Evaluating the utility of anaerobic fungi in cellulosic biofuel production schemes

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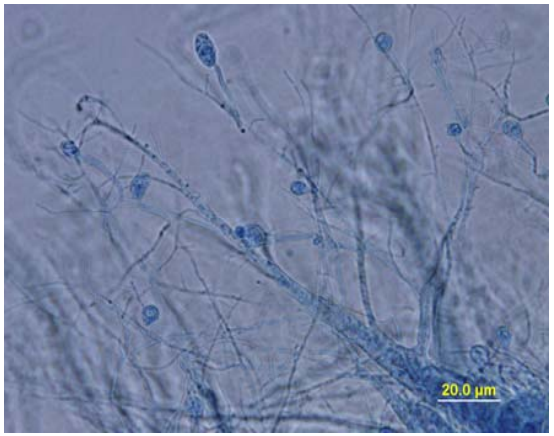
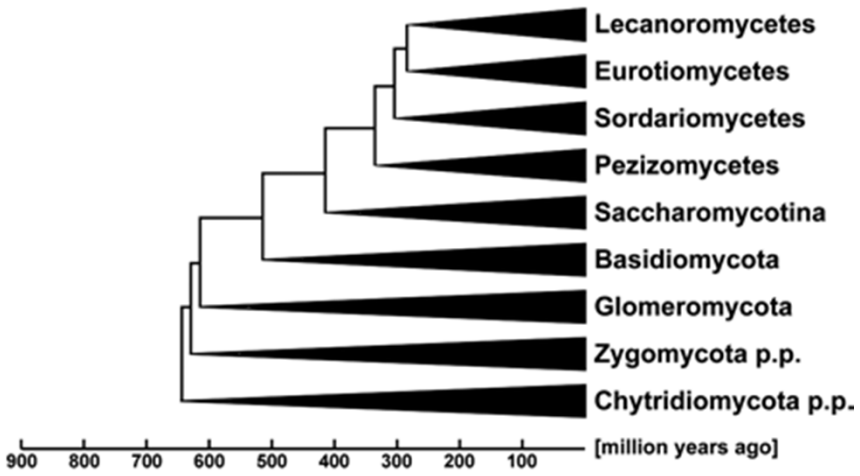
Anaerobic fungi: The neglected fungal phylum

- Separate fungal phylum (Neocallimastigomycota)
- Discovered in sheep in 1975*.
- Restricted habitat: Present in the gut, alimentary tract of many ruminants, herbivores.
- Strict anaerobes.
- Initial colonizers of ***fresh*** plant materials in cow rumen.
- Six Genera, 20 species currently described





*Orpin GC: Studies on the rumen flagellate Neocallymastix frontalis
J. Gen. Microbiol. 91:215-218

Information on anaerobic fungi is sparse after 38 years of their discovery

- Anaerobic and eukaryotic.
- Few interested research groups (Only a handful of laboratories are (semi)-active around the world).
- No high quality genome sequence available.
- No genetic system available.

Poorly researched……. but not insignificant

- Ecological Relevance:
  - Restricted habitat.
  - Scope of diversity not completely understood.
- Evolutionary relevance:
  - Basal fungal lineage.
  - Unique evolutionary trajectory.
- Physiological relevance:
  - Adaptation of fungi and microeukaryotes to strict anaerobiasis.
- Practical relevance:
  - Activity impacts animal host.
  - Potential utilization in biofuel production from plant biomass.

Research thrust: could anaerobic fungi be relevant in cellulosic biofuel production schemes?

I. Ecology/Microbiology

A. Diversity of anaerobic fungi in herbivores.
B. Isolation of robust strains.

II. Genomes/Transcriptomes

A. Sequencing strategies.
B. General genomic features.
C. Comparative genomics.
D. Hydrogenosomal structure.
E. Lignocellulolytic capability.

