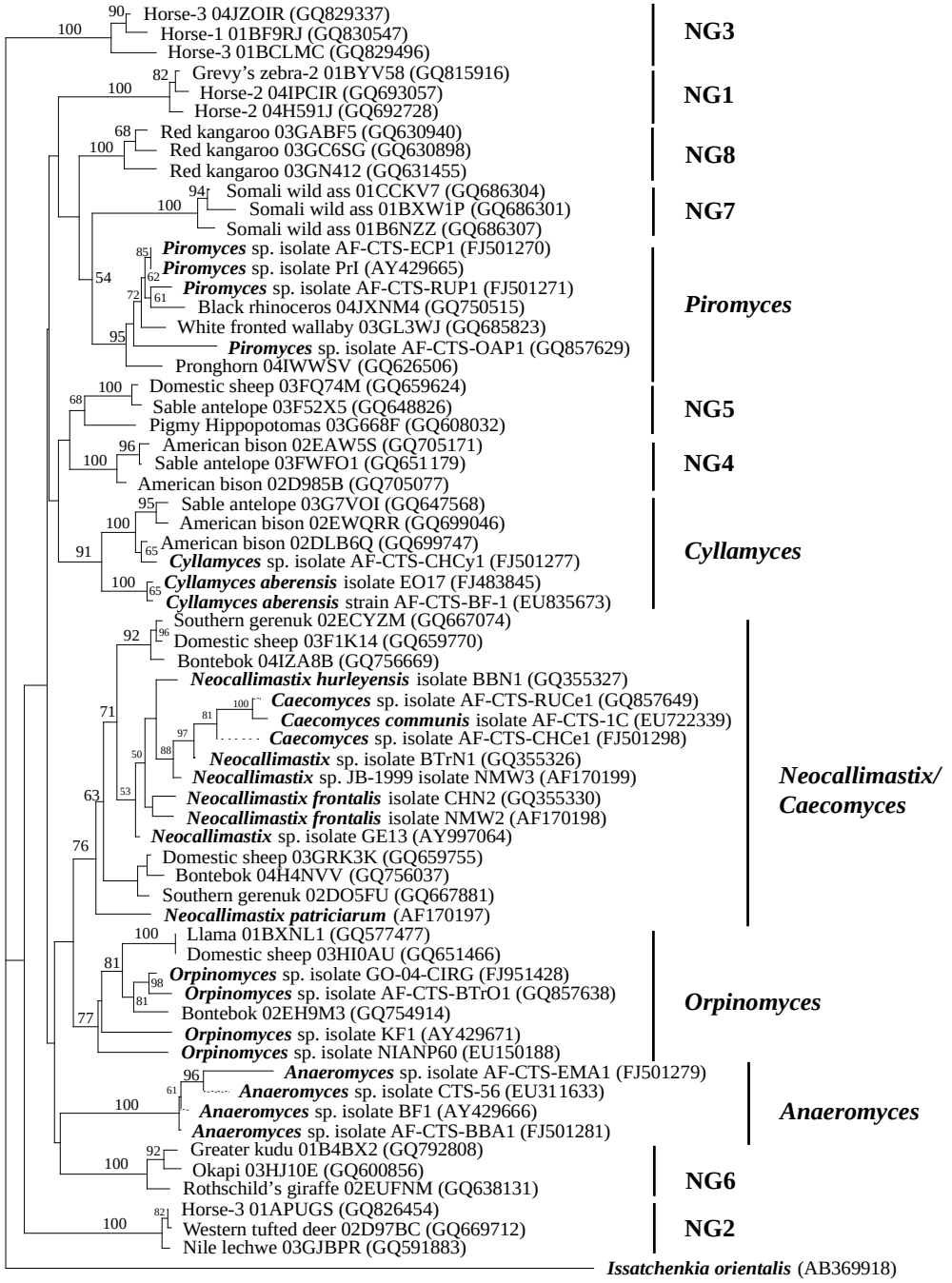
Horse-3 04JZOIR (GQ829337)
Horse-1 01BF9RJ (GQ830547)
Horse-3 01BCLMC (GQ829496)
NG3
Grevy's zebra-2 01BYV58 (GQ815916)
Horse-2 04IPCIR (GQ693057)
Horse-2 04H591J (GQ692728)
NG1
Red kangaroo 03GABF5 (GQ630940)
Red kangaroo 03GC6SG (GQ630898)
Red kangaroo 03GN412 (GQ631455)
NG8
Somali wild ass 01CCKV7 (GQ686304)
Somali wild ass 01BXW1P (GQ686301)
Somali wild ass 01B6NZZ (GQ686307)
NG7
Piromyces sp. isolate AF-CTS-ECP1 (FJ501270)
Piromyces sp. isolate PrI (AY429665)
Piromyces sp. isolate AF-CTS-RUP1 (FJ501271)
Black rhinoceros 04JXNM4 (GQ750515)
White fronted wallaby 03GL3WJ (GQ685823)
Piromyces sp. isolate AF-CTS-OAP1 (GQ857629)
Pronghorn 04IWWSV (GQ626506)
Piromyces
Domestic sheep 03FQ74M (GQ659624)
Sable antelope 03F52X5 (GQ648826)
Pigmy Hippopotomas 03G668F (GQ608032)
NG5
American bison 02EAW5S (GQ705171)
Sable antelope 03FWFO1 (GQ651179)
American bison 02D985B (GQ705077)
NG4
Sable antelope 03G7VOI (GQ647568)
American bison 02EWQRR (GQ699046)
American bison 02DLB6Q (GQ699747)
Cyllamyces sp. isolate AF-CTS-CHCy1 (FJ501277)
Cyllamyces aberensis isolate EO17 (FJ483845)
Cyllamyces aberensis strain AF-CTS-BF-1 (EU835673)
Cyllamyces
Southern gerenuk 02ECYZM (GQ667074)
Domestic sheep 03F1K14 (GQ659770)
Bontebok 04IZA8B (GQ756669)
Neocallimastix hurleyensis isolate BBN1 (GQ355327)
Caecomyces sp. isolate AF-CTS-RUCe1 (GQ857649)
Caecomyces communis isolate AF-CTS-1C (EU722339)
Caecomyces sp. isolate AF-CTS-CHCe1 (FJ501298)
Neocallimastix sp. isolate BTrN1 (GQ355326)
Neocallimastix sp. JB-1999 isolate NMW3 (AF170199)
Neocallimastix frontalis isolate CHN2 (GQ355330)
Neocallimastix frontalis isolate NMW2 (AF170198)
Neocallimastix sp. isolate GE13 (AY997064)
Domestic sheep 03GRK3K (GQ659755)
Bontebok 04H4NVV (GQ756037)
Southern gerenuk 02DO5FU (GQ667881)
Neocallimastix patriciarum (AF170197)
Neocallimastix/
Caecomyces
Llama 01BXNL1 (GQ577477)
Domestic sheep 03HI0AU (GQ651466)
Orpinomyces sp. isolate GO-04-CIRG (FJ951428)
Orpinomyces sp. isolate AF-CTS-BTrO1 (GQ857638)
Bontebok 02EH9M3 (GQ754914)
Orpinomyces sp. isolate KF1 (AY429671)
Orpinomyces sp. isolate NIANP60 (EU150188)
Orpinomyces
Anaeromyces sp. isolate AF-CTS-EMA1 (FJ501279)
Anaeromyces sp. isolate CTS-56 (EU311633)
Anaeromyces sp. isolate BF1 (AY429666)
Anaeromyces sp. isolate AF-CTS-BBA1 (FJ501281)
Anaeromyces
Greater kudu 01B4BX2 (GQ792808)
Okapi 03HJ10E (GQ600856)
Rothschild's giraffe 02EUFNM (GQ638131)
NG6
Horse-3 01APUGS (GQ826454)
Western tufted deer 02D97BC (GQ669712)
Nile lechwe 03GJBPR (GQ591883)
NG2
Issatchenkia orientalis (AB369918)
0.01 substitutions/site

Table 2-2. Correlation coefficients of diversity measures.

Factor	Correlation		
	Div Ordering	Rarefaction	No of genera
Family	0.63	0.60	0.56
Gut Type	0.37	0.30	0.21
Ruminance	0.28	0.37	0.32
Feed Type	0.20	0.29	0.31

Figure 2-2. A-C Network graph highlighting shared OTUs between different anaerobic fungal communities in different animal hosts. The same graph is coded with three different criteria to ease comparison. A. Feed type. B. Gut type and C. Animal host phylogeny (family). Circular nodes indicate animal datasets, whereas smaller square, grey nodes represent individual OTUs. Datasets with a higher proportion of Shared OTUs are pulled to the middle, while datasets with a high proportion of Unique OTUs remain on the periphery. The distance between any two datasets is a function of the number of shared OTUs between the two. D. Nonmetric multidimensional scaling plot of AF datasets obtained in this study.

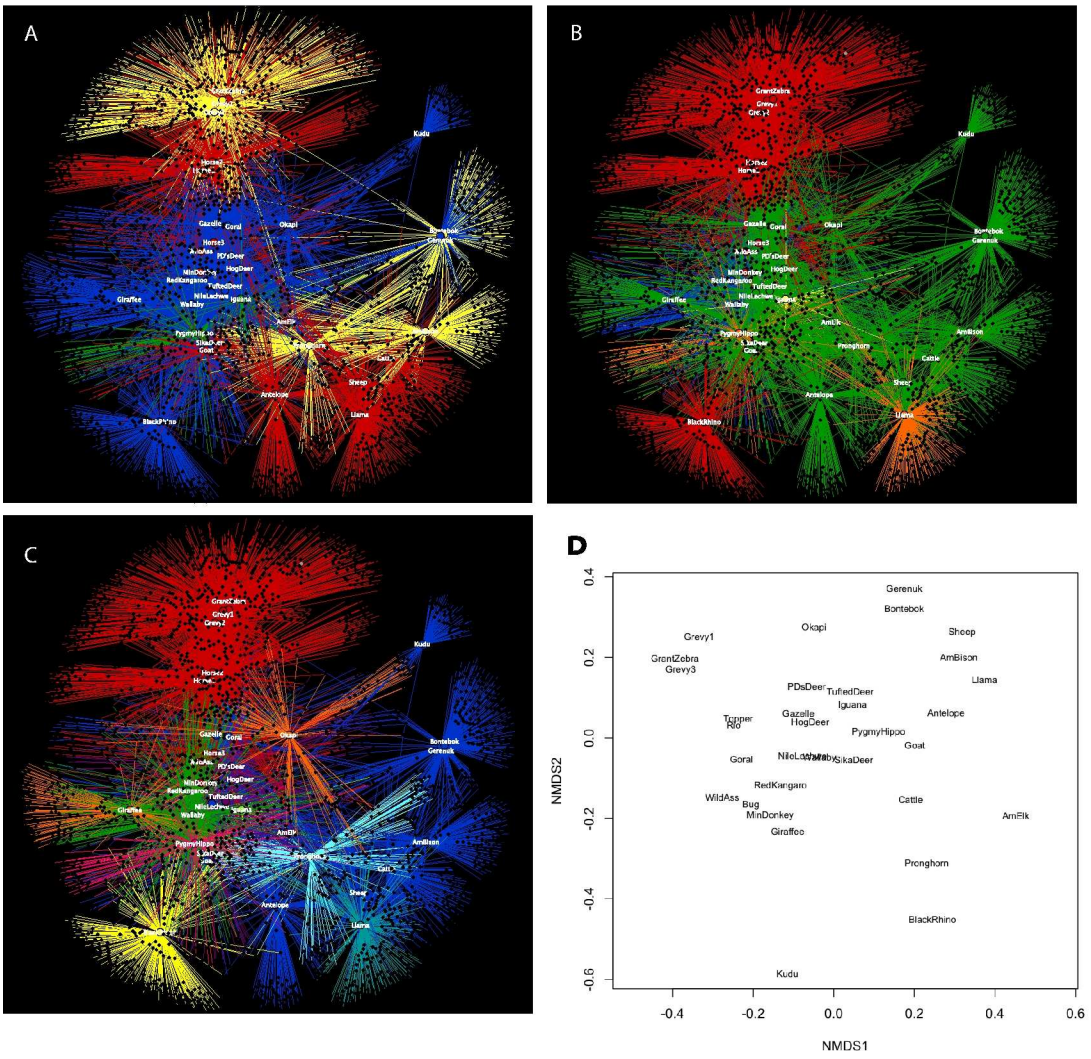

Table 2-3. Detailed information on the herbivores sampled in this study.

