Sugar Cane Biomass Structure and Prospection of new Microoganims Producing Cellulotic Enzymes Aiming Second Generation Ethanol Production

4th International Conference on Biofuels Standards:
Current Issues, Future Trends

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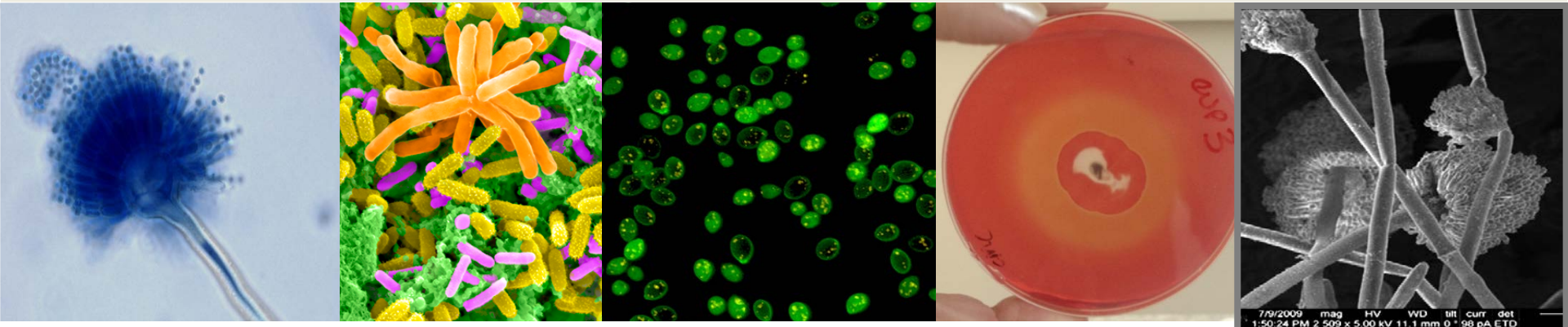
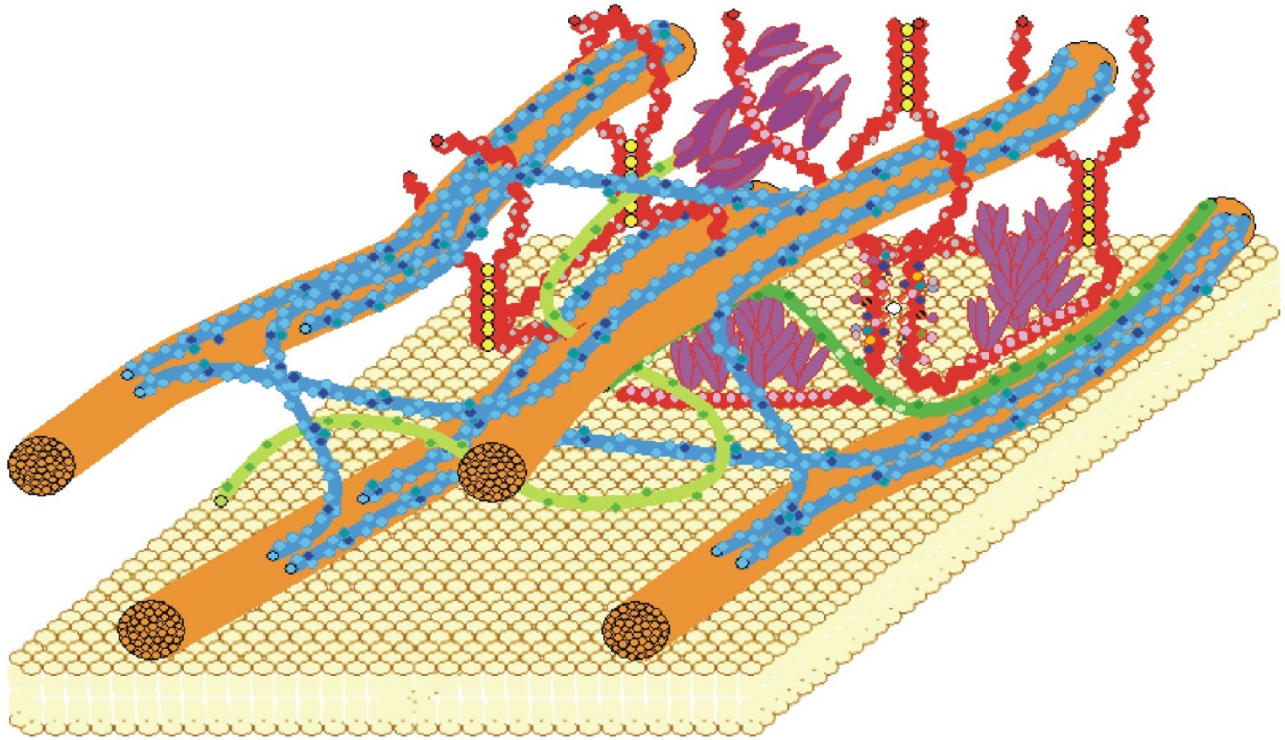


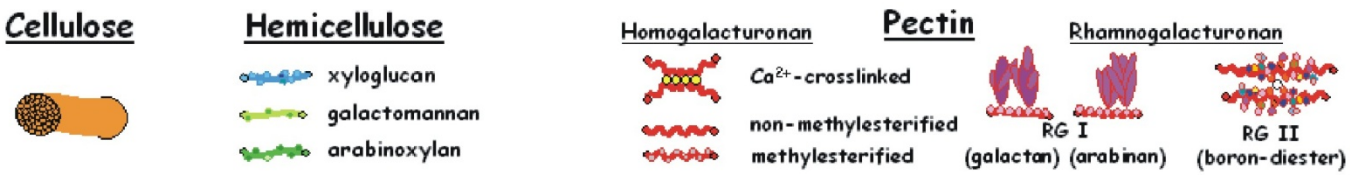
AIMS

- Better characterization of the sugar cane structure
- Isolation of microorganims from animal models (termites and snail *Achatina fulica*) that are able to degradate cellulose from sugar cane bagasse.
- Identification of fungal enzymes that play a key role in bioethanol production process.