III. Metabolic potential

A. Growth on switch grass.
B. Effect of Pretreatments.
C. Versatility.

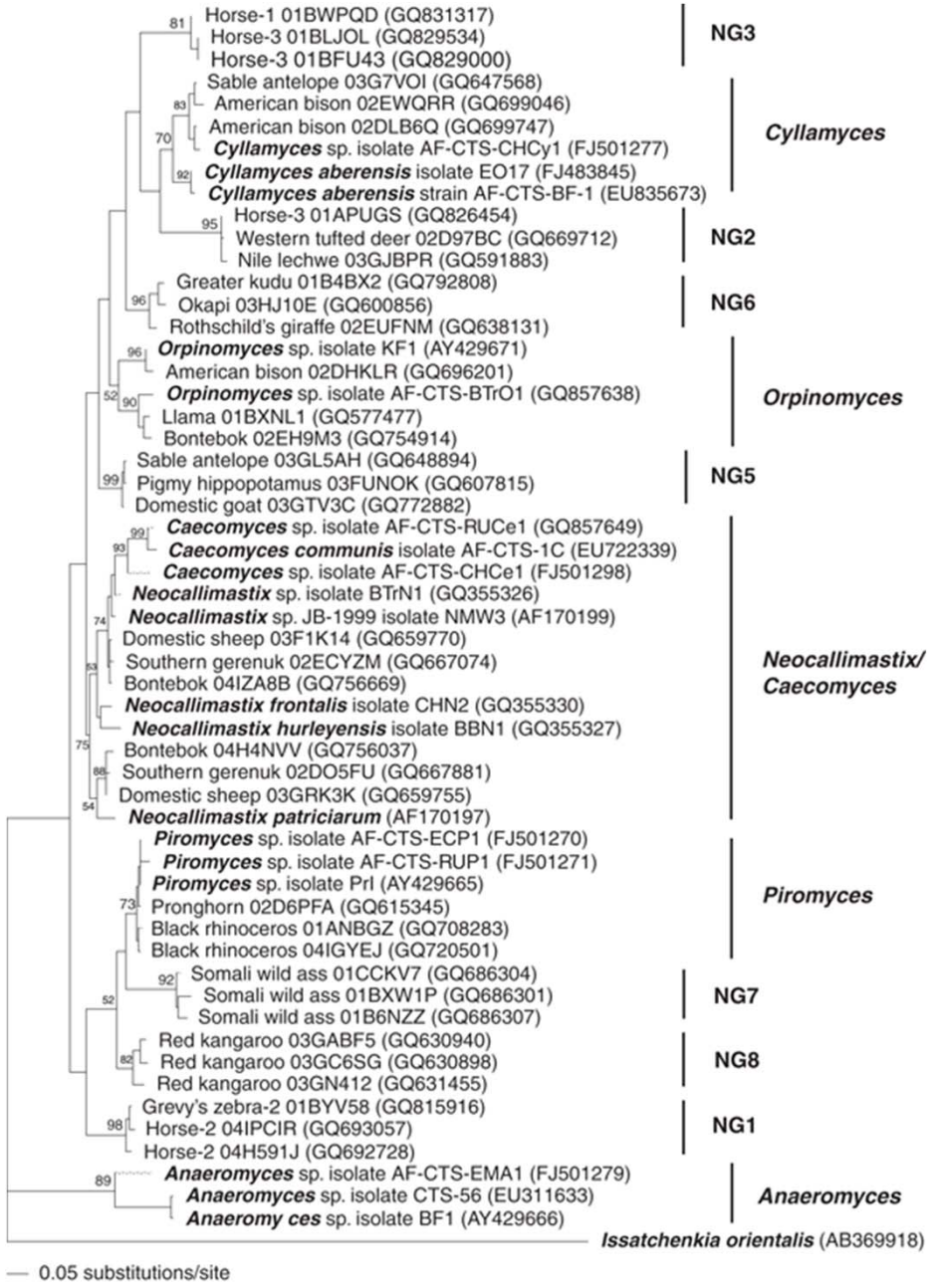
IV. Critical assessment

A. Bioconversion agents in pure culture.
B. Bioconversion agents in Co-culture.

I. Ecology of anaerobic gut fungi

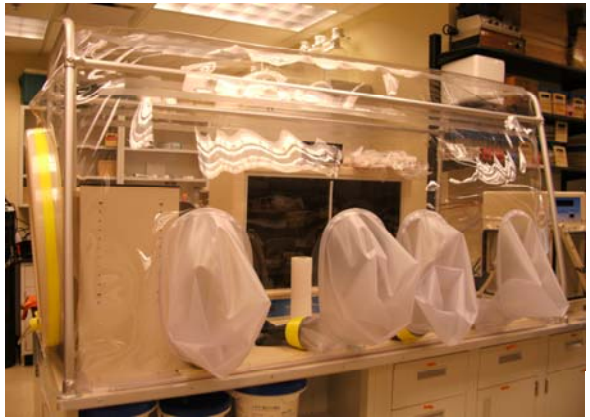
A. Diversity of anaerobic fungi in herbivores

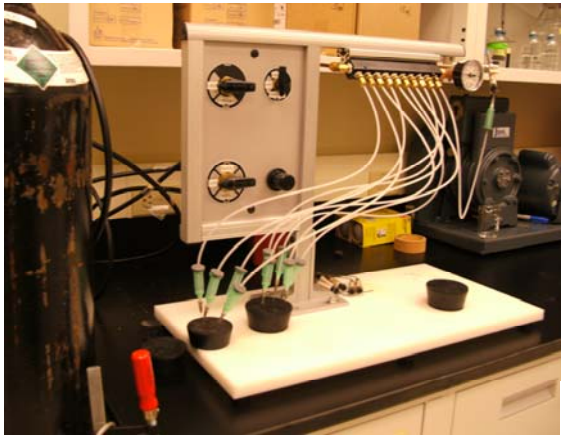
- Survey of anaerobic fungi in 30 different animals using Nuclear ITS spacer culture-independent analysis.
- Discovery of 8 novel putative genera.
- Variations in diversity and community structures between different animal hosts.
- Host species is the most important factor shaping the diversity and community structure of anaerobic fungi.