Common name	Scientific name	Class	Family	Gut Type	Ruminant	Feed type	Location
Horse 1	*Equus caballus*	Mammalia	Equidae	Hindgut	Non ruminant	Prairie	Stillwater
Horse 2	*Equus caballus*	Mammalia	Equidae	Hindgut	Non ruminant	Prairie	Stillwater
Horse 3	*Equus caballus*	Mammalia	Equidae	Hindgut	Non ruminant	Prairie	Cushing
Miniature donkey	*Equus asinus asinus*	Mammalia	Equidae	Hindgut	Non ruminant	Prairie	Oklahoma City Zoo
Somali wild ass	*Equus asinus somalicus*	Mammalia	Equidae	Hindgut	Non ruminant	Prairie	Oklahoma City Zoo
Grants zebra	*Equus burchelli boehmi*	Mammalia	Equidae	Hindgut	Non ruminant	Prairie	Oklahoma City Zoo
Grevy's zebra 1	*Equus grevyi*	Mammalia	Equidae	Hindgut	Non ruminant	Prairie and alfalfa	Oklahoma City Zoo
Grevy's zebra 2	*Equus grevyi*	Mammalia	Equidae	Hindgut	Non ruminant	Prairie and alfalfa	Oklahoma City Zoo
Black rhinoceros	*Diceros bicornis michaeli*	Mammalia	Rhinocerotidae	Hindgut	Non ruminant	Alfalfa	Oklahoma City Zoo
White-fronted wallaby	*Macropus parma*	Mammalia	Macropodidae	Foregut	Non ruminant	Alfalfa	Oklahoma City Zoo
Red kangaroo	*Macropus rufus*	Mammalia	Macropodidae	Foregut	Non ruminant	Alfalfa	Oklahoma City Zoo
Pygmy hippopotamus	*Hexaprotodon liberiensis liberiensis*	Mammalia	Hippopotamidae	Foregut	Pseudoruminant	Lettuce	Oklahoma City Zoo
Llama	*Lama glama*	Mammalia	Camelidae	Foregut	Pseudoruminant	Prairie	Cushing
Rothschild's giraffe	*Giraffa camelopardalis rothschildi*	Mammalia	Giraffidae	Foregut	Ruminant	Alfalfa	Oklahoma City Zoo
Okapi	*Okapia johnstoni*	Mammalia	Giraffidae	Foregut	Ruminant	Alfalfa	Oklahoma City Zoo
Indo-Chinese sika deer	*Cervus nippon pseudaxis*	Mammalia	Cervidae	Foregut	Ruminant	Alfalfa	Oklahoma City Zoo
Indian hog deer	*Axis porcinus*	Mammalia	Cervidae	Foregut	Ruminant	Alfalfa	Oklahoma City Zoo

American elk	*Cervus elaphus canadensis*	Mammalia	Cervidae	Foregut	Ruminant	Alfalfa	Oklahoma City Zoo
Pere David's Deer	*Elaphurus davidianus*	Mammalia	Cervidae	Foregut	Ruminant	Alfalfa	Oklahoma City Zoo
Western tufted deer	*Elaphodus cephalophus cephalophus*	Mammalia	Cervidae	Foregut	Ruminant	Alfalfa	Oklahoma City Zoo
Pronghorn	*Antilocapra americana americana*	Mammalia	Antilocapridae	Foregut	Ruminant	Prairie and alfalfa	Oklahoma City Zoo
Bontebok	*Damaliscus pygargus dorcas*	Mammalia	Bovidae	Foregut	Ruminant	Prairie and alfalfa	Oklahoma City Zoo
Grant's gazelle	*Gazella granti roosevelti*	Mammalia	Bovidae	Foregut	Ruminant	Alfalfa	Oklahoma City Zoo
Southern gerenuk	*Litocranius walleri walleri*	Mammalia	Bovidae	Foregut	Ruminant	Alfalfa	Oklahoma City Zoo
American bison	*Bison bison*	Mammalia	Bovidae	Foregut	Ruminant	Prairie and alfalfa	Oklahoma City Zoo
Greater kudu	*Tragelaphus strepsiceros*	Mammalia	Bovidae	Foregut	Ruminant	Alfalfa	Oklahoma City Zoo
Goral	*Naemorhedus caudatus arnouxianus*	Mammalia	Bovidae	Foregut	Ruminant	Alfalfa	Oklahoma City Zoo
Sable antelope	*Hippotragus niger*	Mammalia	Bovidae	Foregut	Ruminant	Prairie	Oklahoma City Zoo
Nile lechwe	*Kobus megaceros*	Mammalia	Bovidae	Foregut	Ruminant	Alfalfa	Oklahoma City Zoo
Domestic cattle	*Bos tarus*	Mammalia	Bovidae	Foregut	Ruminant	Prairie	Stillwater
Domestic sheep	*Ovis aries*	Mammalia	Bovidae	Foregut	Ruminant	Prairie	Cushing
Domestic goat	*Capra hircus*	Mammalia	Bovidae	Foregut	Ruminant	Prairie	Cushing
Green iguana	*Iguana iguana*	Reptilia	Iguanidae	n/a	n/a	Lettuce	Stillwater

Table 2-4. Cytoscape input file example.

Animal node	Edge	OTU node
Rio	4529	1
Rio	1516	15
Rio	1368	49
Rio	986	48
Rio	511	50
Rio	504	99
Rio	195	54
Rio	185	47
Rio	139	127
Rio	115	133
Rio	81	57
Rio	81	102
Rio	77	101
Rio	77	103
Rio	63	552

The cytoscape input consisted of a text file using the ".shared" file generated in mothur for the entire dataset (all 267,287 sequences). The file contained 3 columns, with the first column specifying the animal node, the third column specifying a particular OTU and the second column indicating that an edge should be created between the two nodes. Actual frequency of the sequences within an OTU for a specific animal were kept in the input file to allow for scaling of the edges (data not shown). However, with respect to Figure 2-2, these values could be omitted (all designated as "1").

Figure 2-3. Read length distribution of sequences generated in this study. Read lengths ranged from 130 to 304 bp, with an average of 236 bp.

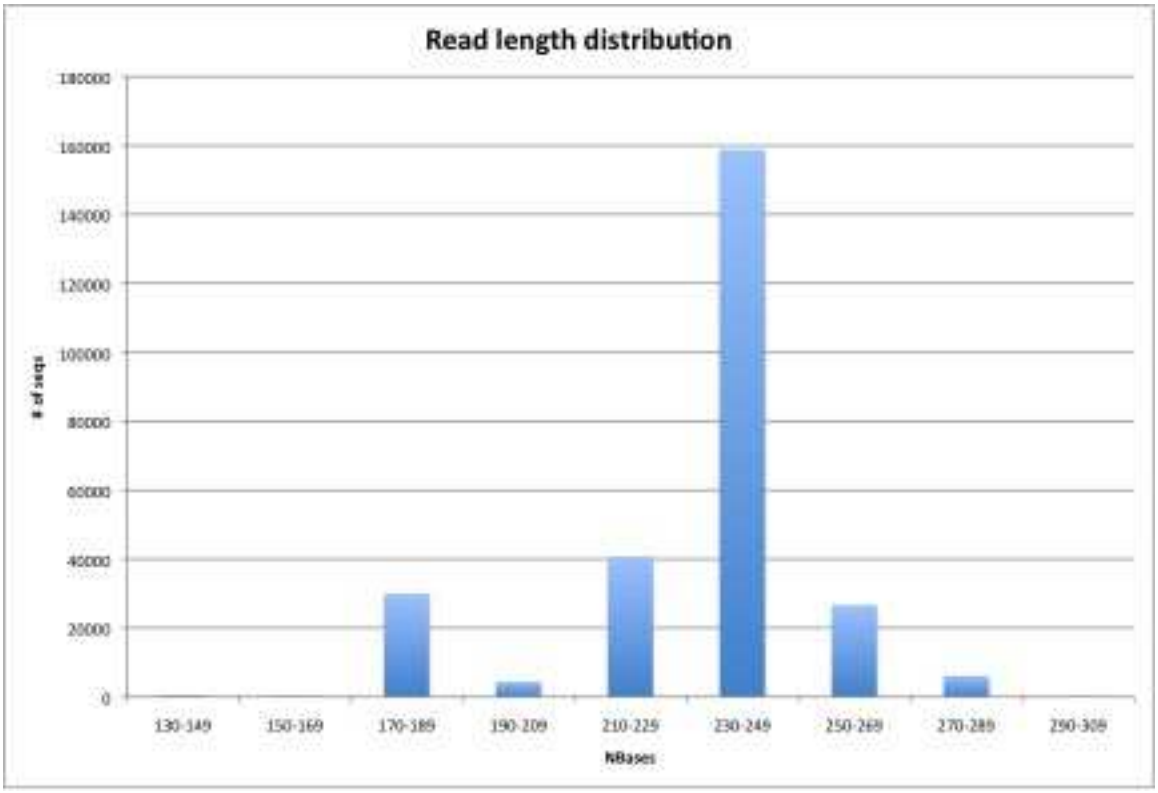

Figure 2-4. Rarefaction curve analysis of AF data generated in this study.

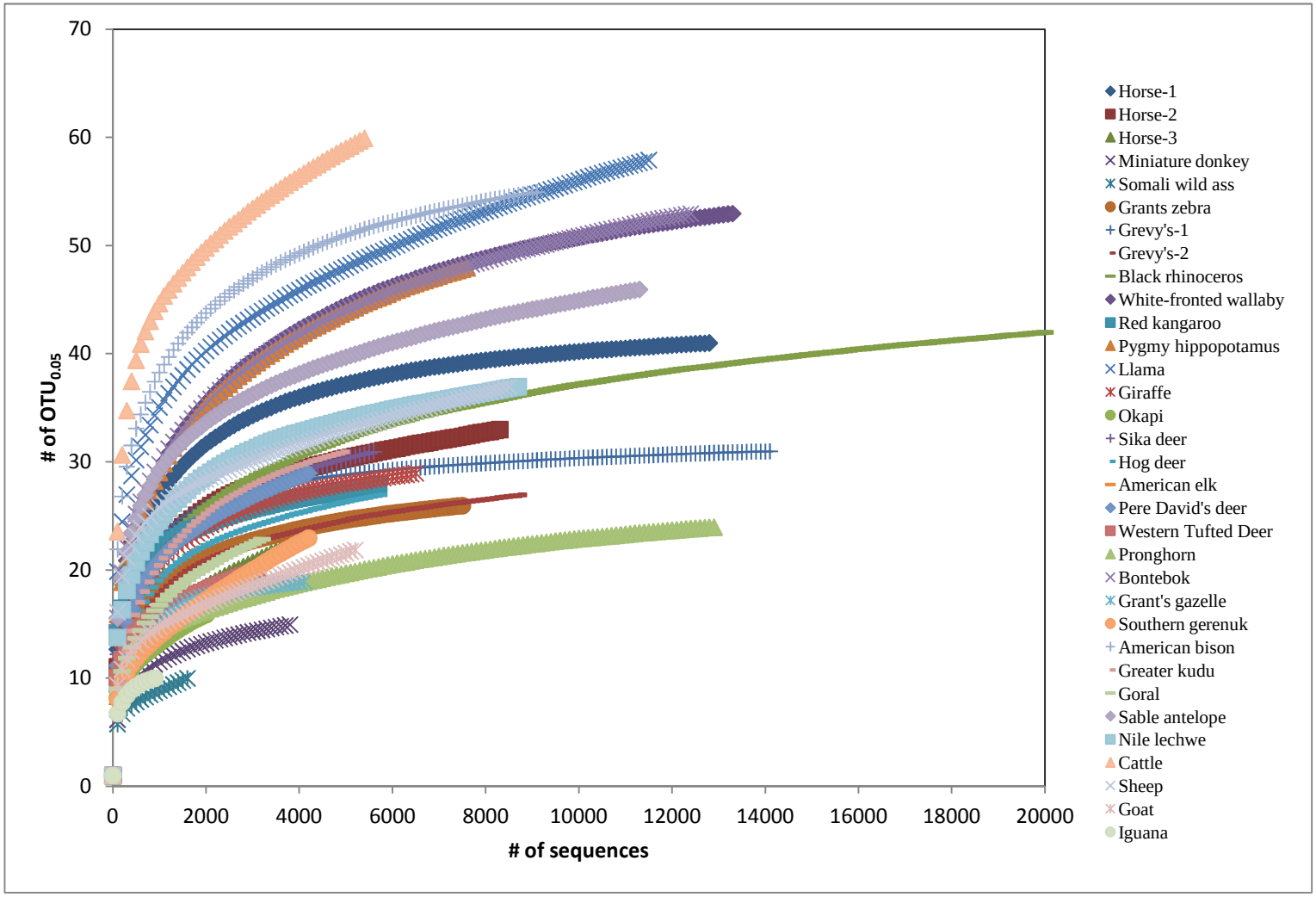

Figure 2-5. Distance dendrogram highlighting the phylogenetic affiliation of anaerobic gut fungi sequences remaining monophyletic regardless of the tree-building algorithm used (parsimony, maximum likelihood, distance) or the exclusion of hypervariable base pairs from the analysis using different stringency options in GBlocks.

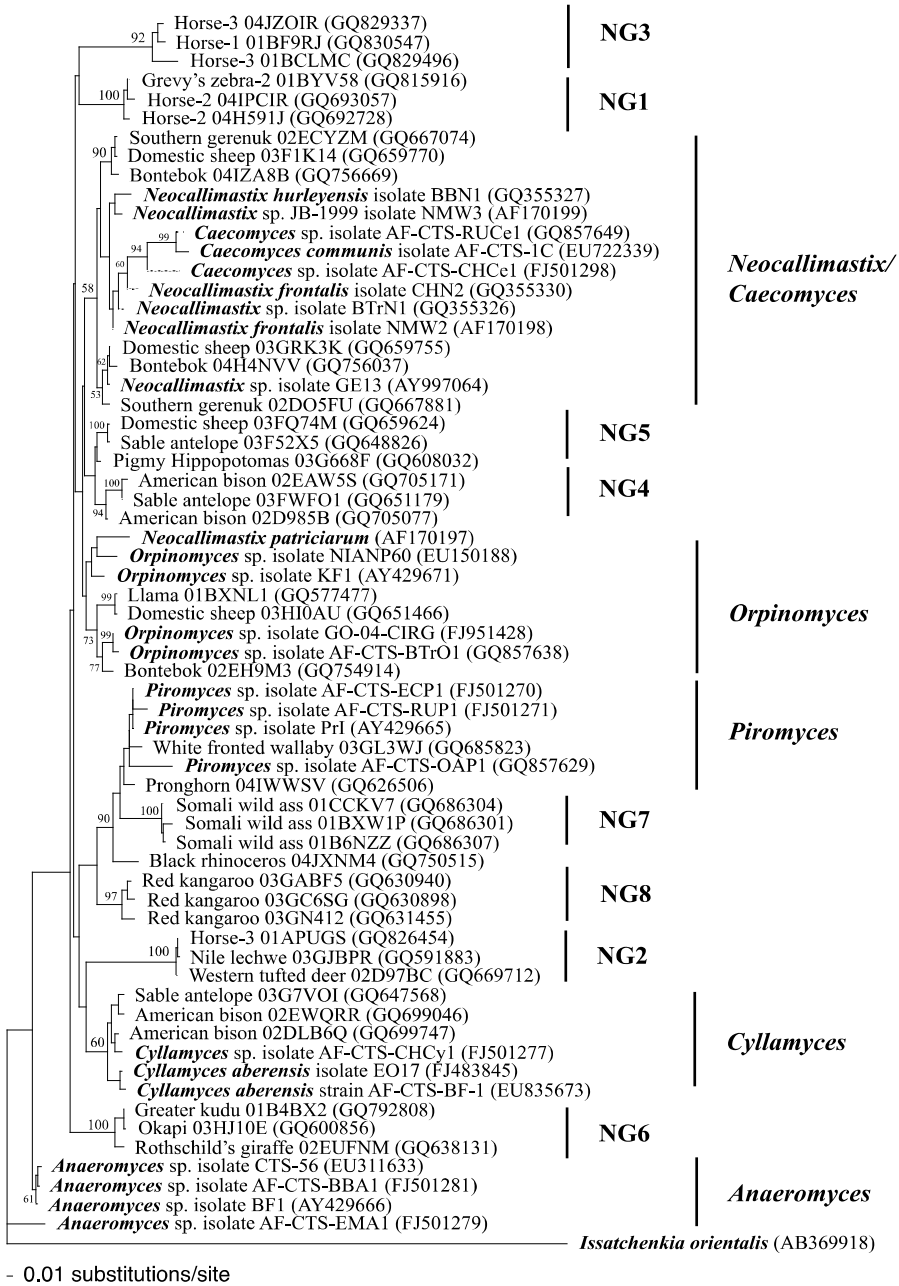

Table 2-5. Diversity ranks of anaerobic fungi datasets using rarefaction curve, diversity ordering, and number of genera. Rankings are from 1 (least diverse) to 33 (most diverse).