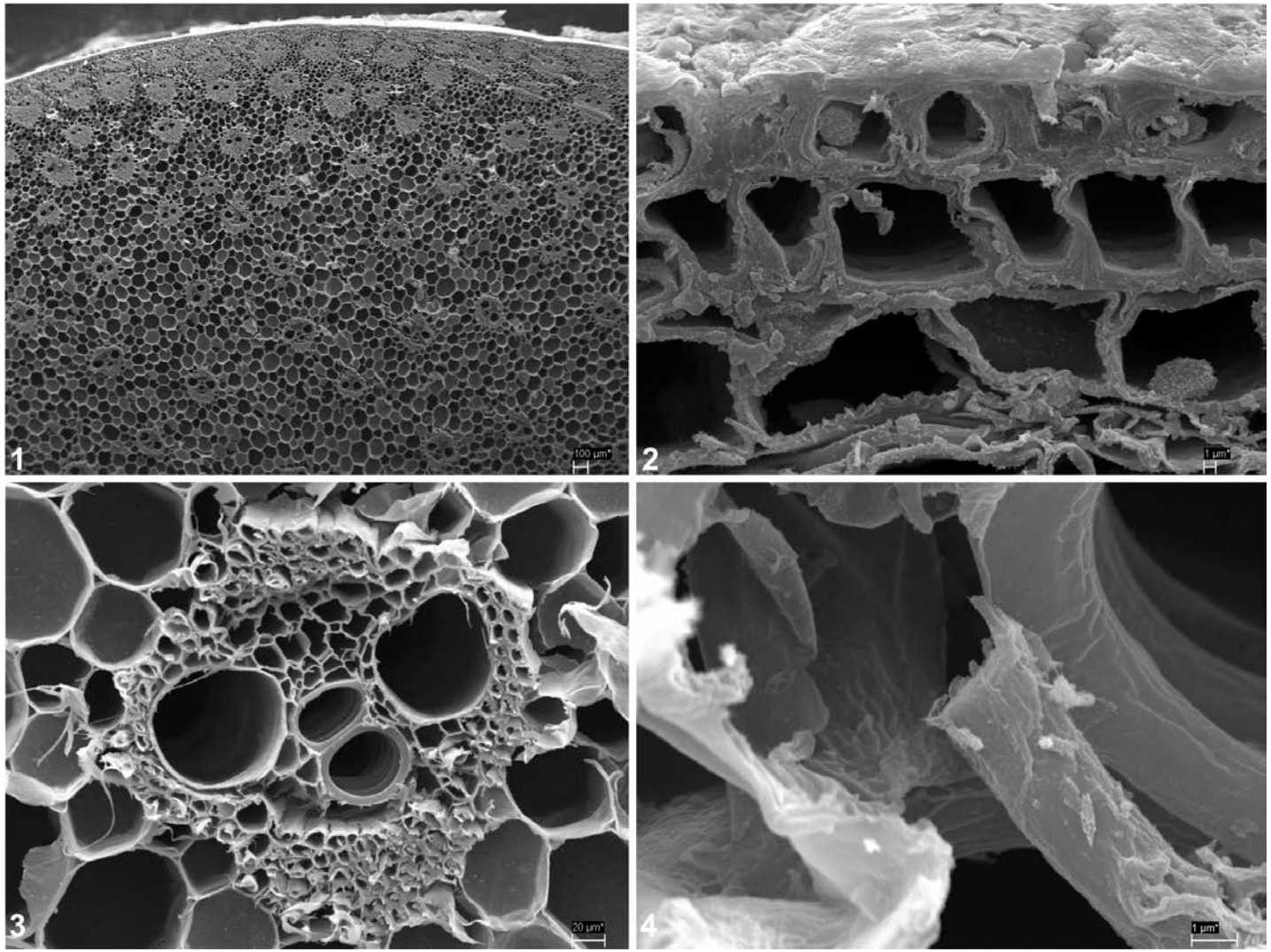
Baixaki

Cell walls are constructed of complex polysaccharides

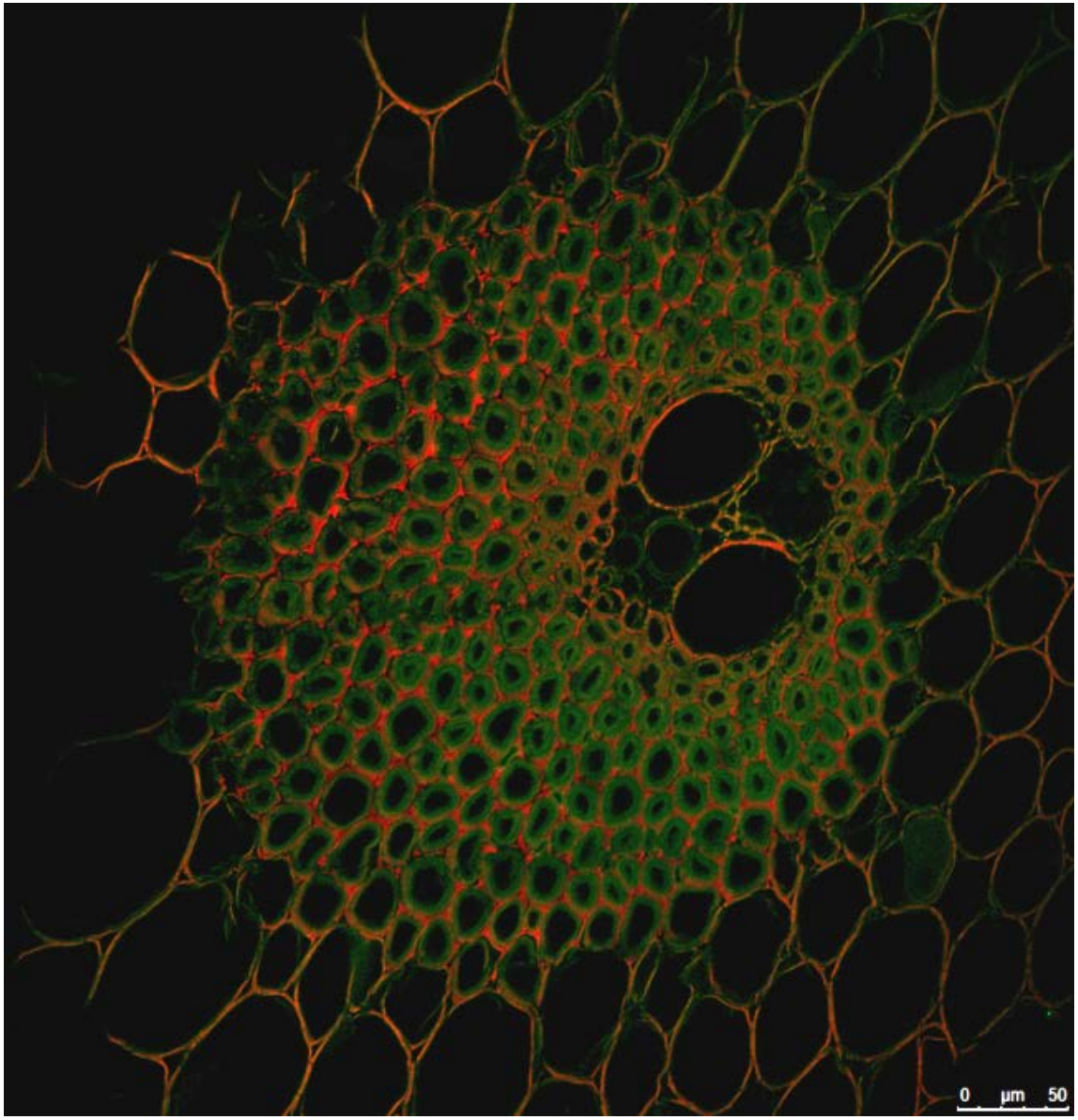


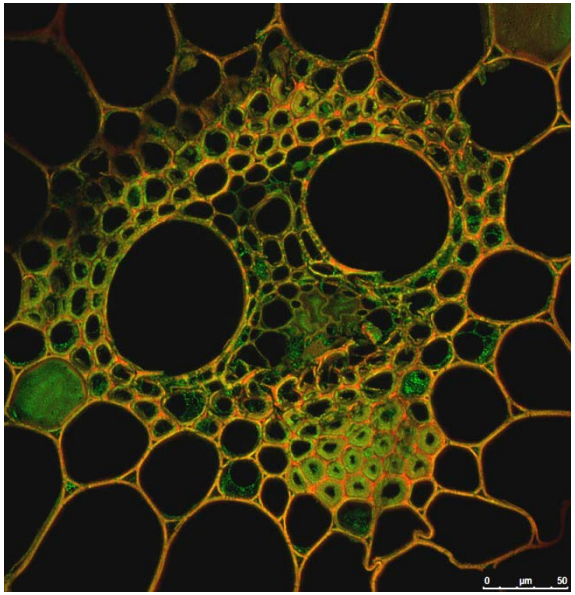


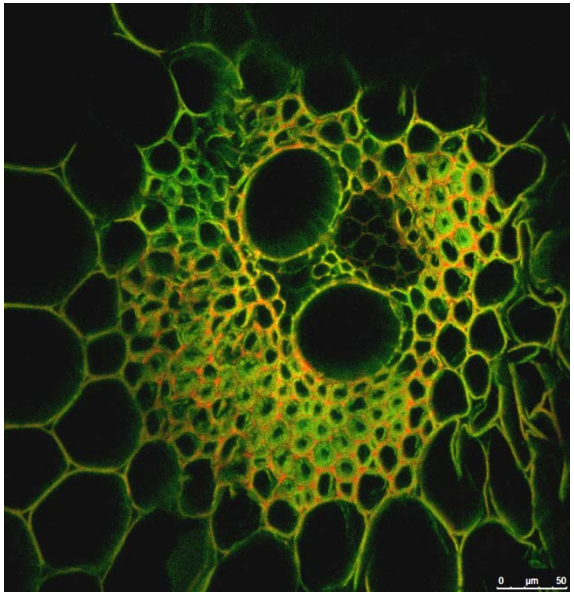
Scanning Electron Microscopy of sugar cane tissue



Microscopy of Localization of cellulose and lignin

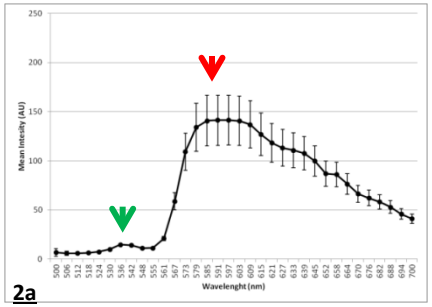


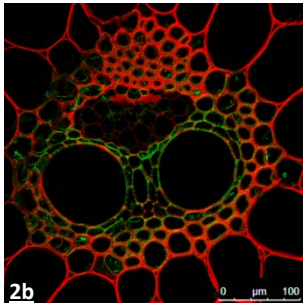


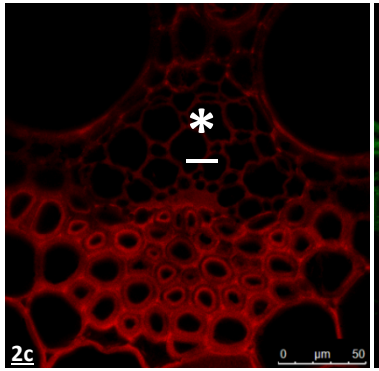


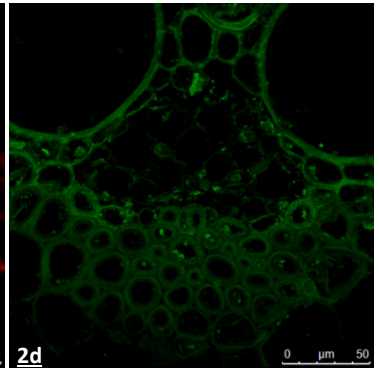
Microscopy Localization of Cellulose And Lignin in Sugar Cane