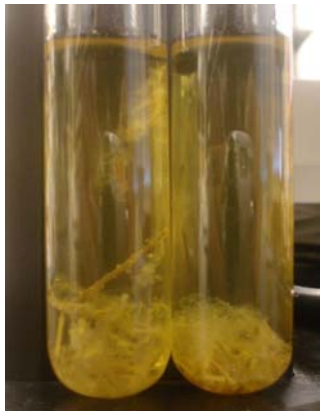
Liggenstoffer et al.2010. The ISME J. **4:**1225–1235

I. Ecology of anaerobic gut fungi
B. Isolation of anaerobic fungi on switch grass



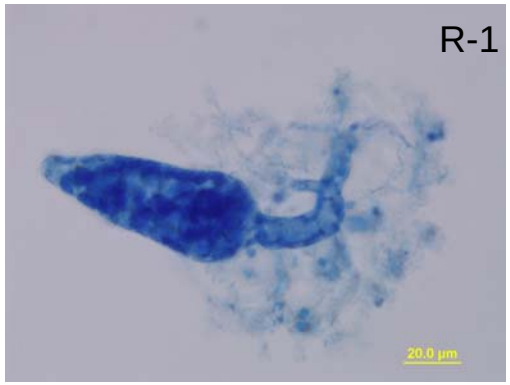


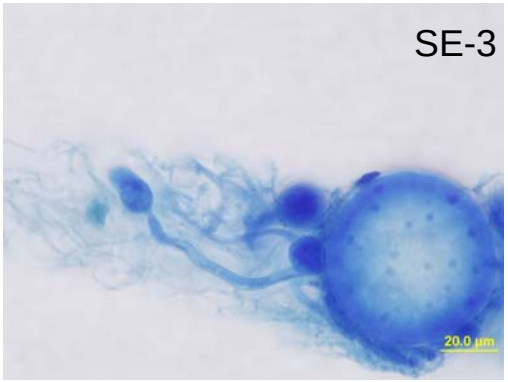
Modified anaerobic enrichment and isolation approaches