Animal	Rarefaction curve rank[a]	Diversity ordering rank	No of genera (rank)[b]
Pygmy hippopotamus	27	33	5 (13.5)
American bison	31	32	8 (31)
Domestic cattle	32	31	8 (31)
White-fronted wallaby	28.5	30	6 (21)
Red kangaroo	15.5	29	5 (13.5)
Indo-Chinese sika deer	19	28	5 (13.5)
Sable antelope	26	27	10 (33)
Pere David's deer	17.5	26	6 (21)
Domestic goat	6.5	25	5 (13.5)
Bontebok	28.5	24	6 (21)
Nile lechwe	23.5	23	8 (31)
Domestic sheep	23.5	22	7 (27.5)
Llama	30	21	6 (21)
Indian hog deer	14	20	7 (27.5)
Horse-Indiv2	20.5	19	6 (21)
Rothschild's giraffe	15.5	18	6 (21)
Horse-Indiv1	25	17	4 (9)
Western tufted deer	9.5	16	7 (27.5)
Grevy's zebra-indiv 1	17.5	15	2 (3)
Okapi	4	14	5 (13.5)
Grants zebra	12.5	13	2 (3)
Grevy's zebra-indiv 2	12.5	12	2 (3)
Goral	11	11	7 (27.5)
Southern gerenuk	6.5	10	5 (13.5)
Greater kudu	20.5	9	2 (3)
Grant's gazelle	6.5	8	3 (6.5)
Green iguana	2	7	6 (21)
Somali wild ass	1	6	6 (21)
Black rhinoceros	22	5	2 (3)
Pronghorn	6.5	4	4 (9)
Horse-Indiv3	9.5	3	6 (21)
American elk	NA	2	3 (6.5)
Miniature donkey	3	1	4 (9)

NA: not applicable due to small dataset.

a: For rarefaction curve ranking, the datasets were ranked from the least diverse (rarefaction curve below) to the most diverse (rarefaction curve above). When two or more rarefaction curves intersected, the corresponding datasets were given an intermediate rank (sum of ranks divided by the number of datasets).

b: For ranking using the number of observed genera, the datasets were ranked form the least diverse (dataset with the least number of observed genera) to the most diverse (dataset with the most number of observed genera). When two or more datasets had the same number of observed genera, they were given an intermediate rank (sum of ranks divided by the number of datasets).

Discussion

In this study, we present a detailed survey of phylogenetic diversity, community structure, and comparative diversity of members of the anaerobic gut fungi using rRNA ITS-1 as a phylogenetic marker. To our knowledge, this represents the first wide scale culture independent sequences analysis of members of the phylum *Neocallimastigomycota*. In addition the work represents the first culture-independent survey of AF community in a reptilian host (Green iguana), and in multiple mammalian species (e.g. American elk, Pronghorn, Bontebok, Southern gerenuk, Goral, and Nile lechwe).

The high level of AF phylogenetic diversity observed in animal hosts surveyed is evident by the fact the average number of species per sample (thirty one) is higher than the total number of AF species currently described (twenty). We acknowledge that our estimates are solely based on sequence divergence values of a single amplicon, rather than a thorough microscopic, biochemical, and sequence analysis. However, this chosen cutoff value (5%) was based on averaging ITS-1 sequence divergence values of known AF isolates. Therefore, although not definitive, this cutoff reflects a reasonable estimate of number of AF species per sample. Another indicator of the highly diverse nature of AF is the identification of multiple novel AF lineages that represented 38.3% of the total sequences obtained. The presence of novel lineages has previously been speculated (25), and unclassifiable patterns in finger printing approaches suggestive of novel genera have subsequently been observed in cow rumen (23). Multiple plausible reasons could account for the inability to previously identify and isolate these novel AF lineages. It is entirely possible that AF affiliated with many of these lineages have thallus and zoospore

structures similar to those of well described AF genera and thus isolates belonging to such lineages would have been classified as members of an already existing AF genus upon isolation. Alternatively, thallus and zoospore morphologies of these novel lineages could possess unique microscopic characteristics that have hence escaped microscopic detection. Finally, regardless of zoospore / thallus morphology, members of such lineages might require unique, yet-unidentified growth media factors or selective substrates for enrichment and growth under laboratory conditions, and are hence unculturable using standard methodologies used for isolating anaerobic fungi (38). It is interesting to note that the choice of substrate indeed appear to have an important influence on the morphology of isolates obtained (13, 15).

Although this study sheds light on the diversity and distribution of anaerobic fungi, we caution against considering the described patterns of AF diversity a definitive description of global AF communities in herbivore hosts. Rather, this study represents a community snapshot of multiple animals from few locations within a single state in a single country. The observed patterns of diversity and community structure for this study may not be maintained within other habitats. Low correlations of diversity to singular measurable factors in this study indicate that either unidentified or inseparable factors may also play a role in shaping the communities within the animals sampled. We reason that only a well-controlled experiment tracking AF community structure in replicates of a single animal species at different age groups, feed regiments, and geographical locations would provide an accurate description of the community dynamics of AF fungi, and factors influencing the community structure within various animal species.

Anaerobic fungi are highly fibrolytic microorganisms, producing a wide array of cell-bound and cell-free cellulolytic, hemicellulolytic, glycolytic, and proteolytic enzymes (21, 32, 41, 42). The anaerobic nature (which deters many mycologists), and eukaryotic affiliation (which deterred anaerobic microbiologists) have limited the number of active research groups investigating these microorganisms to a dedicated but small group of scientists. In 1989, Bauchop (2) concluded a review on the biology of AF by asserting that "*The anaerobic fungi also attract attention as a new group of cellulase- and hemicellulase-producing microorganisms. The challenge of adapting this group of microorganisms in biotechnology will undoubtedly be accepted by scientists in the near future*". With few exceptions (6, 17, 18, 20), we believe that this challenge has not sufficiently been met.

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CHAPTER III

THE ANAEROBIC FUNGUS *ORPINOMYCES* SP. STRAIN C1A IS AN EFFECTIVE, VERSATILE PLANT BIOMASS DEGRADER

Abstract

Anaerobic fungi (AF) have evolved within the intestinal tracts of herbivorous animals to rapidly attack and deconstruct ingested plant materials. This environmental niche provides AF with exposure to mixed, complex lignocellulosic substrates with relatively short retention times for utilization. These constraints have directed the evolution of anaerobic gut fungi, enabling them to jointly possess mechanisms for biomass deconstruction from anaerobic prokaryotes and from aerobic fungi. These characteristics could conceivably be useful for the production of biofuels from lignocellulosic biomass. We report a significant step towards this goal through the isolation and characterization of *Orpinomyces* sp. strain C1A, a polycentric rhizoidal strain that has been maintained for greater than 200 subcultures without loss of culture viability or degradative capacity. Experimental analyses indicated that strain C1A is a remarkable biomass degrader, capable of simultaneous saccharification and fermentation of the cellulosic and hemicellulosic fractions in multiple untreated grasses and crop residues examined, with the process significantly enhanced by mild pretreatments. This capability, acquired during its separate evolutionary trajectory in the rumen, along with its resilience and invasiveness when compared to prokaryotic anaerobes, render anaerobic fungi promising agents for consolidated bioprocessing schemes in biofuels production.

Keywords: Anaerobic fungi, biofuels, consolidated bioprocessing

Introduction

Members of the anaerobic gut fungi were originally discovered in sheep (16), but have subsequently been observed in the rumen, hindgut, and feces of ruminant and non-ruminant herbivorous mammals and reptilian herbivores. The observation of flagellated zoospores of anaerobic fungi was reported as early as 1910 (15). However, the accidental discovery and subsequent proof that these flagellated zoospores were actually spores of a new fungal lineage rather than ciliated protozoa came relatively late (16).

Anaerobic gut fungi belong to the phylum *Neocallimastigomycota*, an early divergent basal fungal lineage, and are adapted to a specific but restricted environmental niche, the intestinal tracts of herbivorous animals. Within the rumen, they are thought to elicit initial attack on ingested plant cell walls through attraction of motile AF zoospores to lignin-rich regions with prolific hyphal penetration and physical disruption. Anaerobic fungi are capable of simultaneous saccharification of both cellulose and hemicellulose structural fractions of intact plant biomass to volatile fatty acids and alcohol, including ethanol. This capability stems from production of multiple lignocellulosic enzymes including various cellulases, hemicellulases, proteases and esterases. Hence, this combination of characteristics, unique to anaerobic fungi, indicates their potential use in consolidated production of biofuels from lignocellulose.

The direct application of AF for consolidated biological processing (CBP) of lignocellulosic biomass will require an isolate that has diverse lignocellulosic capabilities, rapid growth, and is capable of maintaining culture viability throughout extensive subculturing. Further, an isolate should be selected with greater reliance on prolific indeterminate growth of fungal rhizoids for biomass deconstruction (polycentric

rhizoidal spp.) over determinate growth and zoospore production (monocentric rhizoidal spp. and bulbous spp.). The carbon source used in isolating anaerobic fungi has been shown to influence the morphotype recovered, with a higher proportion of polycentric genera obtained on complex, fiber-rich substrates (7). To this end, we applied anaerobic culturing procedures towards isolation from the highly diverse anaerobic fungal community detected previously in domestic cattle (10). Here we report on the isolation and characterization of an AF isolate, *Orpinomyces* sp. strain C1A, on multiple native and pretreated lignocellulosic plant substrates. The implications for biofuel production are discussed.

Materials and Methods

Isolation and maintenance. *Orpinomyces* sp. strain C1A was isolated from the feces of an Angus steer on a switchgrass-cellobiose medium reduced by cysteine-sulfide and dispensed under a stream of 100% CO_2 using previously described protocols (20). Fresh samples were collected, transferred to the lab, and added to anaerobic, rumen-fluid containing basal media within 15 minutes of collection. The medium was amended with penicillin, streptomycin, and chloramphenicol from an anaerobic stock solution with final concentrations of 50µg/ml, 20µg/ml, and 50µg/ ml ofeach antibiotic, respectively. Five grams of fecal material was aseptically transferred to 45 ml of sterile anaerobic switchgrass-cellobiose media and incubated at 39^{o}C for 30 min with gentle shaking at 80 rpm. From this solution, 1 ml was removed and added to 9 ml of pre-warmed (39^{o}C) media and serially diluted down to 10^{-6}. The original fecal suspension and the dilution tubes were used to make roll tubes immediately and after incubating dilution tubes for 3 additional days. To roll tubes containing 4.5 ml of switchgrass-cellobiose agar medium (1.5%), 0.5 ml of fecal suspension was added, incubated at 39^{o}C and examined daily for the presence of fungal growth. Colonies were transferred into fresh switchgrass-cellobiose liquid media anaerobically in an anaerobic glove chamber and examined daily for growth. Tubes showing growth were subjected to two additional rounds of roll tube isolation and transfer into liquid media as described above. Morphology of isolates was determined visually using a phase contrast microscope.

For maintenance, strain C1A was grown in an anaerobic, rumen fluid-free basal medium that was reduced by cysteine-sulfide and dispensed under a stream of 100% CO_2 as previously described (12). Cellobiose (3.75 g/L) was used as the substrate. For nucleic

acid extraction, cultures were incubated at 39°C for approximately 3-4 days and the fungal cells were harvested during late log phase by centrifugation at 10,000 rpm for 30 minutes.

DNA extraction and sequencing. High molecular weight genomic DNA was extracted using a modified CTAB method for isolation of nucleic acids in anaerobic fungi, with some adjustments (3). In brief, ground fungal mycelium was suspended in 10ml of freshly mixed extraction buffer followed by 1ml of 10% sodium lauroylsarcosine. The solution was incubated overnight at 50^{o}C with gentle shaking. Following lysis, 1.02ml of 5M NaCl and 0.81ml of 10% (w/v) CTAB in 0.7M NaCl was added for every 6 ml of extract and incubated at 65^{o}C for 30 min with occasional inverting. After the samples cooled to room temperature, an equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed by gentle inversion until an even, milky white suspension appeared. The mixture was centrifuged for 10 min at 9,700g (4^{o}C). Isopropanol (60% volume) was added and the pellet was spun down and washed with 5mL of 70% ethanol and resuspended in 500ul TE solution. The sample was transferred to a microcentrifuge tube and incubated with 0.1mg RNase A at 37^{o}C for 1 hour. The supernatant containing the DNA was precipitated with 50ul of 3M Na-acetate and 550ul ethanol. The resulting DNA pellet was washed twice with 70% ethanol, drained, and resuspended in 200ul TE. The DNA was used to identify phylogenetic affiliation using ITS-primers MN100 and MNGM2 as previously described (5, 14). The obtained sequences were aligned using ClustalX (21), and a distance matrix was created using PAUP (Version 4.01b10; Sinauer associates, Sunderland, MA, USA).

Lignocellulolytic capabilities of strain C1A.

Plant materials and pretreatment. Samples of mature Kanlow switchgrass (*Panicum virgatum* var. Kanlow), mature *Sorghum bicolor*, and mature energy cane (*Saccharum officianarum* var Ho02) were obtained from Oklahoma State University experimental plots in Stillwater, OK. Dried alfalfa was obtained from a local farm and ranch supplier. Samples of Bermuda grass (*Cynodon dactylon*) were obtained from residential lawn clippings in Guthrie, OK. Samples of corn stover from *Zea mays* were obtained from the Industrial Agricultural Products Center at the University of Nebraska in Lincoln. Untreated wood samples, including cedar (*Juniperus* sp.), oak (*Quercus* sp.), and pine (*Pinus* sp.) were obtained from a local lumberyard in Stillwater, OK. Cottonwood (*Populus deltoides)* and willow (*Salix babylonica)* wood samples were harvested from live trees growing in the Stillwater area. All samples were dried at 45°C overnight, milled, and sieved to a final particle size of 2 mm as previously described (19).