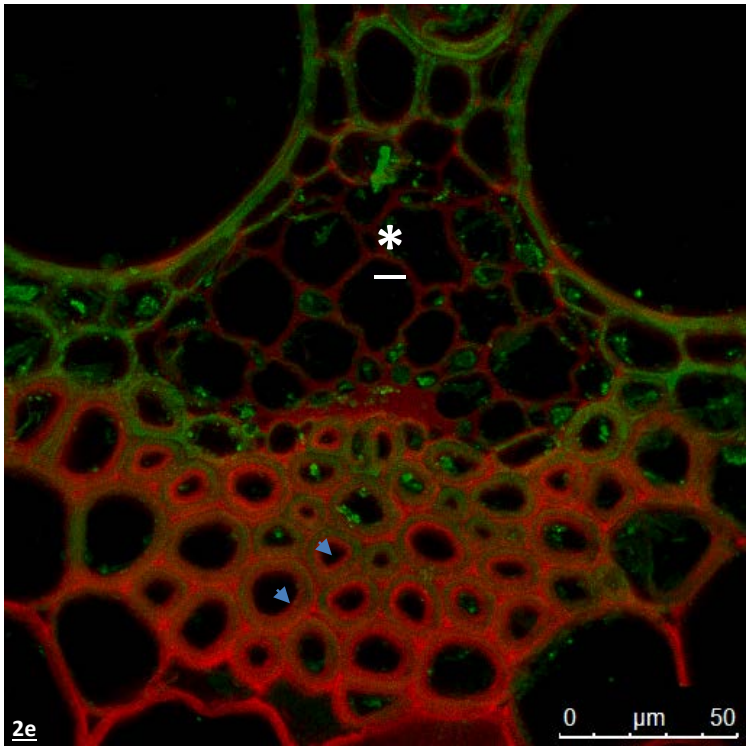
Green – Cellulose
Red - Lignin



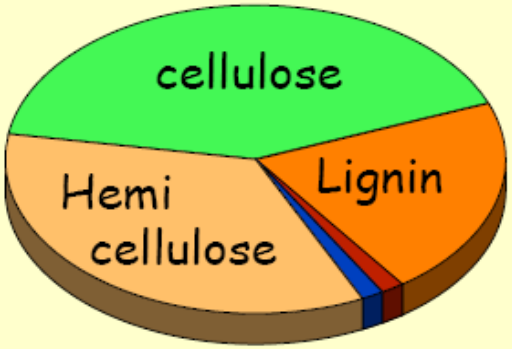






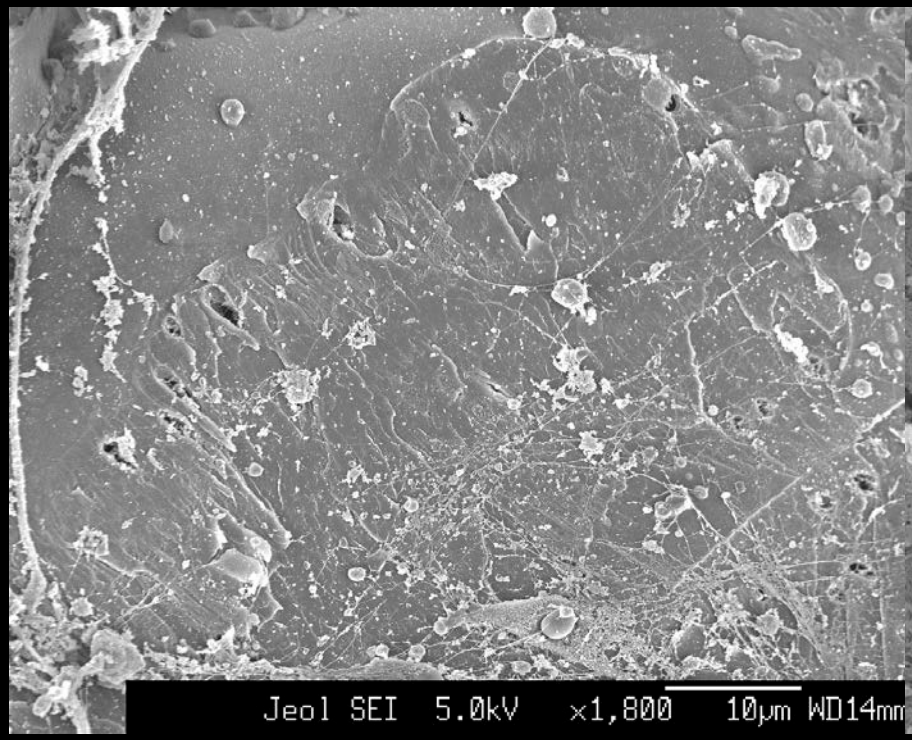


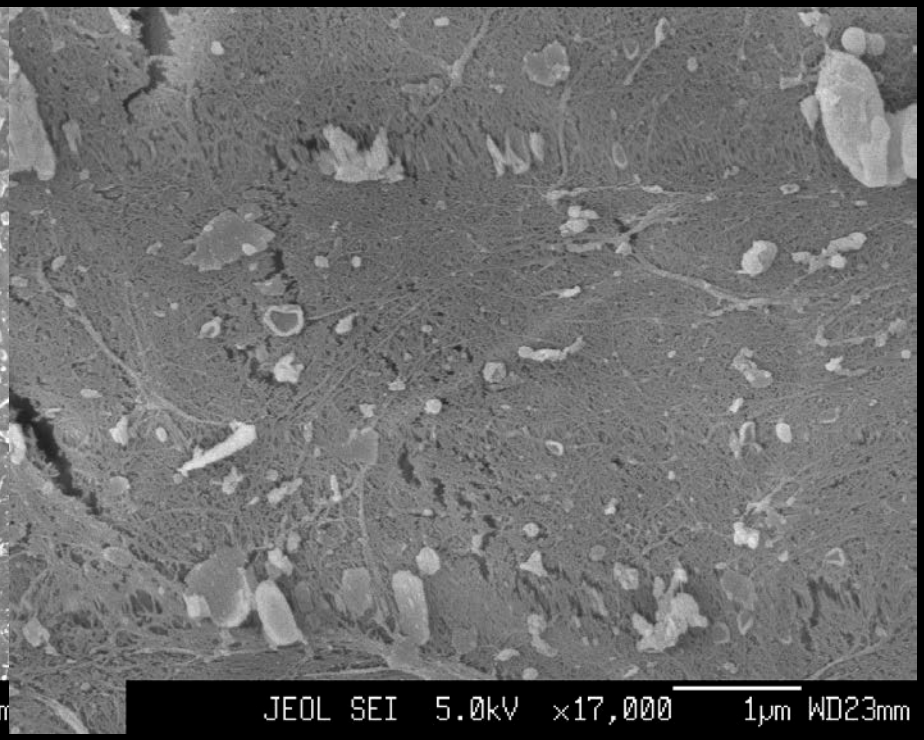
	BAGAÇO
CELULOSE %	46,6
HEMICELULOSE %	25,2
LIGNINA %	20,7