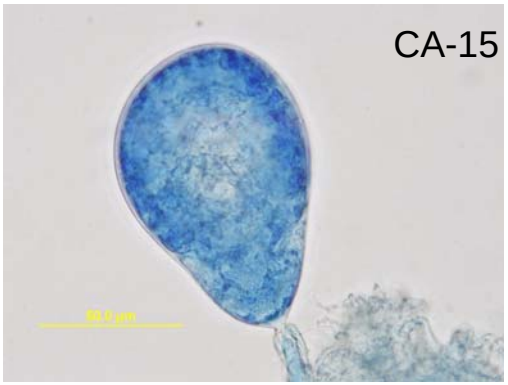


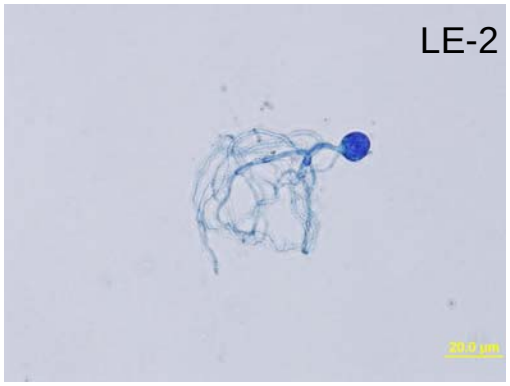
Isolation of anaerobic fungal strains

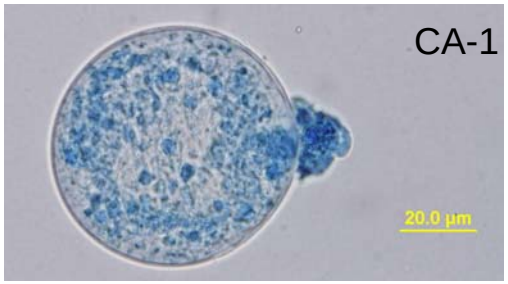
Anaerobic fungal isolates

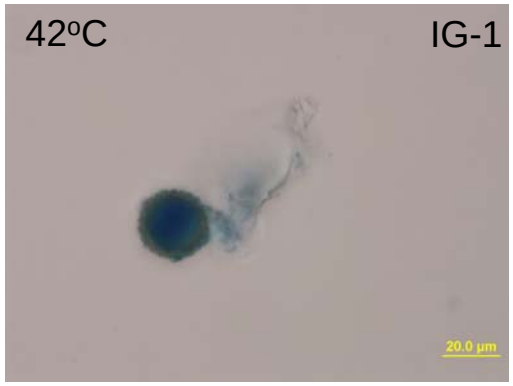


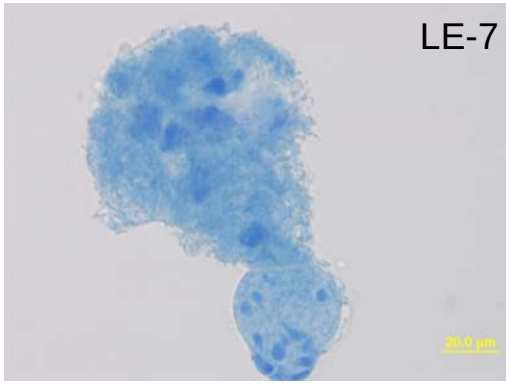


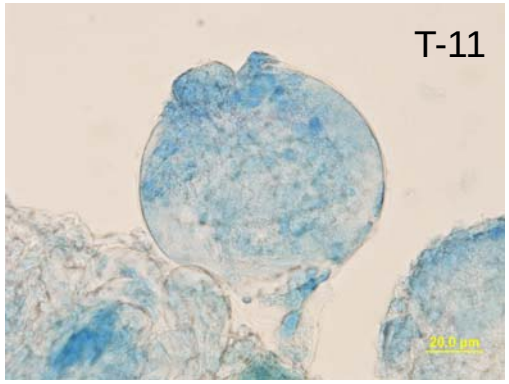


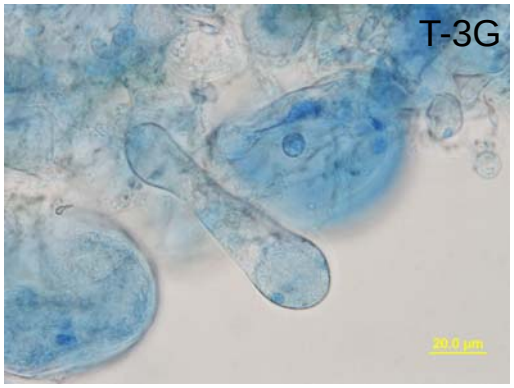






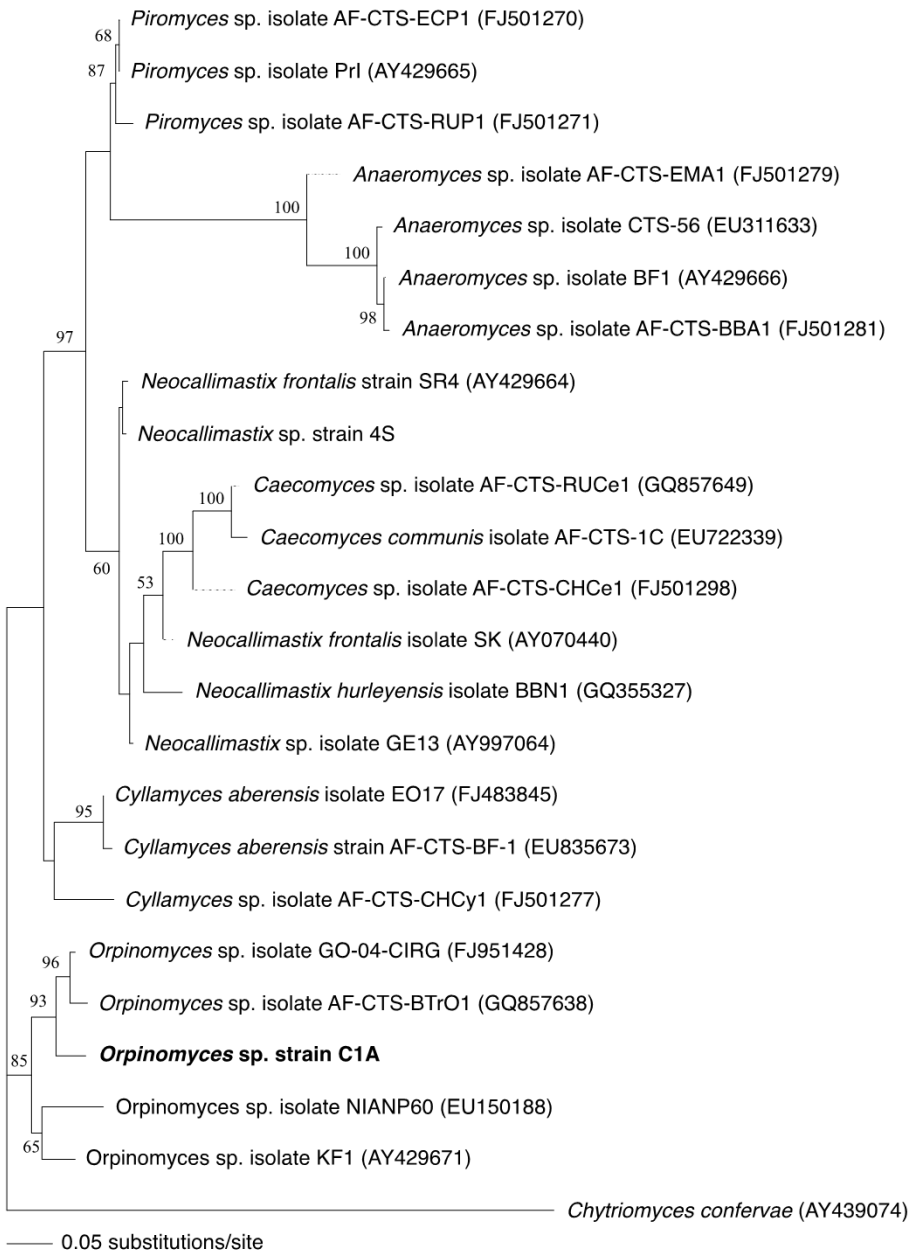




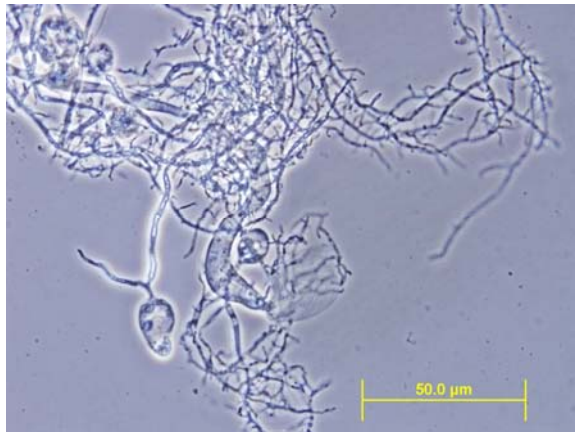


- Multiple monocentric and polycentric isolates obtained.
- Senescence occurred in the majority of isolates obtained.
- Senescence is an issue.

Orpinomyces sp. strain C1A



- Isolated from the feces of an Angus steer on a cellobiose-switchgrass medium.
- 312 transfers during the last three years.
- Polycentric growth.
- Nuclear ITS-2 analysis supports its placement as a member of the genus *Orpinomyces*.



II. Genome of *Orpinomyces* sp. strain C1A

A. Sequencing strategies

A. General genomic features.

A. Comparative genomics: Identification of unique genes/pathways within the C1A genome.

A. Hydrogenosomal structure.