Sodium hydroxide (NaOH) treatments were conducted by heating 4g of dried plant material in 40 ml of a 1% NaOH solution inside a sealed serum bottle at 50^{o}C for 12 hours (24). Acid treatment was conducted by heating 4g of dried plant material in 40 ml of 0.5% H_2SO_4 inside a sealed serum bottle for 1 hour (22, 23). Hydrothermolysis-treated switchgrass was prepared by mixing 60g of switchgrass with distilled water to achieve a 10% dry matter mixture (19). This mixture was placed inside 1L benchtop pressure reactor (Parr Series 4520, Parr Instrument Company, Moline, IL, USA) that was heated to 200°C and agitated at 500 rpm (19). The switchgrass/water mixture was held at 200°C for 10 minutes and then cooled in an ice bath (19). All of the treated switchgrass samples were recovered from pretreatment incubations by filtration. The sodium hydroxide and

acid treated switchgrass were washed with deionized water as previously described (22-24). All of the pretreated switchgrass samples were dried at 45°C for approximately 48 hours before they were used in the experiments described below.

Growth of strain C1A on plant material. Experiments to evaluate the growth of strain C1A on different treated and pretreated plant materials were conducted under strict anaerobic conditions in 160ml serum bottles. All experiments were conducted in triplicate, and unless otherwise specified, 0.5g of plant material was used as the substrate. Experiments were conducted in a previously described rumen fluid-free basal medium (12). The medium was prepared under strict anaerobic conditions using 100% CO_2 and the techniques of Bryant (4), as modified by Balch and Wolfe (2). Once the basal medium was prepared it was autoclaved for 20 minutes at 121°C and 15 psi of pressure and then cooled. Each serum bottle was then amended with the appropriate type of plant biomass inside an anaerobic chamber (Coy Laboratory Products Grass Lake, MI). After the serum bottles were amended with plant materials they were removed from the glove bag and the headspace was re-pressurized with 15 psi of 100% CO_2 (2). Five milliliters of an actively growing culture of strain C1A (approximately 2.6 mg of fungal biomass) was used as an inoculum and added to 45ml media in 160 ml serum bottles. In all experiments, serum bottles were incubated at 39^oC in a non-shaking incubator. Substrate-unamended controls were included in all experiments to account for any product carryover from the inoculum. Triplicate bottles were sacrificed at different time intervals to quantify substrate loss and product formation.

Analytical methods. Fatty acids and ethanol in supernatant fractions were quantified using an HPLC with a refractive index detector (1100 Series, Agilent, Santa Clara, CA,

USA) and an Aminex HPX-87H column (Biorad, Sunnyvale, CA, USA), which was heated to 60^{o}C. The mobile phase was 0.01 N H_2SO_4, with a flow rate of 0.6 ml per minute. Sugars in supernatant fractions were also quantified using an HPLC with a refractive index detector (1100 Series, Agilent, Santa Clara, CA, USA). The HPLC was equipped with an Aminex HPX-87P column (Biorad, Sunnyvale, CA, USA), which was heated to 85°C. Distilled water was used as the mobile phase at a flow rate of 0.6 ml per minute.

The amount of plant material consumed in serum bottles was calculated by subtracting the time final from the time 0 dry weights of each plant material. Since the time final pellets contained a mixture of plant and fungal biomass, the amount of fungal biomass at time final was indirectly quantified using formate concentrations as previously described (11). The amounts of cellulose, xylan, hemicellulose, and lignin in the different plant substrates were determined using the standard NREL procedures (17). The procedure included the addition of 3mL of 72% sulfuric acid to each sample and incubation at 30±3°C for 1 hour, stirring every 5-10 min. The samples were then diluted with 84mL of deionized water, capped, and autoclaved for 1 hour to 121°C. The cooled solution was filtered, and this filtrate was used to determine carbohydrate content and soluble lignin. The remaining solids were washed and dried to constant weight at 105°C to determine acid-insoluble residue (AIR) and then ashed at 575°C for 24 hours (17). Analyses of resulting carbohydrates within the filtrate were done by HPLC with refractive index detection (RID) (Agilent 1100 Series, Santa Clara, CA) on an Aminex HPX-87P column at 85°C with a mobile phase of deionized water pumped at 0.6mL/min for 30 min (17). Twenty microliters of each sample were analyzed for cellobiose,

glucose, xylose, galactose, arabinose and mannose. Contributions of structural constituents to the total biomass composition were determined using the NREL summative mass closure procedure (18). The acid-soluble lignin (ASL) content was determined using a UV spectrophotometer set at a wavelength of 205 nm, as has been previously used to determine ASL in switchgrass (6). As recommended in the NREL procedure, ASL in corn stover was measured at 320 nm, whereas a 240 nm wavelength was used for the remaining biomass types (17).

Results

Isolation and phylogenetic affiliation. Strain C1A was isolated from the feces of an Angus steer on a cellobiose-switchgrass medium. The isolate displayed polycentric growth and effectively colonized switchgrass. Phylogenetic analysis using the nuclear ribosomal internal transcribed spacer I (ITS-I) region supported the placement of strain C1A as a member of the genus *Orpinomyces* (Figure 3-1).

Strain C1A is an effective, versatile biomass degrader. Strain C1A effectively metabolized a variety of sugars and polysaccharides, including crystalline cellulose and xylan. (Figure 3-2). Extensive utilization of cellulose occurred during the first 96 hours of growth in batch culture (Figure 3-2). More importantly, strain C1A grew readily on untreated, as well as mild acid-, mild alkali-, and hydrothermolysis-treated switchgrass, with the concurrent utilization of cellulose and hemicellulose fractions, but not lignin (Figure 3-3, Figure 3-4). Dry weight losses of substrate ranged between 18.6% (28.7% of fermentable sugars) in untreated switchgrass to 40.8% (53.9% of fermentable sugars) in NaOH-treated switchgrass. Further, adjustments to the inoculum/substrate ratios resulted in an increase in the amount of switchgrass metabolized up to 42.8% and 58.4% of the dry weight of untreated and NaOH-treated switchgrass, respectively (Table 3-1). Strain C1A performed extremely well on NaOH-treated switchgrass, since this method of pretreatment retains the majority of the hemicellulose content (1, 13), which is degradable by strain C1A. Strain C1A also grew well on hydrothermolysis-treated switchgrass, presumably due to the fact that the removal of hemicellulose resulted in a greater accessibility to cellulose fibers. Acid pretreatment also removed the hemicellulose fraction from switchgrass, but strain C1A did not perform as well on acid-

pretreated switchgrass as it did on hydrothermolysis-pretreated switchgrass. Previous studies have shown that acid pretreatment often results in the release of inhibitory compounds (8)

End product analysis indicated that lactate, acetate, and formate are the main end product of plant biomass degradation. Only minor amounts of ethanol were produced, ranging between 0.045-0.096 mg ethanol/mg biomass (Figure 3-5, Table 3-2).

In addition to switchgrass, we tested the capability of strain C1A to utilize several other types of energy crops (e.g. alfalfa, sorghum, energy cane), agricultural residues (e.g. corn stover), and grasses (e.g. Bermuda grass). We chose these specific plant materials due to the variations in the percentages of cellulose, hemicellulose, and lignin in these plants (Table 3-3). The results demonstrate the versatility of strain C1A, since it was able to metabolize all different types of examined plant biomass (Table 3-3). Further, strain C1A was capable of degrading multiple types of lignocellulosic biomass without pretreatment ranging from 9.0-40.6% of the starting dry weight, 21.3-60.0% of the glucan, and 3.8-43.0% of the xylan fractions (Figure 3-6). Within both untreated and NaOH-treated experiments, strain C1A was most effective in the metabolism of corn stover, with 40.6% and 62.3% dry weight loss, 51.0% and 75.8% loss in cellulose fraction, and 43.0% and 74.3% loss in hemicellulose fractions in untreated and NaOH-treated corn stover, respectively.

Figure 3-1. (A) *Orpinomyces* sp. strain C1A on anaerobic agar roll tubes. (B) Phase contrast micrograph displaying polycentric growth of strain C1A. (C) Strain C1A growing on and colonizing switchgrass (1), compared to uninoculated control (2) (D) Distance dendogram based on ITS1-1 region highlighting the phylogenetic affiliation of strain C1A within the Neocallimastigomycota. The tree was constructed using Neighbor Joining algorithm with Jukes-Cantor corrections. Bootstrap values shown are based on 1000 replicates.

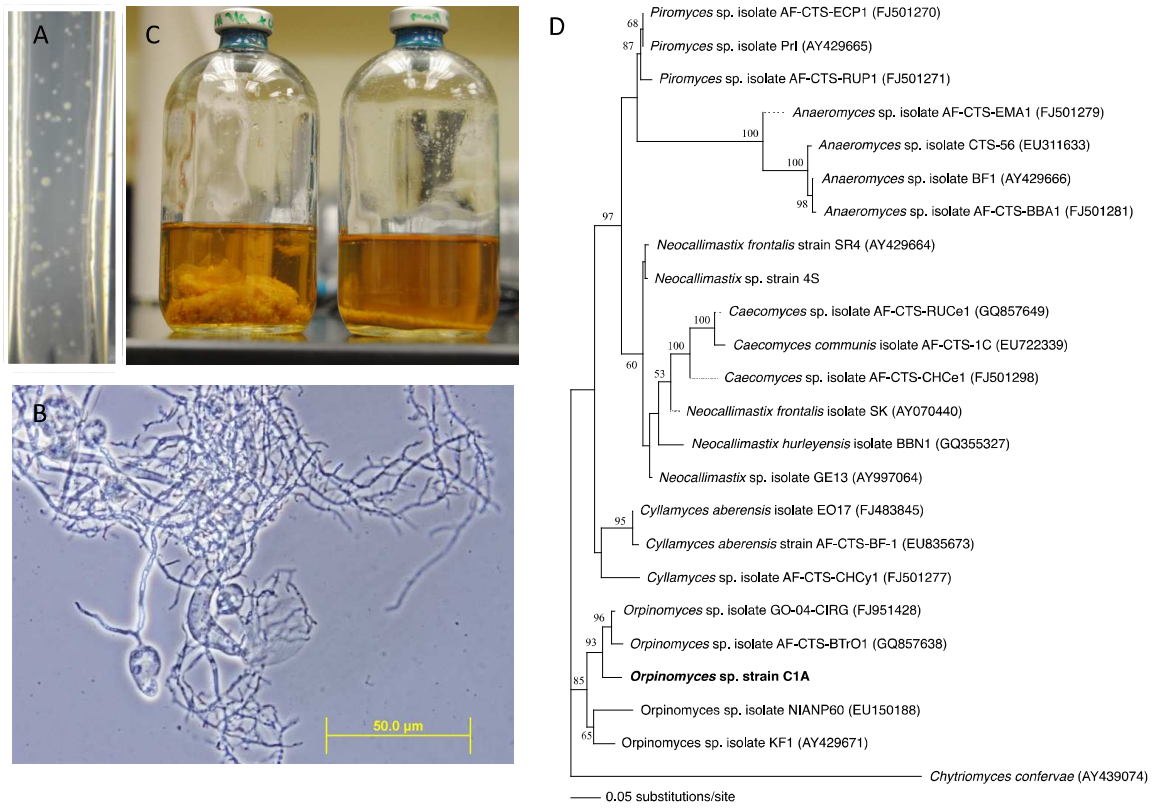


Figure 3-2. Crystalline cellulose and xylan utilization by *Orpinomyces* strain C1A. (A) Cellulose loss (○) and lactate (◊), formate (□), acetate (△), and ethanol (X) production in microcosms that were amended with avicel and strain C1A. (B) Formate (◊), Lactate (□), acetate (△), and ethanol (X) production in microcosms that were amended with xylan and strain C1A

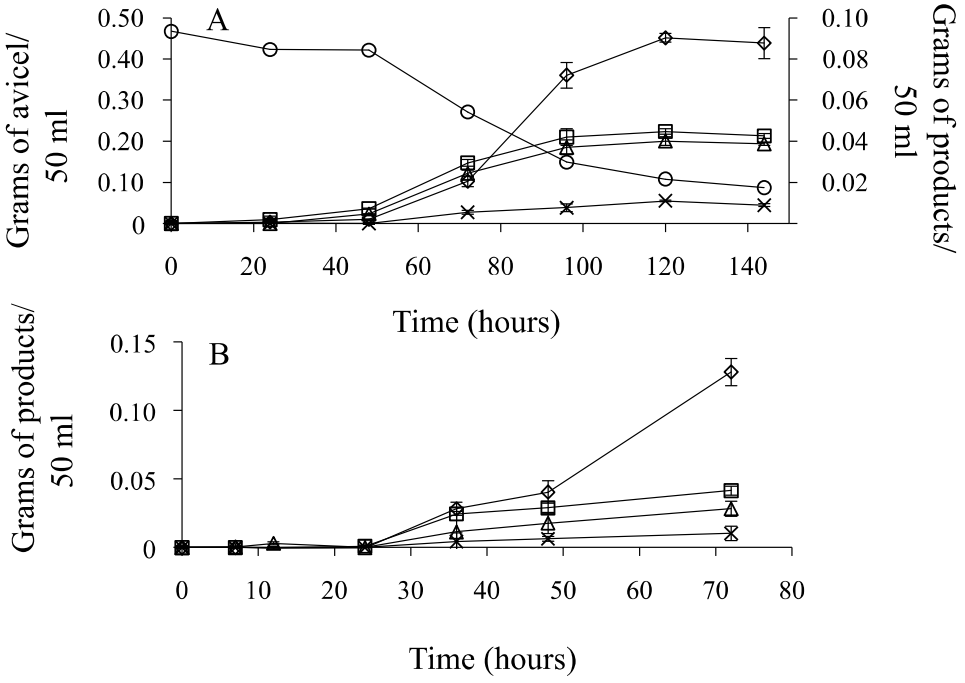


Figure 3-3. Lignocellulosic capabilities of strain C1A. (A to D) % Dry weight (◆), cellulose (■), hemicellulose (▲), and lignin (●) lost in microcosms that contained untreated (A), sodium hydroxide-treated (B), acid-treated (C), and hydrothermolysis-treated (D) switchgrass.