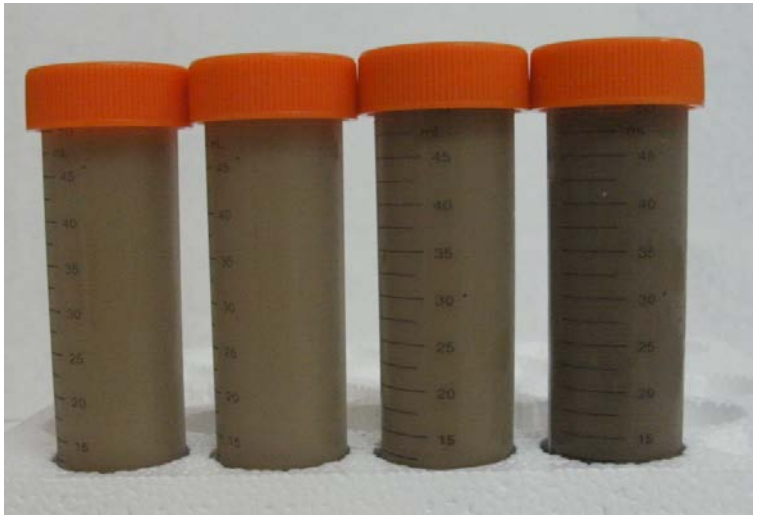


Typical grass composition

Fotos: Carlos Rossel, 2004.
D Vrije et al (2002) Int J Hydrogen Energy 27,1381


Jeol SEI 5.0kV ×1,800 10μm WD14mm

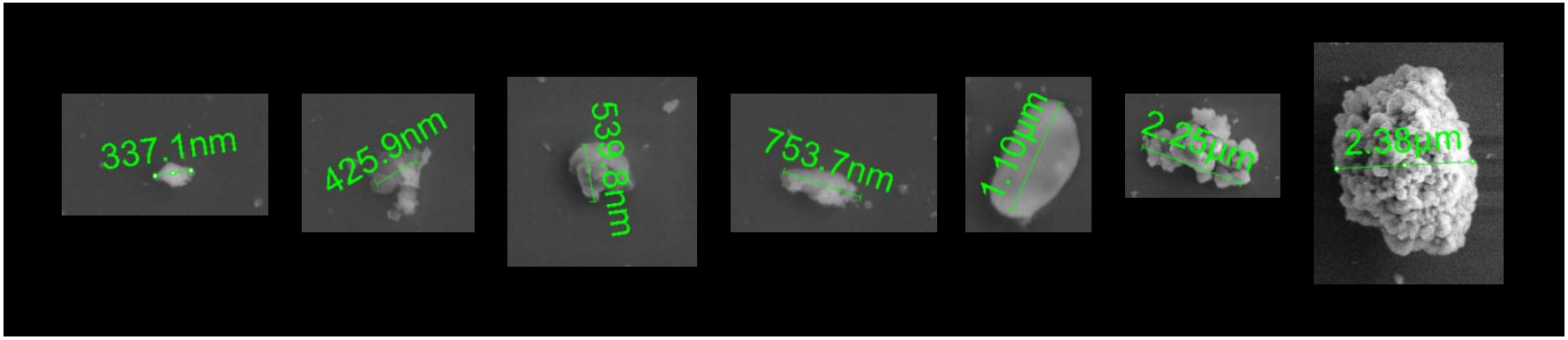

JEOL SEI 5.0kV ×17,000 1μm WD23mm



Powdered sugarcane bagasse

suitable for enzymatic assays of lignocellulosic enzymes

colloidal, micrometer range

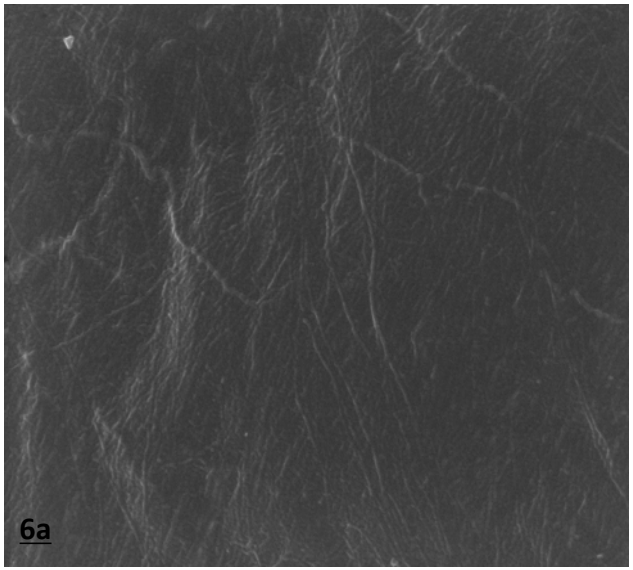


Effect of termochemical pretreatment on sugar cane structure

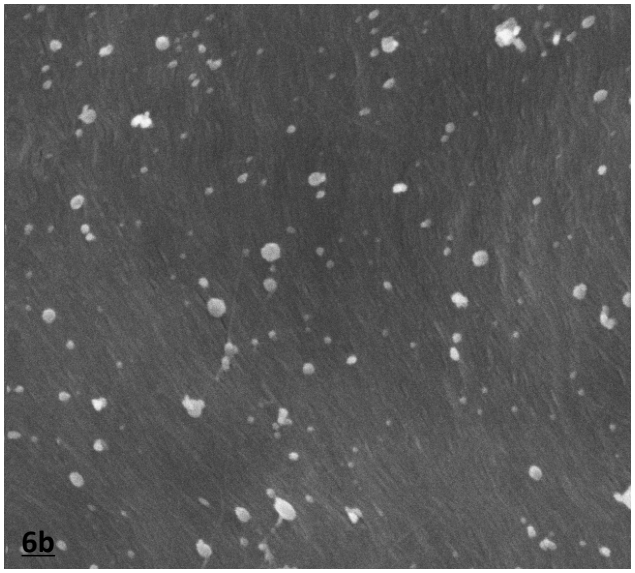
A - Control

B – Pré-treatment