A. Lignocellulolytic genes and transcriptional analysis.

A. C1A genome general genomic features

- CEGMA analysis estimate 94% genome coverage.
- Unique/interesting features in C1A genome:
  - GC content
  - Simple sequence repeats (SSRs)
  - % non coding regions
  - Genome size
  - Number of genes
  - Gene duplication

Genome size	100.95 MB
Number of Contigs	32,574
Protein Coding	20.60%
Non- coding intergenic	73.60%
Non-coding introns	5.10%
rRNA	0.67%
5.8S	183 (30,763 bp)
18S	272 (168,110 bp)
28S	366 (457,301 bp)
tRNA	0.06%
	770 (58,292 bp)
Number of Genes	16,347
Number of Genes with transcripts	14,009
Average Gene Length	1623
Number of Intron	35,697
Introns/gene	2.18
Average Intronlength	163
GC content	17.00%
Protein Coding	26.80%
Intergenic	14.80%
Intron	8.10%
SSR Repeats	4.90%
TE repeats	3.31%

E. Lignocellulolytic capabilities of strain C1A

- Anaerobic fungi are the initial colonizers of plant materials in the herbivorous guts.
- Earlier efforts demonstrated their ability to metabolize cellulose and hemicellulose.
- Multiple genes identified through cDNA library screenings.