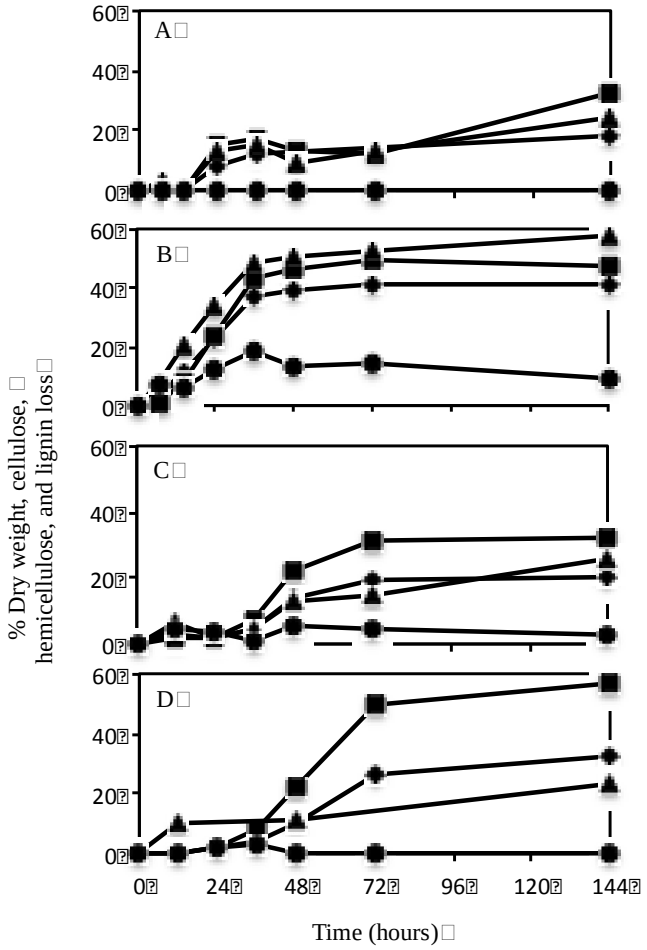


Figure 3-4. Grams of dry weight (♢), cellulose (□), hemicellulose (△), and lignin (X) lost in microcosms that contained untreated (A), sodium hydroxide-treated (B), acid-treated (C), and hydrothermolysis-treated (D) switchgrass.

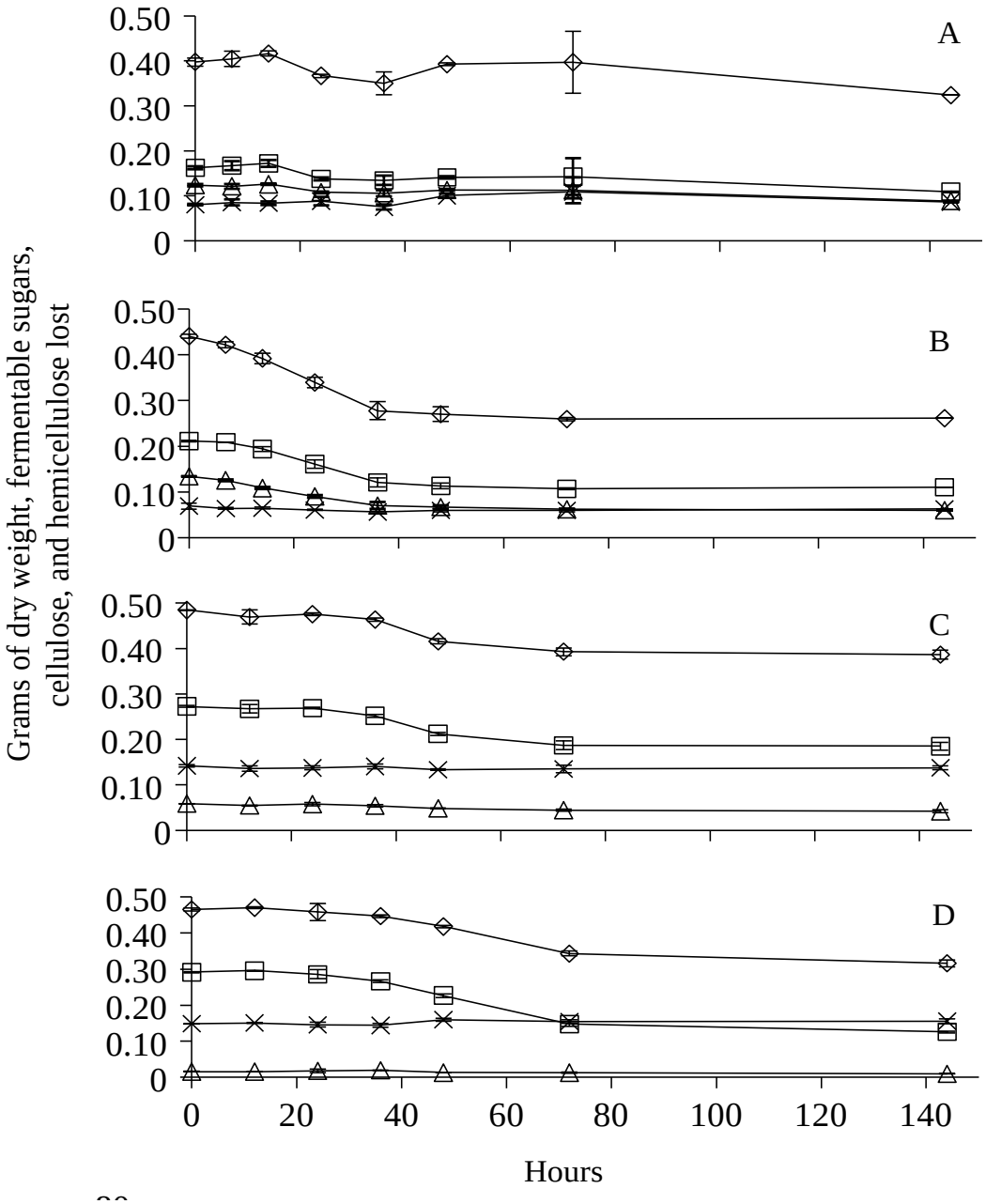


Table 3-1. Average dry weight and non-lignin losses in microcosms with different amounts of untreated, acid-treated, sodium hydroxide-treated, or hydrothermolysis-treated switchgrass.

Treatment	Amount of Switchgrass added to microcosms (mg)	Substrate to inoculum ratio[a]	% dry weight loss[b]	% non-lignin loss[c]
Untreated switchgrass	500	250	25.2 ± 1.3[d]	34.9 ± 1.8[d]
	250	125	28.6 ± 2.6	38.6 ± 2.6
	100	50	42.8 ± 9.5	57.8 ± 0.6
	50	25	33.2 ± 0.4	44.8 ± 0.6
Acid-treated switchgrass	500	250	14.3 ± 2.6	22.7 ± 4.1
	250	125	17.0 ± 1.3	27.0 ± 2.7
	100	50	23.3 ± 5.4	37.1 ± 8.6
	50	25	23.0 ± 1.5	36.6 ± 2.5
NaOH-treated switchgrass	500	250	34.6 ± 3.9	41.8 ± 4.7
	250	125	57.8 ± 2.8	69.9 ± 3.4
	100	50	58.4 ± 1.8	70.6 ± 2.2
	50	25	50.6 ± 17.4	70.3 ± 19.8
Hydrothermolysis-Treated Switchgrass	500	250	30.0 ± 2.5	44.6 ± 3.7
	250	125	48.4 ± 2.4	72.6 ± 3.6
	100	50	28.7 ± 2.0	42.9 ± 2.9
	50	25	26.0 ± 4.8	38.9 ± 7.2

[a] Substrate inoculum ratio obtained by dividing the amount of added switchgrass by the amount of inoculum added to each set of microcosms. Approximately 2.5 mg of C1A fungal cells were added to each microcosm.

[b] % dry weight loss was calculated by subtracting the time final dry weight of each substrate from the time zero dry weight of each substrate and multiplying the value that was obtained by 100.

[c] % non lignin loss was calculated using the following formula: [(T_0 dry weight-T_{final} dry weight)/(T_0 dry weight-T_0 lignin weight)] x 100. T_0 lignin weights were determined for each plant using compositional analysis.

[d] % dry weight and non lignin losses are expressed as the mean ± standard deviation of the % dry weight and % non-lignin losses in triplicate microcosms.

Figure 3-5. Acids and alcohols produced during switchgrass degradation. Lactate (■), formate (●), acetate (▲), and ethanol (◆) production in microcosms that contained untreated (A), acid-treated (B), sodium hydroxide-treated (C), and hydrothermolysis-treated switchgrass (D).

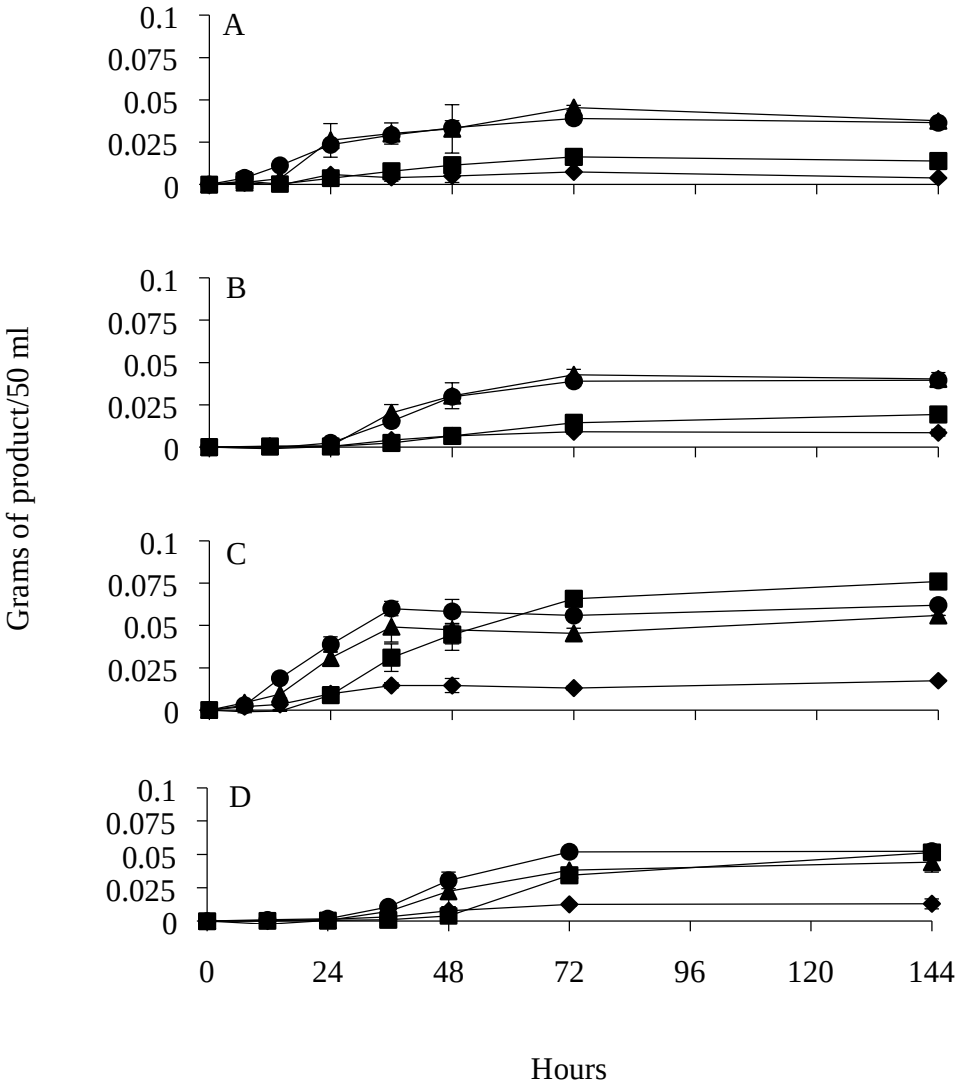


Table 3-2. Ratios of end products produced (grams) per gram of plant biomass consumed by strain C1A.

Substrate	**Product (g)/ plant biomass consumed (g)**			
	Lactate	**Formate**	**Acetate**	**Ethanol**
Unt. Switchgrass	0.187	0.491	0.506	0.053
Acid Switchgrass	0.1982	0.403	0.410	0.086
NaOH switchgrass	0.422	0.345	0.310	0.096
Hydrothermolysis switchgrass	0.344	0.349	0.293	0.087
Unt. Alfalfa	0.298	0.376	0.426	0.074
NaOH alfalfa	0.283	0.272	0.300	0.061
Unt. Bermuda	0.022	0.341	0.500	0.0454
NaOH Bermuda	0.185	0.377	0.400	0.015
Unt. Corn Stover	0.477	0.273	0.284	0.063
NaOH Corn Stover	0.384	0.204	0.176	0.062
Unt. Sorghum	0.404	0.316	0.353	0.066
NaOH Sorghum	0.458	0.233	0.185	0.048
Unt. Energy Cane	0.447	0.494	0.576	0.082
NaOH Energy Cane	0.556	0.295	0.290	0.063

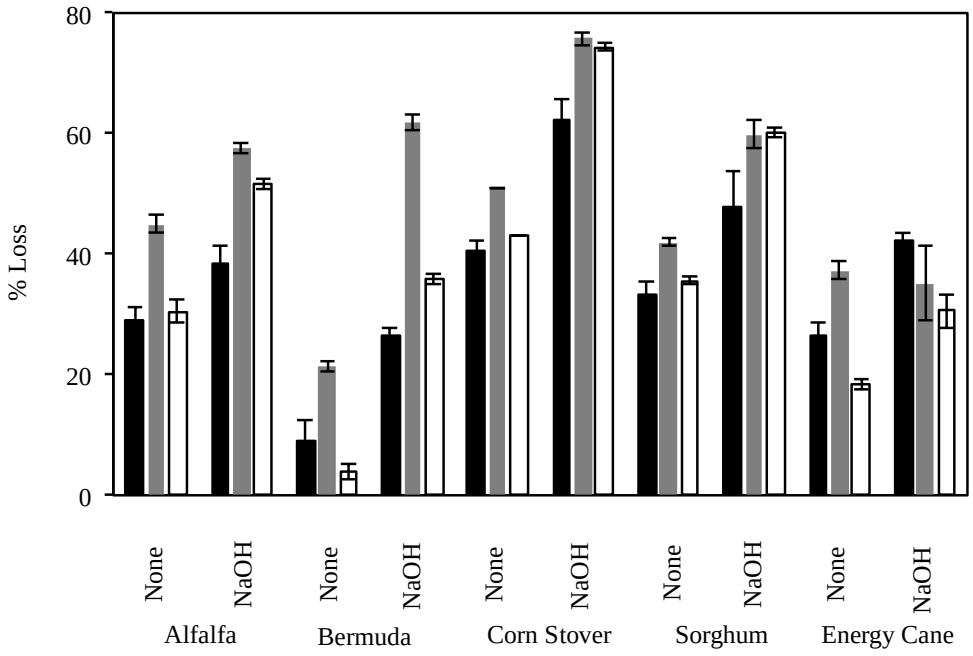
Table 3-3. Summary of dry weight, cellulose, hemicellulose, xylan, lignin, and fermentable sugar losses in microcosms with different types of plant materials.