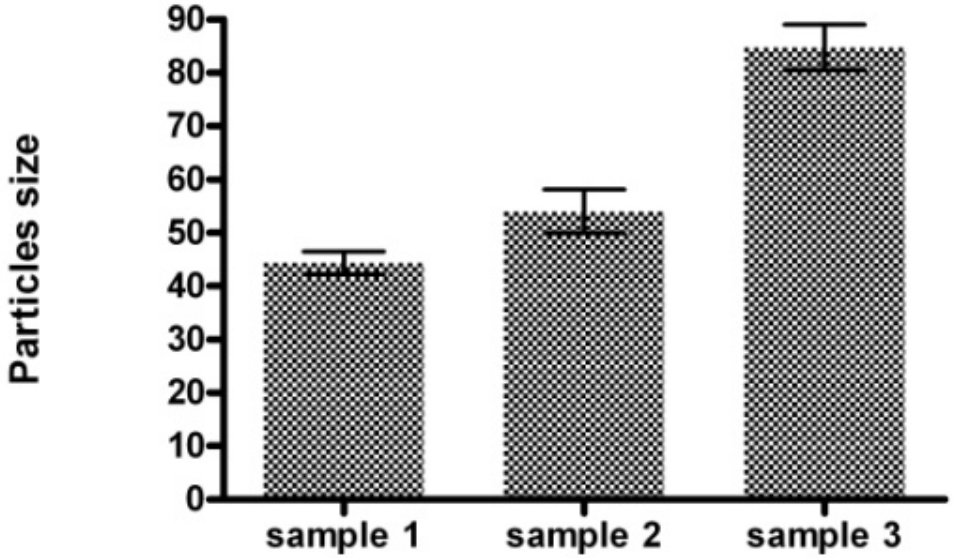
C – Particles Diameter



6a



6b



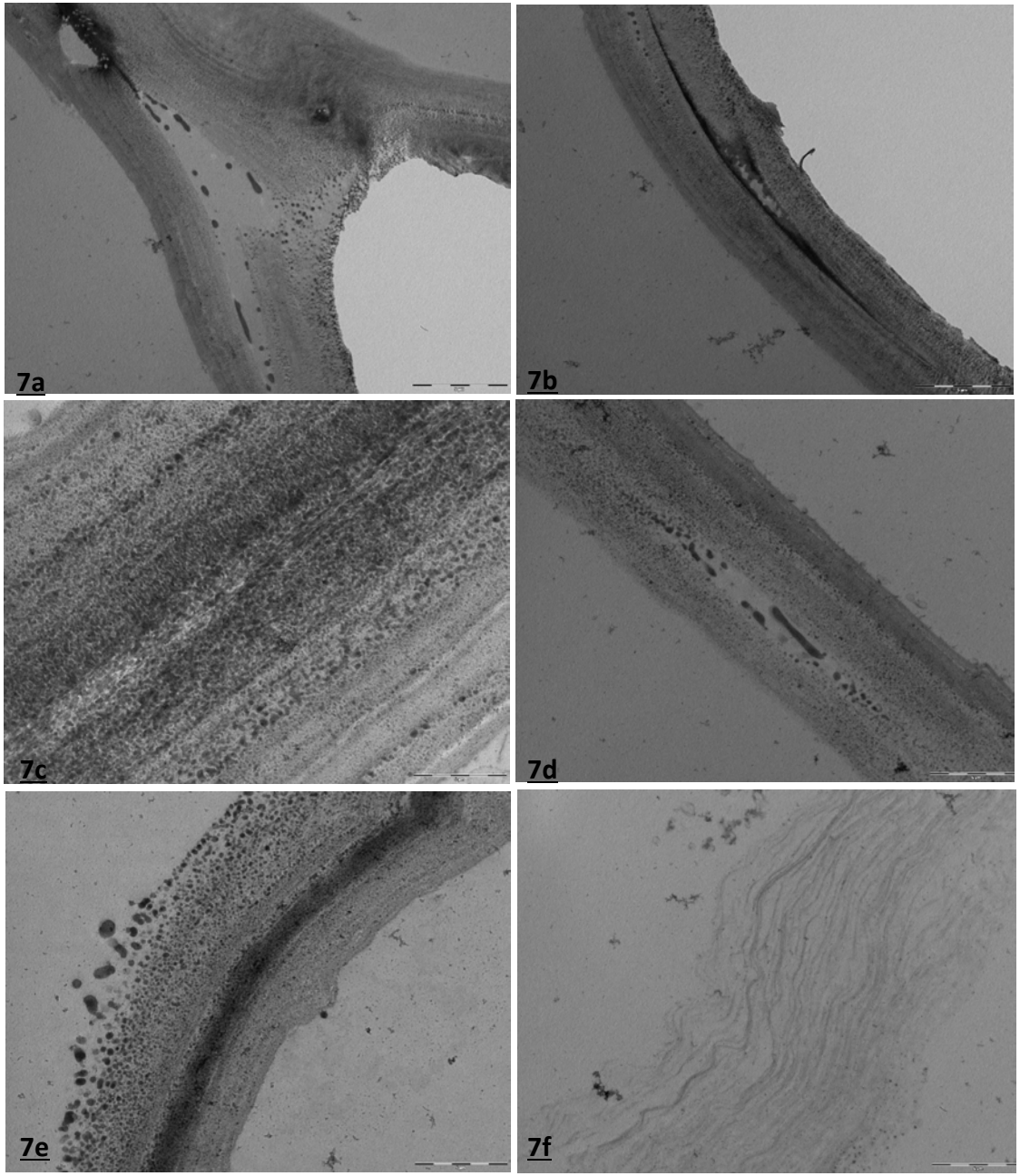
6c

Effect of termochemical pretreatment on sugar cane structure

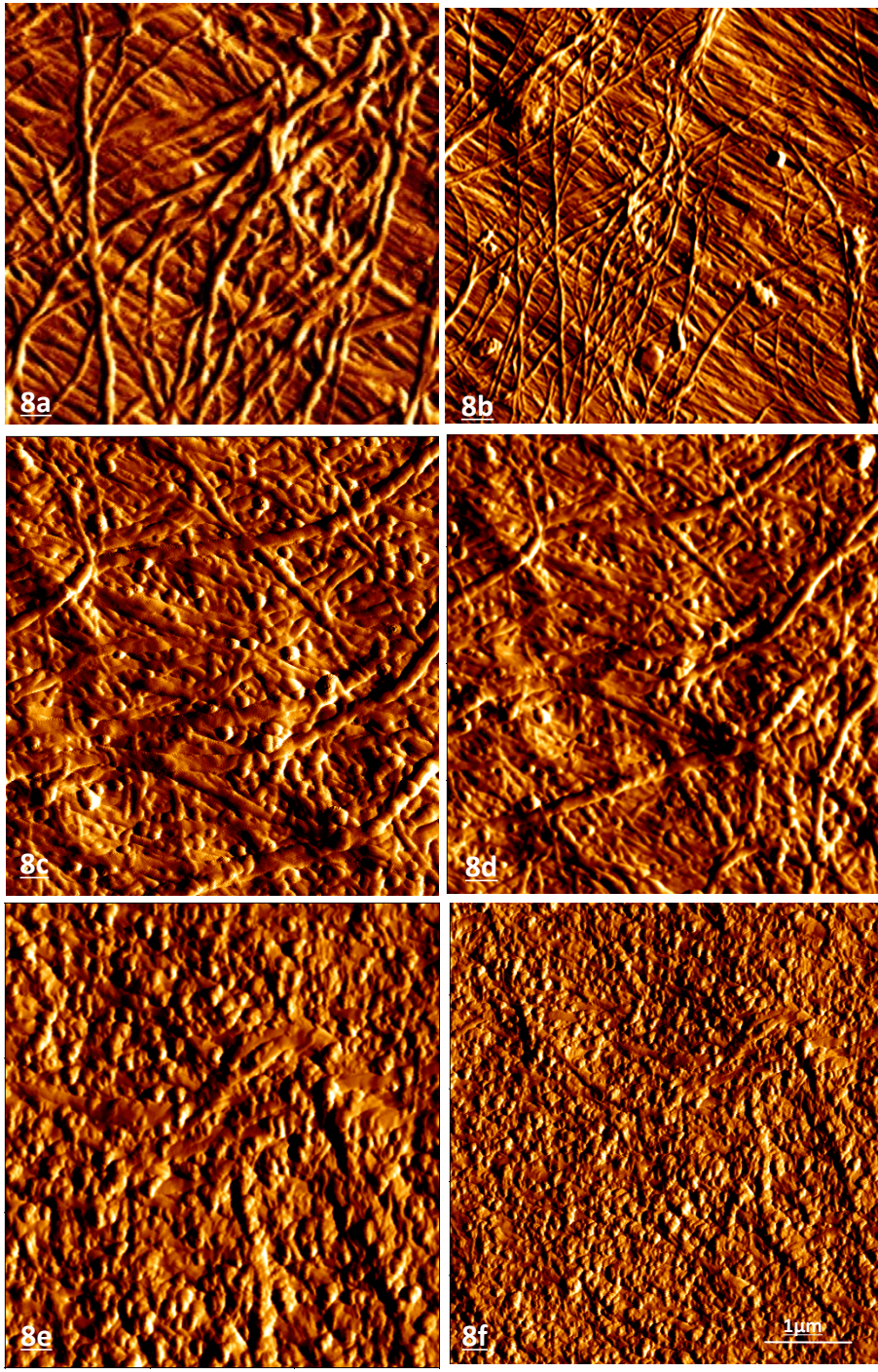
As seen by TEM

1) $KMnO_4$

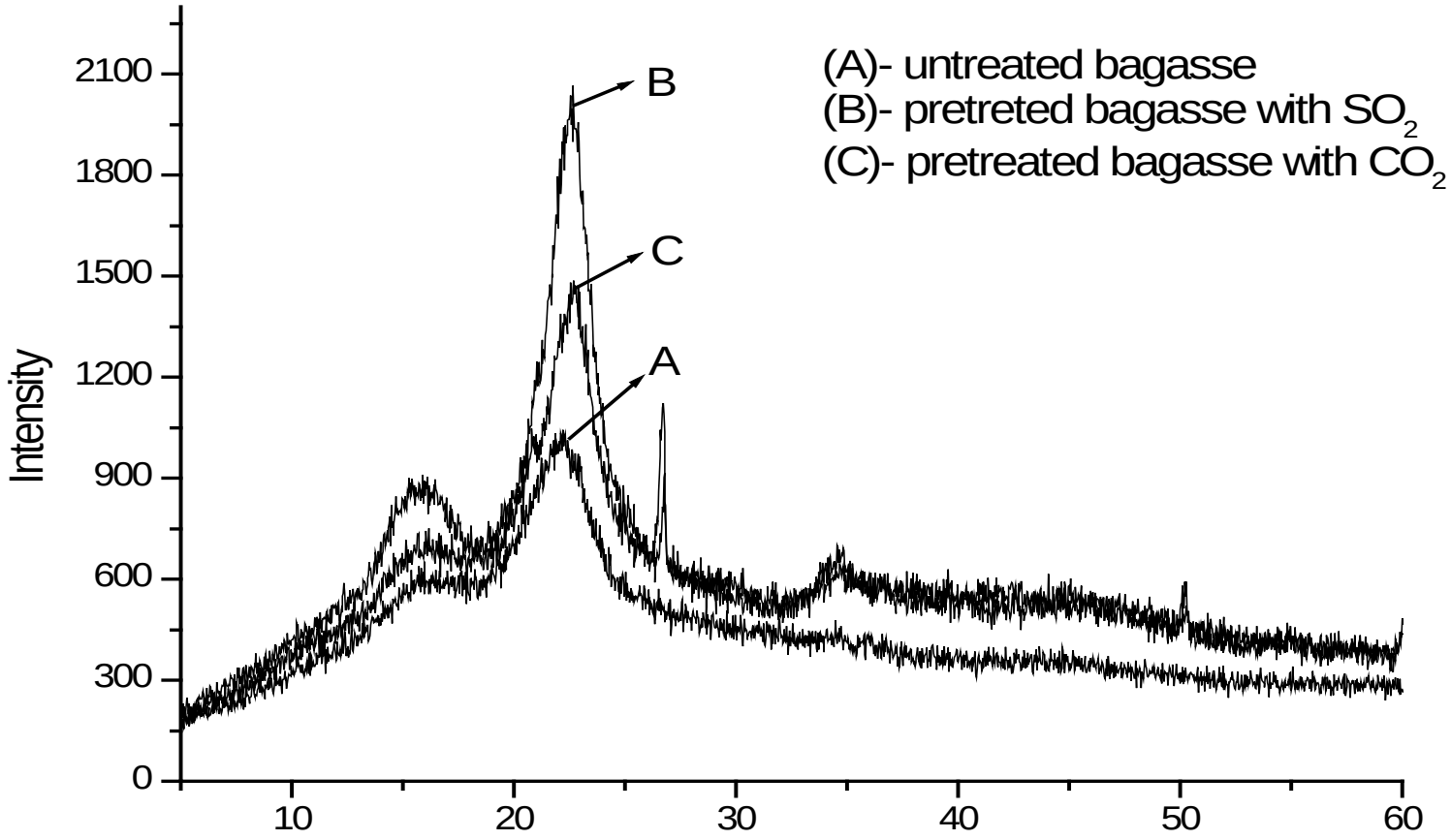
Lignina:–electrondense regions



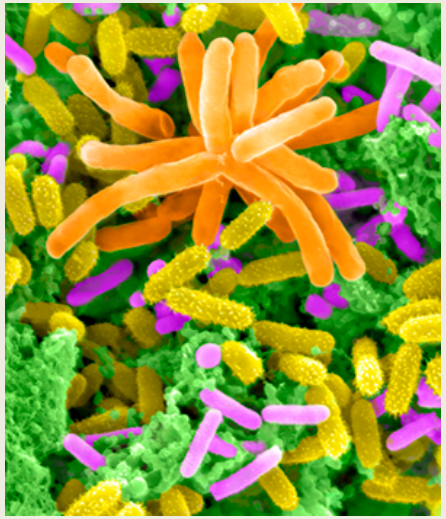
Effect of termochemical pretreatment on sugar cane structure (cellulose fibers) as seen by Atomic Force Microscopy. Notice the desorganization of the fibers and the appearance of particles.