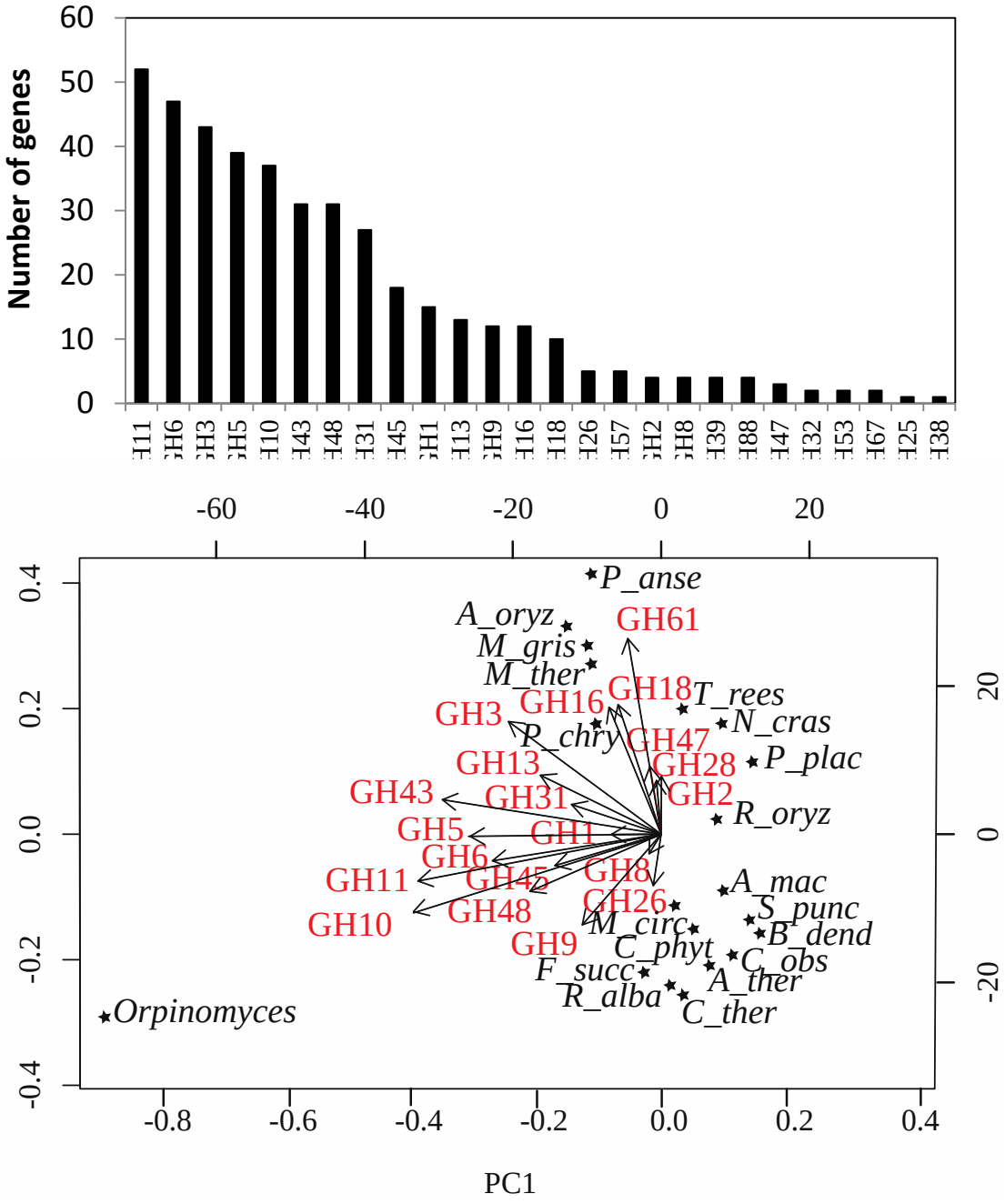
Carbohydrate active enzymes (CAZymes) genes in the C1A genome

-Highest number of Glycoside hydrolase, Carbohydrate esterase, in all sequenced fungal genomes so far.

Phylum	Species Name	GH	CE	PL
Neocallimastigomycota	***Orpinomyces* sp. strain C1A**	**358**	**92**	**24**
Ascomycetes	**Eurotiomycetes**			
	Aspergillus nidulans	259	33	22
	Aspergillus niger	254	26	8
	Aspergillus Oryzae	306	30	23
	Penicillium chrysogenum	223	22	9
	Sordariomycetes			
	Magnaporthe grisea	240	47	5
	Myceliophthora thermophila	215	29	8
	Thielavia terrestris	224	27	4
	Podospora anserina	236	41	7
	Neurospora crassa	197	22	4
	Nectria haematococca	329	44	*33*
	Trichoderma reesei	200	22	5
	Saccharomycetes			
	Saccharomyces cerevisiae	57	3	5
	Schizosaccharomycetes			
	Schizosaccharomyces pombe	54	5	0
	Cryptococcus neoformans	83	7	3
Basidiomycetes	**Agaricomycetes**			
	Laccaria bicolor	163	17	7
	Postia placenta	144	10	6
	Ustilaginomycetes			
	Ustilago maydis	102	1	18
Mucoromycotina	*Mucor circinelloides*	97		
	Rhizopus orizae	116	41	6
Blastocladiomycota	*Allomyces macrogynus*	95	19	4
Chytridiomycota	*Batrachochytrium dendrobatidis*	36	4	1
	Spizellomyces punctatus	54	14	1

Glycoside hydrolases in the C1A genome

- Unique GH composition
- Expansion of:
  - cellulolytic families GH6, GH9, GH45, GH48
  - hemicellulolytic families GH10, GH11, and GH43
- Reduction or absence of:
  - GH7, GH16, GH18, GH28, and GH61.



Cellulose metabolism in C1A genome

Activity	CAZY affiliation	No. of genes in C1A genome	Phylogenetic affiliation of closest relatives
Endoglucanase	GH45	20	E
	GH5	37	RB
	GH8	4	B, RB
	GH9	13	B, RB
Cellobiohydrolase	GH6	49	E
	GH48	32	B, RB

E: Eukaryotic
B: Bacteria
RB: Rumen Bacteria

Hemicellulose metabolism in C1A genome

Substrate	Activity	CAZY affiliation	No. of genes in C1A genome	Phylogenetic affiliation of closest relatives
Xylans (arabinoxylan, arabinoglucoronoxylan)	Endoxylanase	GH10	37	B, RB
		GH11	53	RB
		GH43	2	B, RB
	beta-xylosidase	GH43	9	RB
		GH39	4	RB
	Alpha-glucuronidase	GH67	2	B
	Alpha-N-arabinofuranosidase	GH43	18	B
	Acetyl Xylan esterase	NA	26	B
	Polysaccharide deacetylase	GH57	5	B
		GH5	1	B
		GH11	4	B
		Others	12	B
	Feruloyl esterase/ cinnamoyl esterase	NA	29	B
Mannans/ glucomannans	Mannanase, Mannosidase	GH5	4	B
		GH26	5	B
		GH2	3	B
Mixed linkage beta glucan	Lichenase	GH16	12	RB