Substrate	Treatment	Dry weight		Cellulose		Hemicellulose		Xylan		Lignin		% DW lost	% FS lost
		T_0	T_f	T_0	T_f	T_0	T_f	T_0	T_f	T_0	T_f		
Switch grass	None	0.40 ± 0.01	0.32 ± 0.07	0.16 ± 0	0.11 ± 0	0.15 ± 0	0.11 ± 0.01	0.12 ± 0	0.09 ± 0.03	0.09 ± 0	0.10 ± 0.01	18.6	28.71
	Acid	0.48 ± 0	0.39 ± 0.01	0.27 ± 0	0.19 ± 0.01	0.06 ± 0	0.05 ± 0	0.06 ± 0	0.04 ± 0	0.15 ± 0	0.16 ± 0	20.2	31.25
	NaOH	0.44 ± 0	0.27 ± 0	0.21 ± 0	0.11 ± 0	0.15 ± 0	0.07 ± 0	0.13 ± 0	0.06 ± 0	0.08 ± 0.01	0.09 ± 0	40.82	53.92
	Steam	0.50 ± 0	0.32 ± 0.01	0.29 ± 0	0.13 ± 0	0.02 ± 0	0.02 ± 0	0.02 ± 0	0.01 ± 0	0.16 ± 0	0.18 ± 0.01	32.19	45.66
Corn stover	None	0.44 ± 0.06	0.26 ± 0	0.20 ± 0	0.10 ± 0	0.16 ± 0	0.10 ± 0	0.13 ± 0.01	0.06 ± 0	0.08 ± 0	0.06 ± 0	39.32	47.45
	NaOH	0.47 ± 0.02	0.18 ± 0.04	0.26 ± 0.01	0.06 ± 0	0.16 ± 0	0.04 ± 0	0.13 ± 0	0.03 ± 0	0.04 ± 0	0.04 ± 0.01	60.94	75.19
Sorghum	None	0.41 ± 0.01	0.28 ± 0.01	0.18 ± 0	0.11 ± 0	0.13 ± 0	0.09 ± 0	0.11 ± 0	0.07 ± 0	0.1 ± 0	0.07 ± 0	32.11	39.26
	NaOH	0.48 ± 0.02	0.25 ± 0.03	0.26 ± 0	0.10 ± 0.01	0.15 ± 0	0.06 ± 0	0.12 ± 0	0.04 ± 0	0.07 ± 0	0.07 ± 0.01	46.85	59.92

Energy cane	None	0.32 ± 0.01	0.24 ± 0.01	0.13 ± 0	0.08 ± 0	0.11 ± 0	0.07 ± 0	0.08 ± 0	0.06 ± 0	0.08 ± 0	0.07 ± 0.01	34.05	28.85
	NaOH	0.49 ± 0.01	0.29 ± 0.01	0.16 ± 0	0.10 ± 0.01	0.16 ± 0	0.07 ± 0	0.12 ± 0	0.05 ± 0	0.10 ± 0	0.09 ± 0.01	41.14	33.35
Alfalfa	None	0.33 ± 0.02	0.24 ± 0.01	0.14 ± 0	0.08 ± 0	0.10 ± 0	0.07 ± 0	0.05 ± 0	0.05 ± 0	0.08 ± 0	0.07 ± 0	27.52	38.85
	NaOH	0.47 ± 0.02	0.29 ± 0.01	0.23 ± 0	0.10 ± 0	0.13 ± 0	0.06 ± 0	0.07 ± 0	0.03 ± 0	0.11 ± 0	0.12 ± 0	37.18	53.39
Bermuda	None	0.49 ± 0.001	0.45 ± 0.01	0.11 ± 0	0.09 ± 0	0.16 ± 0	0.15 ± 0	0.09 ± 0.	0.08 ± 0	0.2 ± 0	0.2 ± 0	7.96	11.1
	NaOH	0.49 ± 0.01	0.37 ± 0	0.15 ± 0	0.06 ± 0	0.12 ± 0.01	0.07 ± 0	0.08 ± 0	0.03 ± 0	0.23 ± 0	0.22 ± 0	25.51	50.33
Willow	None	0.46 ± 0.01	0.45 ± 0	0.21 ± 0	0.20 ± 0	0.11 ± 0.01	0.11 ± 0	0.09 ± 0	0.09 ± 0	0.14 ± 0	0.14 ± 0.01	2.39	5.66
	NaOH	0.50 ± 0.01	0.40 ± 0.01	0.21 ± 0	0.15 ± 0	0.12 ± 0.01	0.08 ± 0	0.09 ± 0	0.05 ± 0	0.18 ± 0	0.15 ± 0	19.56	26.28

T_0: Value at time zero.
T_f: Value at time final.
DW: dry weight
FS: fermentable sugar

Figure 3-6. Lignocellulosic capabilities of strain C1A on multiple types of untreated and sodium hydroxide pretreated plant materials. Percentages of dry weight (black bars), cellulose (grey bars), and hemicellulose (white bars) lost.



Discussion

In this work, we report on the isolation of *Orpinomyces* sp. strain C1A and describe its degradative capabilities on multiple substrates, including untreated lignocellulosic plant biomass. Morphological and ITS1 sequence analyses indicated it as a member of the *Orpinomyces* genus of anaerobic fungi. Strain C1A has been maintained for greater than 200 subcultures in a cellobiose medium supplemented with rumen fluid, without loss of culture viability or degradative capacity. This polycentric strain exhibited extensive hyphael growth on cellulose, xylan, switchgrass, alfalfa, bermuda grass, corn stover, forage sorghum, and energy cane within 72 hours after inoculation.

The lignocellulosic abilities described for strain C1A is further reflected in the observed structural, metabolic, and genomic traits for this fungus. Many of these are not shared with other basal fungal relatives or non-fungal Opisthokonts, and hence could be regarded as Neocallimastigomycota-specific adaptations to the anaerobic gut environment. Further, the development of cellulosomes, and the acquisition of many GH enzymes could be viewed as an adaptation to improve the access, speed, and efficacy of biomass degradation.

Our results suggest that the lignocellulolytic capabilities of strain C1A could be exploited outside the rumen for the production of biofuels from plant biomass. The most promising approach for lignocellulosic biofuel production involves consolidated bioprocessing, which combines the saccharification of lignocellulose and the fermentation of the resulting sugars in a single step, and is carried out by a single microorganism or microbial consortia (25). Here, we show that strain C1A simultaneously couples the saccharification of the cellulosic and hemicellulosic fractions

of plants to the fermentation of the resulting hexose and pentose sugars. Further, the invasive nature and filamentous growth pattern of these anaerobic fungi allows plant biomass degradation to proceed without pretreatment, but the process was significantly enhanced using mild pretreatments and through optimizing the amount of starting fungal inoculum to the amount of substrate present. To our knowledge, the extent of lignocellulosic biomass degradation by strain C1A has not been reported for a single microorganism in the absence of saccharification enzymes. Comparisons to the thermophilic anaerobic bacteria *Caldicellulosiruptor bescii* and *Anaerocellum thermophilum*, which are recognized for their leading capabilities on untreated lignocellulosic substrates, including switchgrass, revealed that strain C1A was able to match the amounts reported for switchgrass degradation by both bacterial species (9, 26). Further, this degradation occurred under mesophilic conditions without the higher temperature requirements of *C. bescii* or *A. thermophilum*. Anaerobic fungi thus represent extremely promising microorganisms for exploitation in direct lignocellulolytic schemes.

As part of its fermentative metabolism, strain C1A is capable of producing ethanol as a minor end product during pyruvate metabolism. Indeed, 1 copy of alcohol dehydrogenase has been identified, and C1A can tolerate up to 3% ethanol (data not shown). However, given its relatively low ethanol productivity and relatively low ethanol tolerance, efforts towards improving alcohol production and tolerance via physiological and genetic manipulations are needed to improve ethanol productivity in this remarkable plant biomass-degrading anaerobic fungal strain.

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CHAPTER IV

EFFECT OF HYDROTHERMOLYSIS PRETREATMENT ON LIGNOCELLULOSIC BIOMASS DEGRADATION BY THE ANAEROBIC FUNGUS *ORPINOMYCES* SP. STRAIN C1A

Abstract

Members of the anaerobic fungi (Phylum Neocallimastigomycota) are efficient biomass degraders and represent promising agents for fuel and chemical production from lignocellulosic biomass. Pretreatment of lignocellulosic biomass is considered an unavoidable first step in enzyme-based saccharification schemes, but its necessity in any proposed anaerobic fungi-based schemes is still unclear. Here, we evaluated the effect of hydrothermal pretreatments on the extent of corn stover and switchgrass degradation by an anaerobic fungal isolate, *Orpinomyces* sp. strain C1A. Using a factorial experimental design, we evaluated the effect of three different temperatures (180, 190, and 200°C) and three hold times (5, 10, and 15 min). Pretreated corn stover and switchgrass were more amenable to degradation by strain C1A than was untreated biomass, as evident by the higher proportion of plant biomass degraded compared to untreated controls. However, when factoring in the proportion of biomass lost during the pretreatment process (ranging between 25.78 and 58.92% in corn stover and 28.34 and 38.22% in switchgrass), hydrothermolysis provided negligible or negative improvements to the extent of corn stover and switchgrass degradation by strain C1A. Product analysis demonstrated a shift towards higher ethanol and lactate production and lower acetate production associated with increase in pretreatment severity, especially in switchgrass incubations. The results are in stark contrast to the requirement of pretreatment in enzyme-based schemes for biomass saccharification, and their implications on the potential utility of anaerobic fungi in biofuel and biochemical production are discussed.

Introduction

Lignocellulosic biomass is defined as the raw, non-edible plant biomass that is mainly composed of sugar (cellulose and hemicellulose) and aromatic (lignin) polymers. Generally, lignocellulosic biomass could be classified as virgin biomass (the naturally occurring vegetation within an environment), crop-residue biomass (i.e. the inedible fraction of various crops such as corn stover and wheat straw), or dedicated energy crops (planted for the sole purpose of harvesting for energy production, e.g. switchgrass) (6). Collectively, lignocellulosic biomass is a vast and underutilized resource for the production of sugars, biofuels, and other value-added chemicals.

Production of fuels and chemicals from lignocellulosic biomass is technically feasible, but is currently too expensive for widespread utilization and commercialization. One of the most studied processes for making fuels and chemicals from lignocellulosic biomass is enzymatic hydrolysis and fermentation. In this process, exogenously supplied enzyme preparations are utilized to extract sugar from plant polymers, and the produced sugars are then converted to fuels and chemicals using dedicated sugar-metabolizing microorganisms (3). The main plant polymers targeted for biofuel production in lignocellulosic biomass are cellulose and hemicellulose, both of which are structural components of plant cell walls and are chemically bound to a variety of complex macromolecules, mainly lignin (21). Enzymatic treatment of lignocellulosic biomass to depolymerize cellulose and hemicellulose is a complex and costly endeavor requiring the concerted actions of multiple enzymes to depolymerize cellulose and hemicellulose (4, 11). Enzymes represent a substantial part of the overall cost of fuel production from lignocellulosic biomass (8).

One promising alternative is the use of microorganisms for breakdown of lignocellulosic biomass rather than exogenous enzymes (14). We are currently exploring the utility of a special group of microorganisms, the anaerobic fungi (Phylum Neocallimastigomycota), as promising agents for biofuel and biochemical production. Anaerobic fungi reside in the rumen and gut of herbivores where they play an important role in the initial steps of plant biomass degradation in these habitats. Anaerobic fungi are highly fibrolytic microorganisms, producing a wide array of cell-bound and cell-free cellulolytic, hemicellulolytic, glycolytic, and proteolytic enzymes (10, 12, 25). By attaching themselves to plant materials, they colonize and excrete extracellular enzymes that degrade structural plant polymers to be available to other microbes. Therefore, many of the capabilities acquired by anaerobic fungi during their evolutionary history and adaptation to the herbivorous guts represent extremely desirable traits for direct conversion of lignocellulosic biomass to sugars, fuels and chemicals.

In addition to potential cost savings associated with eliminating the need for expensive enzymes for biomass degradation, anaerobic fungi could potentially provide cost savings by eliminating or simplifying the pretreatment process. Pretreatment of biomass utilizes physical, chemical or physio-chemical approaches to overcome biomass recalcitrance and render it more amenable to enzyme degradation (3). Physio-chemical pretreatments provide physical disruption of the substrate with alteration of biomass either through added or generated acids/bases (3). Hydrothermal pretreatment is one type of physio-chemical approach that uses elevated temperatures and pressure to generate acidic reaction conditions within the reactor (3, 16). This results in substantial removal of hemicellulose and dislocation of lignin from the biomass (9).

While pretreatment is an unavoidable process in enzyme-based saccharification of lignocellulose, its value in enhancing plant biomass degradation by anaerobic fungi is less clear. Anaerobic fungi exhibit an invasive growth pattern, with their hyphae readily penetrating plant cell walls during growth; hence, improving access and allowing localized delivery of lignocellulolytic enzymes. Further, in addition to cellulases and hemicellulases, anaerobic fungi produce a wide array of accessory enzymes that aid in exposing cellulose and hemicellulose molecules for degradation. Examples of such enzymes include acetyl xylan esterase for debranching hemicellulose polymer chains to sugars and feruloyl/cinnamoyl esterases for mobilizing hemicellulose from lignin (17) . Also, non-catalytic proteins, such as swollenin, for physical disruption of cell wall structures are produced by anaerobic fungi.