Effect of termochemical pretreatment on the cristalinity
As seen by X-ray diffraction



Search for microorganisms and enzymes that can convert lignocellulose biomass into monosaccharides



The traditional methodologies

Selection of cellulase-producing microorganisms

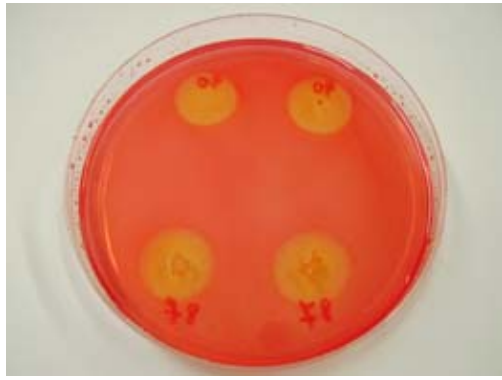
Growth in in cellulose rich medium

↓

Limited to cultivable microorganisms
(1%-15% of the environmental sample)

↓

Total potential is not being exploited
(uncultured microorganisms)



In many environments, about 99% of the microorganisms can not be cultured by standard techniques, and non-cultivated fraction includes various organisms which are somewhat related to the cultivatable fraction

What is metagenomics?

The term "metagenomics" was first used by Jo Handelsman, Jon Clardy, Robert M. Goodman, and others, and appeared for the first time in publications in 1998.
Definition: direct genetic analysis of the genomes contained in an environmental sample

Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products

Jo Handelsman[1], Michelle R Rondon[1], Sean F Brady[2], Jon Clardy[2] and Robert M Goodman[1]

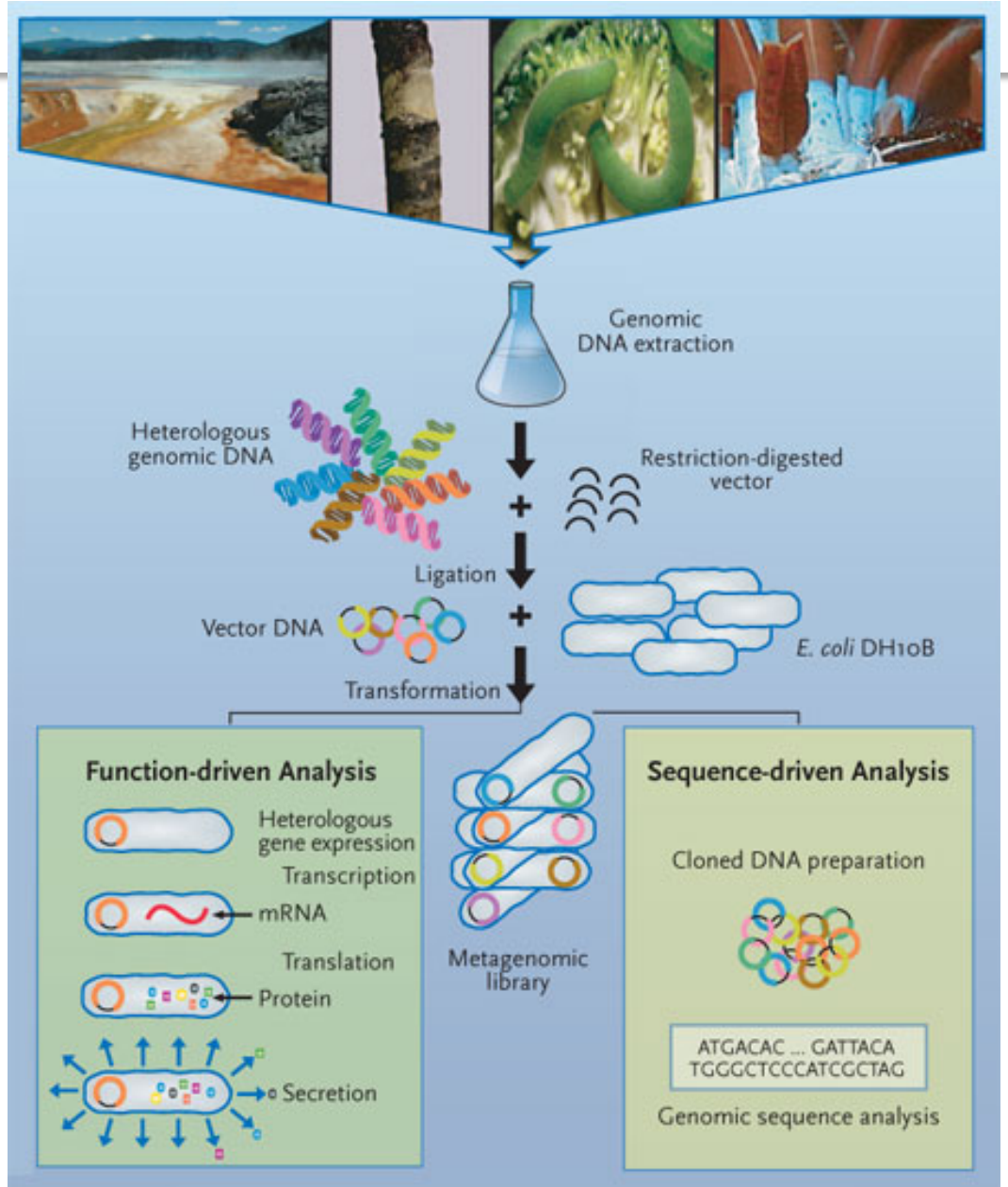
Cultured soil microorganisms have provided a rich source of natural-product chemistry. Because only a tiny fraction of soil microbes from soil are readily cultured, soil might be the greatest untapped resource for novel chemistry. The concept of cloning the metagenome to access the collective genomes and the biosynthetic machinery of soil microflora is explored here.

Chemistry & Biology October 1998, **5**:R245–249
http://biomednet.com/elecref/10745521005R0245

Addresses: [1]Department of Plant Pathology, University of Wisconsin–Madison, 1630 Linden Drive, Madison, WI 53706, USA. [2]Department of Chemistry and Chemical Biology, Baker Laboratory, Cornell University, Ithaca, NY 14853, USA.

Metagenomics

Functional analysis and based on sequences of microbial genomes contained in a collective environmental sample



Experimental model:
Giant African Snail (*Achatina fulica*)

Aims: Identification and exploitation of the non-cultivable microorganisms (method based on 16S rRNA gene sequencing); search for genes for cellulases of found in the intestinal tract of African Achatina fulica carmujo

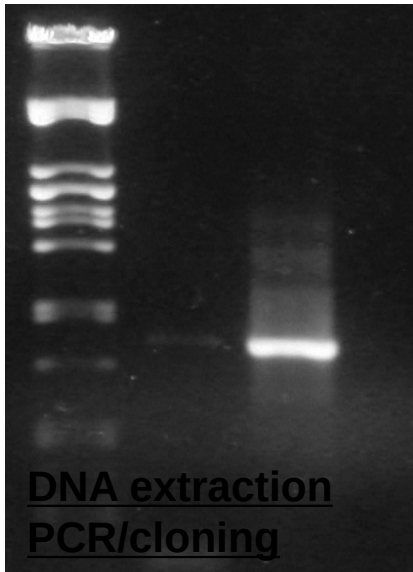
FIOCRUZ

Laboratório Nacional de Computação Científica

A. Fulica **is considered one of the most destructive pests affecting subtropical and tropical areas, causing large damages to farms, commercial plantations, and domestic gardens. It can also be found on trees, decaying materials in decomposition, and next to garbage deposits.**

Crop juice

Faeces



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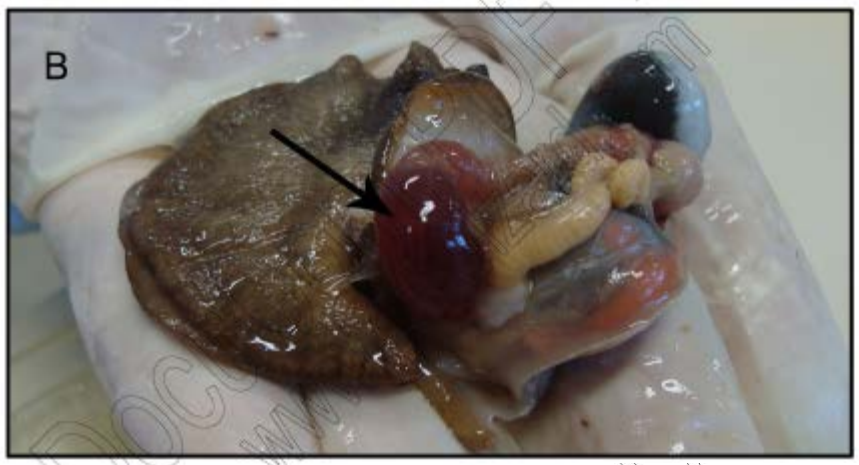
PLoS one

Gut Bacterial Communities in the Giant Land Snail *Achatina fulica* and Their Modification by Sugarcane-Based Diet