E: Eukaryotic
B: Bacteria
RB: Rumen Bacteria

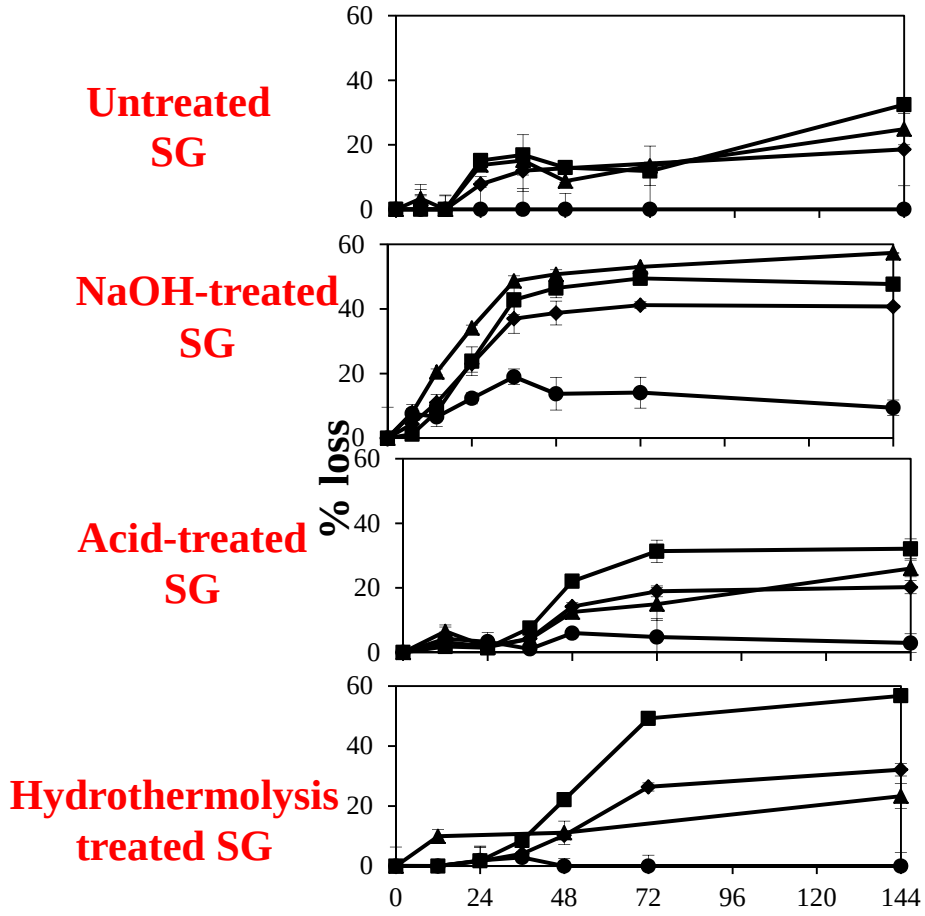
Role of HGT in shaping lignocellulolytic capabilities of strain C1A

- 247 (69.2%) of GH genes were most closely related to bacterial orthologs
- 141 (39.5%) of GH genes were most closely related to bacterial orthologs from lineages that are prevalent in the bovine rumen:
  - Families Lachnospiraceae, Clostridiaceae, Eubacteriaceae, and Ruminococcaceae within the order Clostridiales
  - Family Streptococcaceae within the order Bacillales,
  - Family Prevotellaceae within the order Bacteroidetes,
  - Phylum Fibrobacteres.

Strain C1A has evolved from an ancestor with a week cellulolytic capabilities to a robust cellulose and hemicellulose degrader by acquiring genes from bacterial donors through HGT.

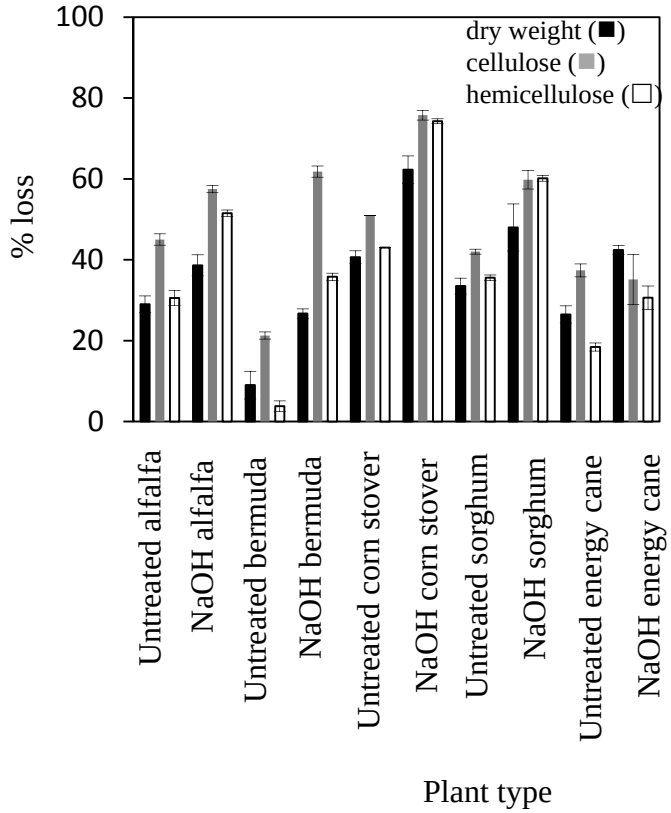
III. Metabolic analysis: Switchgrass degradation by strain C1A