Here, we tested the utility of hydrothermolysis pretreatment in biomass degradation by *Orpinomyces* sp. strain C1A. Strain C1A is an anaerobic fungal strain that was isolated and has been maintained and routinely subcultured in our laboratory for the last 4 years. As such, it does not exhibit senescence as previously observed in multiple anaerobic fungal strains (15, 20). Strain C1A is also considerably less fastidious than other anaerobic fungal strains since it can survive prolonged storage at room temperature, and can readily be stored on agar roll tubes for prolonged periods of time. Our results suggest that many of the improvements in plant biomass degradation realized by hydrothermolysis pretreatment of corn stover and switchgrass are not offset by losses of plant polymers encountered during the pretreatment process. The implications of these results on proposals to utilize anaerobic fungi for biofuel and biochemical production are discussed.

Materials and Methods

Microorganism. The anaerobic fungal strain *Orpinomyces* sp. strain C1A was isolated from the feces of an Angus steer as described previously (26). Strain C1A was grown in an anaerobic basal media containing clarified rumen fluid reduced by cysteine-sulfide and dispensed under a stream of 100% CO_2 as previously described (26). Starting cultures were grown on cellobiose (3.75 g/liter) and then added to either untreated or hydrothermal pretreated plant material.

Plant materials. Samples of mature Kanlow switchgrass (*Panicum virgatum* var. *Kanlow*) were obtained from Oklahoma State University experimental plots in Stillwater, OK, USA. Samples of corn stover (*Zea mays*) were obtained from the Industrial Agricultural Products Center at the University of Nebraska in Lincoln, NE, USA. All samples were dried at 45°C overnight, milled, and sieved to a final particle size of 2 mm as previously described (24). All pretreated samples were dried at 45°C for approximately 48 h before use.

Pretreatment. A three-factorial design was employed testing pretreatment temperatures of 180, 190, and 200°C and reaction hold times of 5, 10, and 15 min. Hydrothermal pretreatment was prepared by mixing 60 g of dry switchgrass or corn stover with distilled water to achieve a 10% dry matter mixture (24). This mixture was placed inside a 1-liter benchtop pressure reactor (Parr Series 4520; Parr Instrument Company, Moline, IL, USA) that was heated to a specific temperature (180-200°C) for a set hold time (5-15 min) (24). After the specified hold time, the mixture was cooled in an ice bath to 55°C (24). The insoluble reside was separated from the hydrolysate by filtration. The pretreated material was subjected to four washes with 500 ml of milliQ water at 60±5°C.

Experimental set up and design. Experiments to evaluate the growth of strain C1A on different treated and pretreated plant materials were conducted under strict anaerobic conditions in 160-ml serum bottles. All experiments were conducted with 0.5 g of plant material as the substrate. Each serum bottle was then amended with the appropriate type of plant biomass inside an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). The serum bottles were then removed from the glove bag and the headspace was flushed with 100% CO_2. Five ml of an actively growing culture of strain C1A (approximately 2.6 mg of fungal biomass) was used as an inoculum and added to 45 ml medium in 160-ml serum bottles. In all experiments, serum bottles were incubated at 39°C in a non-shaking incubator. Controls without substrate were included in all experiments to account for any product carryover from the inoculum.

The effects of the pretreatment variables (reaction temperature and hold time) on the extent of utilization of two lignocellulosic biomass substrates (switchgrass and corn stover) were examined. A factorial experimental design in which pretreatment temperatures of 180, 190, and 200°C combined with hold times of 5, 10, and 15 min was implemented, as well as untreated corn stover and switchgrass controls. These nine different pretreatment combinations per substrate result in R_0 severity indices from 3.05 to 4.12 (as calculated by the formula of Overend and Chornet for severity (18). For similar processes, R_0 severity values between 3.0 and 4.5 were needed for efficient saccharification of wheat straw using commercial enzymes preparations (3). All experiments were conducted in triplicate. In each of the 18 different conditions tested (9 corn stover and 9 switchgrass pretreatments), the effect of pretreatment on biomass

composition, the amount of plant biomass utilized by C1A, and the patterns of product formation were measured.

Analytical methods. The amount of plant material consumed in serum bottles was calculated by subtracting the final dry mass from the initial dry mass of each plant material. Since the pellets at the end of incubation contained a mixture of plant and fungal biomass, the amount of fungal biomass at the end of incubation was indirectly quantified using formate concentrations as previously described (13). The amounts of glucan and xylan in untreated and pretreated substrates were determined using a standard National Renewable Energy Laboratory (NREL) procedure (22). Briefly, the procedure included the addition of 3 ml of 72% sulfuric acid to each sample and incubation at 30±3°C for 1 h, with stirring every 5 to 10 min. The samples were then diluted with 84 ml of deionized water, capped, and autoclaved for 1 h at 121°C. The cooled solution was filtered, and this filtrate was used to determine carbohydrate content and soluble lignin. The remaining solids were washed and dried to constant weight at 105°C to determine acid-insoluble residue (AIR) and then converted to ash at 575°C for 24 h. Analyses of resulting carbohydrates within the filtrate were done by HPLC with refractive index detection (RID) (Agilent 1100 series; Santa Clara, CA, USA) on an Aminex HPX-87P column at 85°C with a mobile phase of deionized water pumped at 0.6 ml/min for 30 min. Twenty µl of each sample were analyzed for cellobiose, glucose, xylose, galactose, arabinose, and mannose. Contributions of structural constituents to the total biomass composition were determined using the NREL summative mass closure procedure (23). The acid-soluble lignin (ASL) content was determined using a UV spectrophotometer set at a wavelength of 205 nm, as has been previously used to determine ASL in switchgrass

(7). As recommended in the NREL procedure, ASL in corn stover was measured at 320 nm, whereas a 240-nm wavelength was used for forage sorghum (22).

Final end products of C1A metabolism (fatty acids and ethanol) in supernatant fractions were quantified using a high-pressure liquid chromatograph (HPLC) with a refractive index detector (1100 series; Agilent, Santa Clara, CA, USA) and an Aminex HPX-87H column (Bio-Rad, Sunnyvale, CA, USA), which was heated to 60°C. The mobile phase was 0.01 N H_2SO_4, with a flow rate of 0.6 ml per minute (26).

Results

Effect of hydrothermolysis pretreatment temperature and hold time on biomass composition. Hydrothermolysis resulted in the removal of the xylan fraction of biomass and the subsequent increase in glucan and lignin fractions in pretreated biomass (Table 4-1). In general, pretreatment severity increased the proportion of xylan removed, and hence increased the glucan and lignin within the pretreated biomass (Table 4-1). Severity index was positively correlated to glucan precentage (Pearson correlation coefficient r = 0.65, and 0.84 for corn stover, and switchgrass, respectively), and negatively correlated to xylan percentage (Pearson correlation coefficient r = -0.91, and -0.9 for corn stover, and switchgrass, respectively). The xylan fraction of corn stover was more easily solubilized than that of switchgrass at lower temperatures (180°C). However, at higher temperatures (190°C and 200°C), comparable levels of xylan removal were obtained for both biomass types, and the most severe pretreatment (200°C for 15 minutes) resulted in comparable removal of xylan for both biomass types (93.3% in corn stover and 93.0% in switchgrass).

Loss of dry mass associated with various pretreatment conditions was also quantified (Table 4-1). Higher percentages and a wider range of dry weight losses were observed due to the pretreatment process in corn stover when compared to switchgrass (Table 4-1). Dry weight loss associated with the pretreatment process ranged between 25.78 and 58.92% in corn stover and 28.34% and 39.36% in switchgrass. Interestingly, overall loss of dry weight decreased with increasing severity in corn stover. This trend could be explained by the loss of a large fraction of water-soluble compounds from corn stover by pretreatments at milder conditions, with these soluble components converted to

char or pseudolignin at higher severities, a process typically associated with an increase in hydrothermolysis severity (9). On the other hand, overall dry weight loss from switchgrass increased with increasing severity of the pretreatment conditions (Table 4-1). The small reduction in dry weight loss observed for the highest pretreatment severities may indicate the formation of char or pseudolignin under these conditions. The lower amount of solubilization in all switchgrass pretreatments, especially milder pretreatment conditions, in switchgrass as compared to corn stover suggests that a lower proportion of soluble components is removed by pretreatments in switchgrass, and that the majority of dry weight loss in switchgrass is due to hemicellulose removal.

Effect of pretreatment on corn stover and switchgrass degradation by *Orpinomyces* sp. strain C1A. We evaluated whether hydrothermal pretreatments render corn stover and switchgrass more amenable to degradation by strain C1A, and whether any realized increases in the extent of biomass degradation in pretreated biomass justify the energy and cost of the process, as well as the dry weight loss realized during the pretreatment process (Table 4-1). Strain C1A was capable of metabolizing 23.60% of untreated corn stover (equivalent to 28.70% of the non-lignin fraction of untreated biomass) (Table 4-2). Pretreated corn stover was more amenable to degradation, with 31.99%-37.99% of pretreated biomass metabolized by strain C1A (equivalent to 52.6%- 56.30% of non-lignin fraction in pretreated biomass) (Table 4-2). The highest dry weight loss percentage was obtained in 190°C for 15 min pretreatment. The majority of the reported increases in percentage dry weight loss of pretreated corn stover were statistically significant (Table 4-2).

For switchgrass, strain C1A was capable of metabolizing 24.69% of untreated switchgrass (equivalent to 32.0% of the non lignin fraction of untreated biomass). Pretreatment slightly improved the proportion of dry weight degraded, although the improvements realized were lower than those for corn stover (Table 4-2). The increase in dry weight loss of pretreated switchgrass was positively correlated to pretreatment severity (R_0) (Pearson correlation coefficient = 0.66). Highest values were observed at 200°C with a hold time of 5 minutes. However, with the exception of one pretreatment condition (200°C for 15 minutes), the realized improvements in switchgrass degradation were not significant.

Analysis of variance (ANOVA) was used to identify which factor (temperature or hold time) plays an important role in increasing proportion of hydrothermolysis-pretreated biomass by strain C1A. The results showed that for corn stover both the increase in pretreatment temperatures and the increase in hold time significantly improved the extent of biomass degradation (p-value = 0.0004 for temperature and 0.0005 for hold time), while for switchgrass, only the increase in pretreatment temperature had a significant effect on biomass degradation (p-value = 0.0018).

While the above results clearly demonstrate that pretreated biomass is more amenable to degradation than untreated biomass, pretreatment is associated with energy expenditure as well as operational cost. More importantly, hydrothermolysis pretreatment results in significant loss in plant biomass weight due to the substantial solubilization of hemicellulose (Table 4-1), a substrate that is readily utilized by strain C1A (26). Further, our results clearly demonstrate the capability of strain C1A to metabolize untreated switchgrass and corn stover (Table 4-2). Therefore, to determine whether various

hydrothermolysis pretreatments deliver actual improvements to biomass degradation by strain C1A, we readjusted our calculations of percentage dry weight loss of corn stover and switchgrass by strain C1A by taking into account the amount of plant biomass lost during pretreatment (eq 1 and 2):

$$BL_{adj} = (m_0 - m_f) * BL_p \quad (1)$$

$$BL_p = \frac{(m_{bp} - m_{ap})}{m_{bp}} \quad (2)$$

where, BL_{adj} is the adjusted biomass loss fraction, BL_p is the biomass loss fraction from pretreatment, m_0 is the mass before fungal treatment, m_f is the mass after fungal treatment, m_{bp} is the mass before pretreatment, and m_{ap} is the mass after pretreatment. These adjusted values (plant biomass loss due to strain C1A metabolism as a percentage of original plant biomass weight) were then compared to those obtained from untreated plant biomass degradation by strain C1A. Our results (Table 4-2) strongly suggest that improvements in dry weight loss observed in pretreated biomass are not offset by the loss of hemicellulose and water soluble compounds occurring during the pretreatment process. All adjusted values either showed negligible or no improvements in the extent of biomass degradation by strain C1A when compared to untreated plant biomass (Table 4-2).

Product formation patterns. In addition to biomass loss and product formation, we examined the effect of various pretreatments on product formation by strain C1A. Strain C1A utilizes a mixed acid fermentation scheme for sugar metabolism with the main products being acetate, formate, lactate, and ethanol (26). Product formation patterns in

pretreated switchgrass show a general trend in which the proportion of ethanol and lactate produced by strain C1A increased, while that of acetate decreased with pretreatment severity (Pearson correlation coefficient = 0.82, 0.59, and -0.56, for ethanol, lactate, and acetate, respectively). In addition to pretreatment severity, these trends in product formation showed a strong correlation with glucan content (Pearson correlation coefficient = 0.67, 0.57, and -0.78 for ethanol, lactate, and acetate respectively), glucan:xylan ratio (Pearson correlation coefficient = 0.61, 0.44, and -0.64, for ethanol, lactate, and acetate, respectively), and xylan content (Pearson correlation coefficient = -0.73, -0.58, and 0.81, for ethanol, lactate, and acetate, respectively). Ethanol values increased from 2.2% of products in untreated to 9.39% in 200ºC for 15 minutes pretreatment in switchgrass. On the other hand, changes in product patterns in corn stover were less pronounced with only positive correlations between ethanol proportion and pretreatment severity (Pearson correlation coefficient = 0.57). However, the extent of increase in ethanol proportion with the severity of pretreatment was lower for switchgrass (ethanol proportion increased from 8.86% with untreated corn stover to 11.18% in the 200ºC for 15 minutes pretreatment) (Table 4-3).