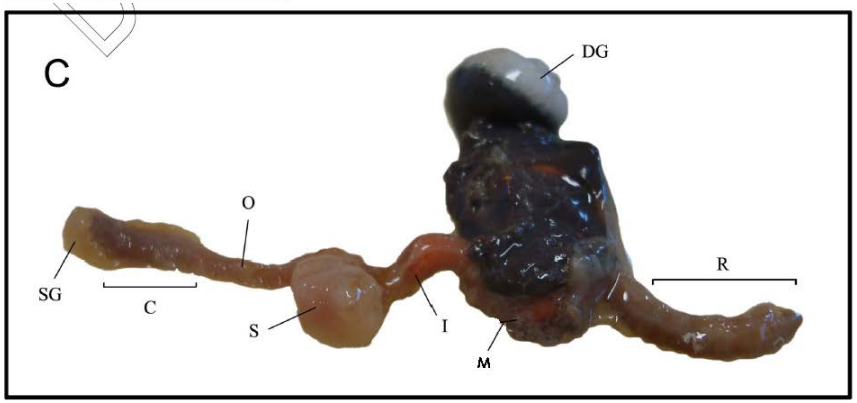
Alexander M. Cardoso[1*], Janaína J. V. Cavalcante[1], Ricardo P. Vieira[2], Joyce L. Lima[2], Maria Angela B. Grieco[1], Maysa M. Clementino[3], Ana Tereza R. Vasconcelos[4], Eloi S. Garcia[1,5], Wanderley de Souza[1,6], Rodolpho M. Albano[7], Orlando B. Martins[1,2]

1 Instituto Nacional de Metrologia, Qualidade e Tecnologia, Rio de Janeiro, Brazil, **2** Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, **3** Instituto Nacional de Controle da Qualidade em Saúde, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil, **4** Laboratório Nacional de Computação Científica, Rio de Janeiro, Brazil, **5** Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil, **6** Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, **7** Departamento de Bioquímica, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil

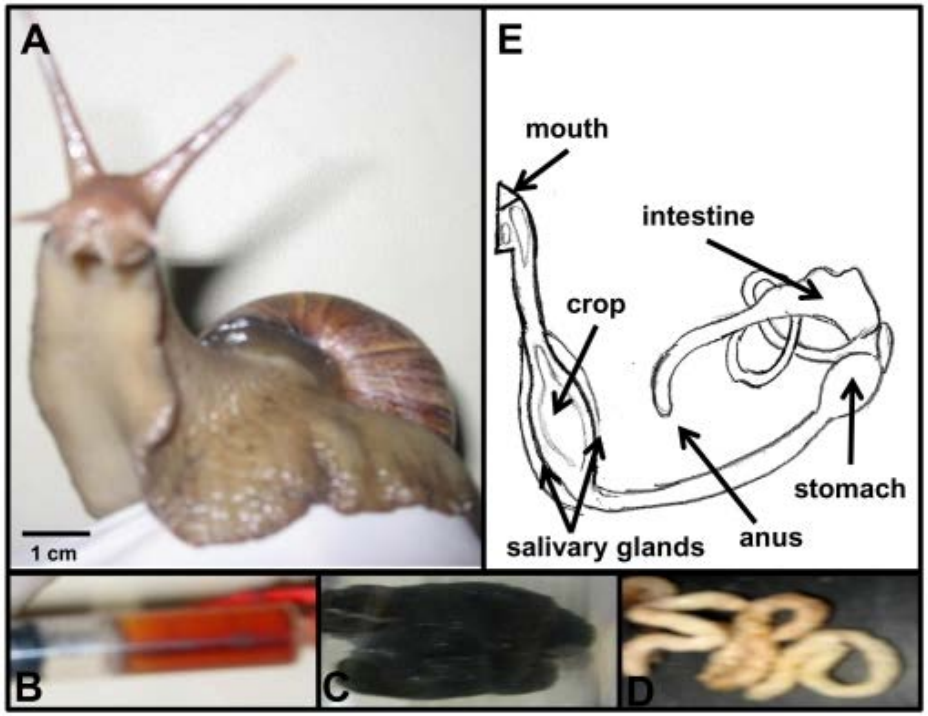
***A. fulica* snail**



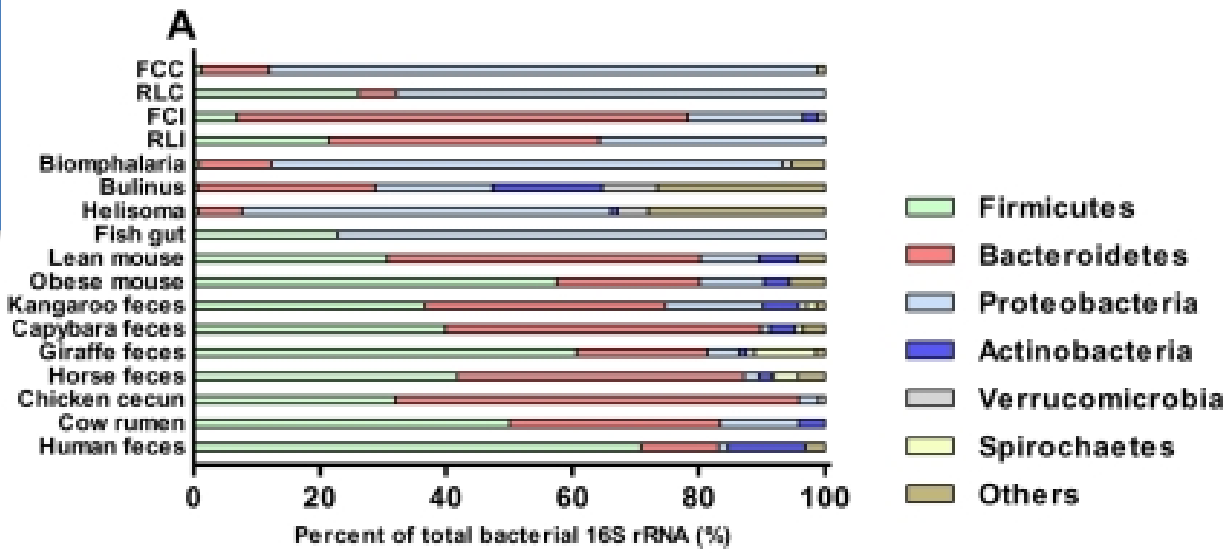
Unshelled snail, with digestive juice-filled crop shown on detail (arrow)



Dissected GIT showing buccal mass (SG), crop (C - empty) , oesophagus (O), stomach (S), foregut (I), digestive gland (DG), midgut (M) and rectum (R)



Snail (A) and its crop fluid (B), feces from field (C) or reared (D) animals are shown.



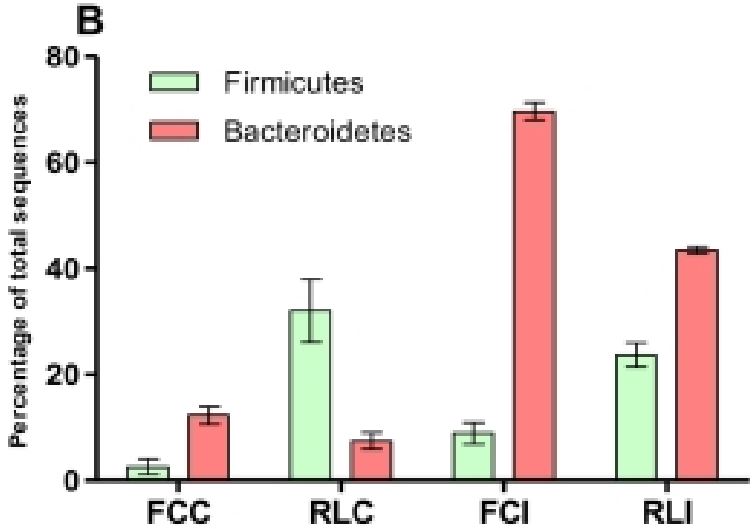
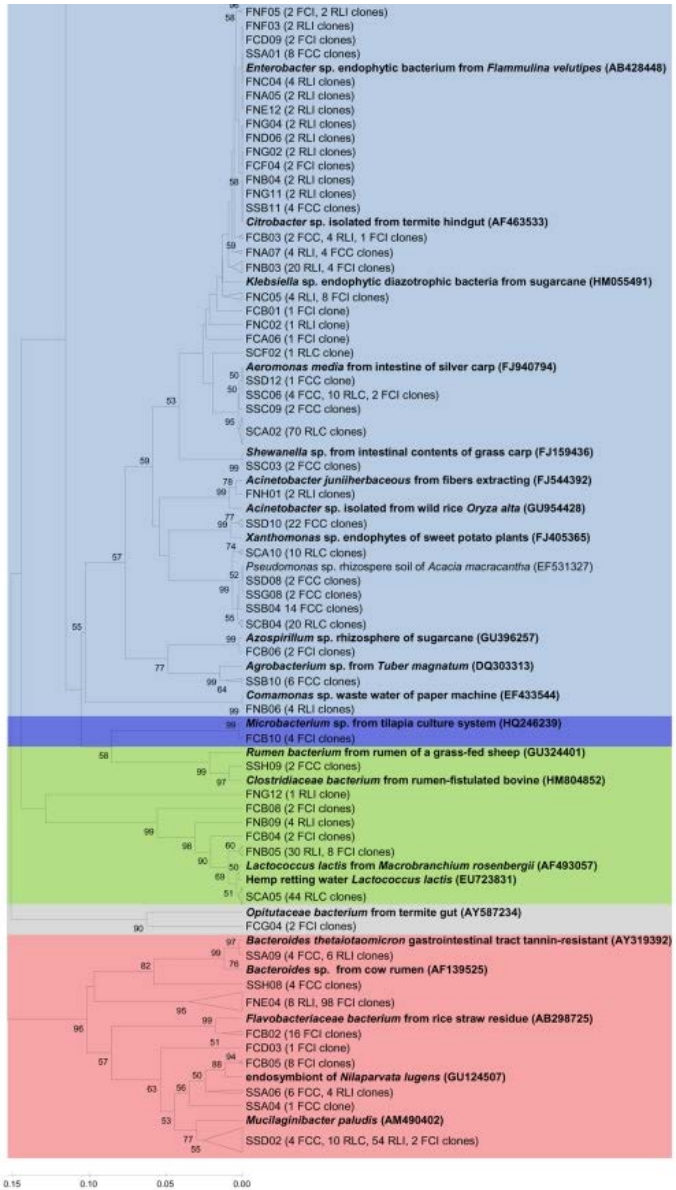