- Strain C1A was capable of degrading untreated, acid-, alkaline-, and hydrothermolysis-treated switchgrass.
- Strain C1A performed extremely well in alkaline- and hydrothermolysis-treated switch grass.



Versatility of strain C1A

- Capable of degrading a wide range of bioenergy plants and crop residues:
  - AlfaAlfa
  - Bermuda grass
  - Sorghum
  - Energy Cane
  - Corn stover
- Exceptional capability in Corn stover:



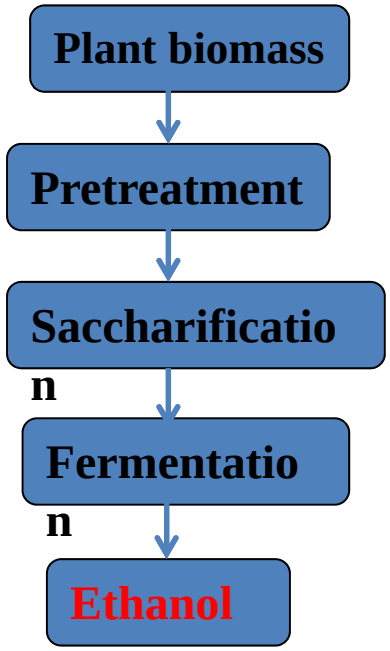
% loss	Untreated	NaOH treatment
Dry weight	40.9	61.7
Cellulose	50	61.5
Hemicellulose	37.5	75
Fermentable sugars	47.5	75.2

Patterns of products formed

Substrate	Grams per gram plant biomass consumed			
	Lactate	Formate	Acetate	Ethanol
Unt. Switchgrass	0.19	0.49	0.51	0.05
Acid Switchgrass	0.20	0.40	0.41	0.09
NaOH switchgrass	0.42	0.35	0.31	0.10
Hydrothermolysis switchgrass	0.34	0.35	0.29	0.09
Unt. Alfalfa	0.30	0.38	0.43	0.07
NaOH alfalfa	0.28	0.27	0.30	0.06
Unt. Bermuda	0.02	0.34	0.50	0.05
NaOH Bermuda	0.19	0.38	0.40	0.02
Unt. Corn Stover	0.48	0.27	0.28	0.06
NaOH Corn Stover	0.38	0.20	0.18	0.06
Unt. Sorghum	0.40	0.32	0.35	0.07
NaOH Sorghum	0.46	0.23	0.19	0.05
Unt. Energy Cane	0.45	0.49	0.58	0.08
NaOH Energy Cane	0.56	0.30	0.29	0.06

Although an extremely promising agent, the amount of ethanol produced is minimal

Anaerobic fungi for biofuel production



Current Schemes	Anaerobic Fungi
Crops (mostly corn)	Grasses, crop residues
Harsh, absolutely necessary	Mild, not absolutely necessary*
Purified enzymes	Anaerobic Fungi***
Fermentative microorganisms	Anaerobic fungi***
Major product	Minor product

Consolidated bioprocessing**

* Highly invasive: minimum pretreatment necessary.
** Capable of simultaneous saccharification and fermentation.
*** Metabolizes cellulose and hemicellulose to acids and alcohols.

Current efforts: Optimization of strain C1A for biofuel production

- ***Improving ethanol production:***
  - Genetic engineering: RNAi, *Agrobacterium*-mediated transformation
  - Co-culturing with a dedicated fermentor
- Optimizing plant biomass: fungal inoculum ratio.
- Improving ethanol tolerance.
- Evaluating air tolerance of strain C1A: Unique capability to grow after up to 9 hours of air exposure.
- Optimizing pretreatments.
- Testing capabilities in mixed prairies, grasses in marginal lands.
- Improving degradation through growth on solid surfaces.
- Working with OSU-TDP towards isolate and process patenting.

Conclusions: Anaerobic fungi display:

- **High phylogenetic diversity.**
- **Unique genomic features.**
- **Remarkable biomass degradation capabilities.**
- **Multiple processes previously unrecognized in the Mycota.**
- **Multiple adaptations to the herbivorous gut.**

Acknowledgments

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