Table 4-1. Composition of untreated and hydrothermally pretreated corn stover and switchgrass[a]

Substrate	Pretreatment		R_0	Glucan (%)	Xylan (%)	Lignin (%)	Dry wt Loss from pretreatment (%)
	Temperature (^oC)	Hold Time (min)					
Corn stover	Untreated			45.70 ± 0.58	36.56 ± 1.21	17.80 ± 0.44	0.00
	180	5	3.05	52.47 ± 0.99	12.82 ± 0.30	31.04 ± 1.93	58.92
	180	10	3.36	51.20 ± 1.64	11.42 ± 0.41	34.27 ± 0.61	56.94
	180	15	3.53	50.87 ± 1.76	10.00 ± 0.44	35.95 ± 1.17	55.47
	190	5	3.35	56.32 ± 0.20	8.16 ± 0.10	34.11 ± 0.29	51.62
	190	10	3.65	58.54 ± 0.59	6.58 ± 0.02	33.87 ± 0.75	48.03
	190	15	3.83	63.40 ± 0.84	5.89 ± 0.32	29.41 ± 1.65	38.35
	200	5	3.64	63.13 ± 0.78	4.34 ± 0.27	30.99 ± 0.32	25.78
	200	10	3.94	58.53 ± 0.80	2.48 ± 0.08	36.14 ± 0.53	34.90
	200	15	4.12	59.61 ± 0.11	2.45 ± 0.11	37.09 ± 0.26	33.01
Switchgrass	Untreated			40.10 ± 0.72	36.10 ± 0.49	23.80 ± 2.46	0.00
	180	5	3.05	51.34 ± 0.26	21.37 ± 0.15	25.15 ± 0.11	28.34
	180	10	3.36	53.57 ± 0.35	18.53 ± 0.38	26.28 ± 0.47	29.82
	180	15	3.53	56.55 ± 0.11	14.44 ± 0.17	27.40 ± 0.21	30.94
	190	5	3.35	58.96 ± 0.18	11.63 ± 0.10	28.16 ± 0.36	40.58
	190	10	3.65	61.52 ± 0.22	7.84 ± 0.12	29.17 ± 0.33	42.72
	190	15	3.83	61.23 ± 0.41	4.59 ± 0.10	30.49 ± 0.13	38.08
	200	5	3.64	61.36 ± 0.24	3.61 ± 0.15	31.08 ± 0.37	39.36
	200	10	3.94	61.20 ± 0.86	2.02 ± 0.09	33.27 ± 1.35	38.18
	200	15	4.12	61.87 ± 0.07	2.55 ± 0.11	34.66 ± 0.23	38.22

[a]All values are the mean of three replicates ± one standard deviation

Table 4-2. Initial and adjusted dry weight (DW) losses and resulting improvements by strain C1A.

Substrate	Pretreatment		R_0	Initial DW loss (%)	Adj DW loss (%)	Improvement (%)	Total Products (g)	Adj Products (g)	Improvement (%)
	Temperature (^oC)	Hold Time (min)							
Corn stover	Untreated			23.60	23.60	-	0.396 ± 0.072	0.396	-
	180	5	3.05	34.79*	14.29*	-9.31	0.409 ± 0.057	0.168	-57.61
	180	10	3.36	32.97*	14.20*	-9.40	0.443 ± 0.026	0.191	-51.83
	180	15	3.53	31.99*	14.25*	-9.35	0.421 ± 0.050	0.187	-52.65
	190	5	3.35	36.57*	17.69*	-5.91	0.487 ± 0.064	0.236	-40.47
	190	10	3.65	35.30*	18.35*	-5.25	0.495 ± 0.053	0.257	-34.98
	190	15	3.83	37.98*	23.41	-0.19	0.544 ± 0.022	0.335	-15.33
	200	5	3.64	37.99*	28.20*	4.60	0.502 ± 0.046	0.373	-5.87
	200	10	3.94	33.15*	21.58	-2.02	0.513 ± 0.012	0.334	-15.71
	200	15	4.12	27.19	18.21	-5.39	0.457 ± 0.107	0.306	-22.65
Switchgrass	Untreated			24.69	24.69	-	0.221 ± 0.003	0.221	-
	180	5	3.05	25.44	18.23*	-6.46	0.289 ± 0.016	0.207	-5.93
	180	10	3.36	25.11	17.62*	-7.07	0.297 ± 0.013	0.208	-5.34
	180	15	3.53	26.60	18.37*	-6.32	0.313 ± 0.015	0.216	-1.70
	190	5	3.35	25.02	14.87*	-9.82	0.321 ± 0.020	0.191	-13.36
	190	10	3.65	28.18	16.14*	-8.55	0.333 ± 0.034	0.191	-13.31
	190	15	3.83	27.22	16.86*	-7.83	0.345 ± 0.019	0.213	-3.02
	200	5	3.64	30.95	18.77*	-5.92	0.373 ± 0.039	0.226	2.72
	200	10	3.94	27.35	16.91	-7.78	0.353 ± 0.024	0.218	-0.74
	200	15	4.12	29.51*	18.23*	-6.46	0.340 ± 0.025	0.210	-4.54

*: Denotes significant difference (Student T-test p-value ≤0.01) between the pretreatment dry wt loss compared to the untreated plant material.

Table 4-3. Ratio of acids and alcohols produced by strain C1A.

Substrate	Pretreatment		Percentage of total products			
	Temperature (°C)	Hold Time (min)	Lactate	Formate	Acetate	Ethanol
Switchgrass	Untreated		19.49 ± 0.39	29.74 ± 0.17	48.54 ± 0.39	2.22 ± 0.06
	180	5	27.21 ± 0.49	27.93 ± 0.29	38.75 ± 0.30	6.11 ± 0.23
	180	10	29.75 ± 0.45	25.85 ± 0.17	36.25 ± 0.19	8.15 ± 0.37
	180	15	23.63 ± 0.19	28.98 ± 0.28	39.76 ± 0.38	7.63 ± 0.12
	190	5	32.59 ± 0.27	25.23 ± 0.29	34.54 ± 0.31	7.64 ± 0.25
	190	10	33.29 ± 0.54	26.12 ± 0.47	33.25 ± 0.57	7.34 ± 0.15
	190	15	33.33 ± 0.41	25.70 ± 0.28	32.23 ± 0.38	8.73 ± 0.15
	200	5	36.20 ± 0.41	24.32 ± 0.52	30.85 ± 0.52	8.63 ± 0.53
	200	10	35.64 ± 1.38	24.86 ± 0.38	31.53 ± 0.30	7.97 ± 0.28
	200	15	27.32 ± 0.53	28.18 ± 0.34	35.11 ± 0.38	9.39 ± 0.06
Corn stover	Untreated		31.19 ± 1.12	22.37 ± 0.47	37.46 ± 1.50	8.97 ± 0.51
	180	5	36.52 ± 1.06	21.07 ± 0.33	32.86 ± 1.15	9.55 ± 0.44
	180	10	35.47 ± 0.25	20.09 ± 0.23	34.24 ± 0.62	10.20 ± 0.21
	180	15	35.62 ± 0.33	20.41 ± 0.48	33.49 ± 1.31	10.47 ± 0.41
	190	5	43.62 ± 1.07	18.40 ± 0.47	28.82 ± 1.37	9.16 ± 0.50
	190	10	40.66 ± 1.40	19.87 ± 0.12	29.75 ± 0.93	9.72 ± 0.20
	190	15	43.82 ± 0.45	18.30 ± 0.16	28.50 ± 0.39	9.38 ± 0.12
	200	5	41.33 ± 1.02	20.45 ± 0.14	29.02 ± 0.81	9.20 ± 0.41
	200	10	37.57 ± 0.33	20.21 ± 0.13	31.53 ± 0.27	10.69 ± 0.31
	200	15	31.16 ± 1.53	22.01 ± 0.80	35.56 ± 2.30	11.27 ± 0.73

Discussion

In this work, we evaluated the utility of hydrothermal pretreatment for improving corn stover and switchgrass degradation and its impact on product formation patterns by the anaerobic fungus *Orpinomyces* sp. strain C1A. Our results indicate that strain C1A is capable of metabolizing untreated as well as hydrothermolysis-treated corn stover and switchgrass. Pretreated corn stover and switchgrass were more amenable to degradation than untreated plant materials. However, the improvements do not offset the loss of biomass weight resulting from the pretreatment process. Finally, pretreatment was associated with a shift in end product formation pattern, resulting in an increase of the proportion of ethanol and lactate and a decrease in the proportion of acetate and formate in pretreated samples compared to untreated controls.

Anaerobic fungi possess many unique properties that could theoretically alleviate the requirement for pretreatments. In addition, hydrothermolysis pretreatments could lead to results that minimize the efficacy of plant biomass degradation by anaerobic fungi. For example, hydrothermolysis results in the loss of water-soluble substrates and insoluble polymers, e.g. hemicellulose, that could readily be depolymerized and converted to sugar monomers by anaerobic fungi. Further, the high temperature and pressure employed in the process could lead to the formation of soluble monomeric sugar degradation products (e.g. furfural, hydroxymethyl furfural) and creation of acids (levulinic, formic), of which the impact on anaerobic fungal growth is uncertain. Further degradation of these compounds can also result in the formation of insoluble carbon-enriched substances, termed char or pseudolignin, which could impact the access of anaerobic fungi to cellulose and hemicellulose (9). Given the above factors, a critical evaluation of the

impact of pretreatment on biomass degradation by anaerobic fungi is warranted. To our knowledge, only one brief paper examined the effect of alkaline pretreatment on plant biomass degradation by anaerobic fungi (19), and no reports on the effect of hydrothermolysis pretreatment on anaerobic fungal degradation are available.

Strain C1A was able to utilize 24.69% and 23.60% (32.0% and 28.7% non-lignin fraction) of untreated switchgrass and corn stover, respectively (Table 4-2). Pretreated plant materials were more amenable to degradation than untreated material, with dry weight losses of 30.95% and 37.99% achieved for switchgrass (200°C for 5 min) and corn stover (190°C for 15 min), respectively (Table 4-2). However, biomass losses occurring during pretreatments were considerable, with up to 42.7% and 58.9% of starting biomass lost in the hydrothermolysis pretreatment of switchgrass and corn stover respectively (Table 4-1). These losses are mainly due to hemicellulose loss, as well as loss of various soluble components and chemical moieties removed during hydrothermolysis. When substrate losses from pretreatment are taken into account, it becomes clear that hydrothermolysis pretreatment achieves no significant improvement in biomass loss compared to untreated controls (Table 4-2). Our results indicate that the observed negligible to negative benefits of pretreatment are mainly due to two factors: 1) The considerable amount of plant biomass degradation already realized in untreated controls; and 2) The fact that hydrothermolysis removes hemicellulose, a substrate that is readily utilizable by strain C1A. Therefore, while the inability of purified enzyme preparations to attack untreated biomass, and the prevalent sole dependence on cellulases in enzymatic-based plant biomass degradation schemes renders pretreatments (e.g. hydrothermolysis) absolutely necessary; the metabolic and growth characteristics of

anaerobic fungi limits or even nullifies the benefits realized from hydrothermolysis pretreatment of plant biomass (Table 4-3).

Interestingly, pretreatment was associated with a shift towards higher proportions of ethanol and lactate and a lower proportion of acetate as products of switchgrass metabolism by strain C1A when compared to untreated controls. Strain C1A is an efficient metabolizer of hemicellulose, and the process involves removal of acetyl moieties from xylan backbone hemicellulose using acetyl xylan esterase enzymes, with the produced acetate accumulating in the medium. Hydrothemolysis pretreatment removes a large proportion of the hemicellulose fraction of plant biomass, hence reducing the amount of acetate released during C1A degradation of pretreated biomass. It is also plausible that the observed shift is driven by changes in the proportion of pyruvate, produced from sugar degradation in C1A, allocated to cytosolic and hydrogenosomal metabolism. Prior biochemical (5), and genomic (26) studies have demonstrated that pyruvate metabolism in anaerobic fungi is a complex process that occurs both in the cytosol as well as in the fungal hydrogenosome. Cytosololic pyruvate metabolism results mostly in the formation of ethanol and lactate, while hydrogenosomal pyruvate metabolism results in the formation of acetate, formate, and succinate (1, 2). The increase in the proportion of ethanol and lactate and the decrease in the proportion of acetate produced due to pretreatment indicate that a higher proportion of produced pyruvate is channeled through cytosolic metabolism. The rationale for such a shift is not clear but could imply a reduced requirement for regeneration of reduced electron carriers, which are partly mediated through proton reduction to hydrogen within the hydrogenosome using hydrogenase enzymes.

In conclusion, this study provides a critical evaluation of the utility of a commonly utilized approach for improving biomass degradation. To identify the optimum strategy for employing anaerobic fungi for biofuel production, similar evaluations of other pretreatment approaches are needed, as well as efforts to improve alcohol production and tolerance via physiological and genetic manipulations.

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Conclusions

The characteristics of anaerobic fungi as described in this dissertation make them promising candidates for enhancing the saccharification of lignocellulose in the subsequent production of liquid biofuels. They are capable of consolidating the pretreatment and saccharification steps needed for untreated lignocellulose, demonstrated for strain C1A on the energy crops of switchgrass, alfalfa, bermuda grass, corn stover, forage sorghum, and energy cane. This is achieved by strain C1A through invasive, filamentous polycentric growth and utilization of both the cellulose and hemicellulose fractions to both acids and alcohols. Further, our results showed that pretreatment of the substrate enhanced degradation by strain C1A but that it also removed a substantial portion of potentially fermentable substrates and increased the energy demands of the process. When the losses and needs of pretreatment were considered, it was found that there was no longer any improvement gained from pretreating the biomass.

A major drawback in the direct use of strain C1A, and all other anaerobic fungi isolated to date, for generating biofuels is the low ratio of ethanol to acid fermentation products. When grown on pretreated lignocellulosic biomass, the ratio of ethanol produced by strain C1A was increased. However, this did not translate to industrially relevant amounts and could not alone justify the use of biomass pretreatment in AF degradation schemes. To overcome this product limitation and render them more ideal candidates for the consolidated production of bioethanol, future efforts should target improvements in alcohol production and tolerance via physiological and genetic manipulations.

Successful growth of anaerobic fungi on lignocellulosic biomass occurs optimally at steady mesophilic temperatures (optimal of 39^{o}C) and under consistently anaerobic conditions. Each of these characteristics could also be exploited to enhance part of the current scheme of biofuel production. They provide two separate means of control over the growth of strain C1A. To cease unwanted growth and utilization of produced sugars to less desirable acid products, aeration and/or temperature elevations would allow for continual, and perhaps increased, enzymatic processing of the biomass. Combined with subsequent fermentation using a dedicated homofermentative microorganism provides an attractive alternative to the costs and consequences of chemical/physical pretreatment of lignocellulose and the excessive application of exogenous enzymatic cocktails. Overall, the discovery and application of strain C1A provides several means of improving the productions of liquid biofuels from lignocellulosic plant biomass.

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