Figure 2. Distribution of sequences in bacterial phyla classified by the RDP Database (A) and proportion (B) of *Firmicutes* and *Bacteroidetes* in the crop and intestine (feces) microbiota of wild vs. reared snails. Clones were designated FC to indicate field-collected snails; RL, reared in the laboratory; C, crop; and I, intestine (feces). The datasets were compared against the following MG-RAST (metagenomics.anl.gov) metagenomic projects: Fish gut (4441695.3); Lean (4440463.3) and Obese Mouse (4440464.3); Red kangaroo (4461325.3); Capybara (4461352.3); Giraffe (4461358.3); Horse (4461321.3); Chicken cecum (4440285.3); Cow rumen (4441682.3) and Human (4440941.3). The sequences from planorbid snails *Biomphalaria pfeifferi* (FJ228890–FJ229104), *Bulinus africanus* (FJ228740–FJ228889), and *Helisoma duryi* (FJ229105–FJ229355) were obtained from NCBI dataset.

doi:10.1371/journal.pone.0033440.g002

Phylogenetic tree of bacterial clones obtained within the snail gut.



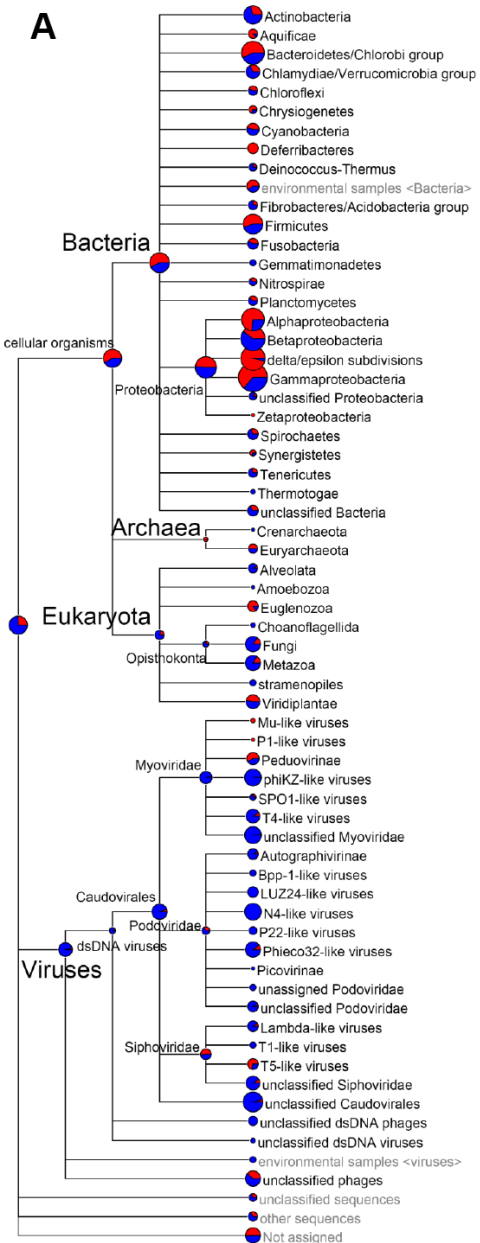
Crop fluid samples showed a higher abundance of Proteobacteria while fecal samples were dominated by Bacteroidetes and Firmicutes, abundant microorganisms in the feces of warm-blooded animals including humans. Within the Epsilon-proteobacteria, a profuse OTU with 72 sequences was related to *Sulforospirillum* spp. Interestingly, in the gutless marine oligochaete *Olavius algarvensis*, endosymbiotic sulphate-reducing bacteria serve as an energy source to the host and may participate within the host in the removal of the end products of fermentation . Other bacterial taxa closely related to herbivore and plant-associated bacteria included *Clostridiaceae, Lactococcus, Bacteroides, Flavobacteriaceae, Mucilaginibacter, Citrobacter, Klebsiella, Aeromonas, Acinetobacter, Pseudomonas,* and *Comamonas*

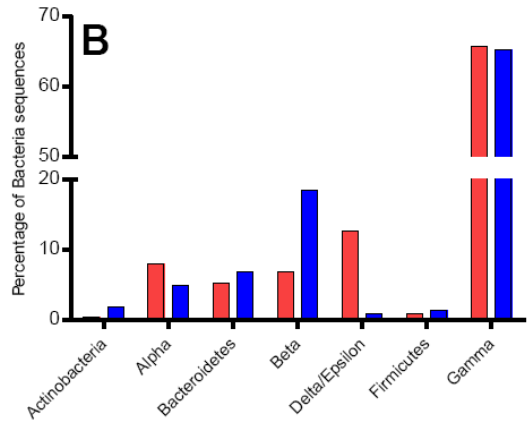
Metagenomic analysis of the microbiota from the crop of an invasive snail reveals a rich reservoir of novel genes
Article submitted for Plos One and accepted in september 2012

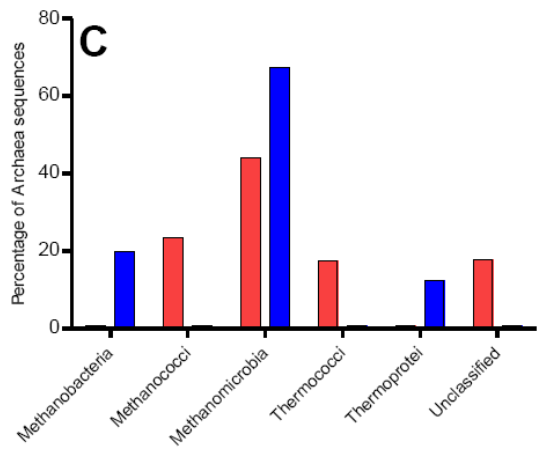
Summary of metagenomic data obtained from the Achatina fulica crop microbiome.

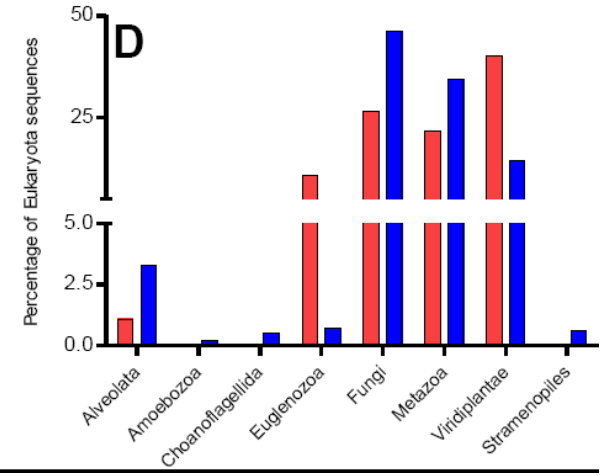
Parameters	Filtered	Non-filtered	Total	Assembly
MG-RAST ID	4460151	4481175	4480501	4482672
No. of sequences	739,381	558,217	1,297,598	
	525,841*	384,126*	909,967*	426,017*
Avg. length (bp)	347 ± 80*	352 ± 82*	349 ± 81*	370 ± 912*
Total length (bp)	182,716,265	135,384,210	318,100,475	158,000,172
Predicted proteins†	281,690	219,893	473,379	294,404
No. of rDNA hits‡				
Bacteria (R/S/G)	(975/982/798)	(479/415/322)	(1498/1389/1141)	(81/71/58)
Archaea (R/S/G)	(0/0/0)	(0/0/0)	(0/0/0)	(0/0/0)
Eukarya (R/S/G)	(0/2/0)	(0/16/0)	(0/18/0)	(0/4/0)
Unassigned (R/S/G)	(151/304/264)	(44/183/164)	(173/466/391)	(9/35/23)
LCA¥				
Bacteria (%)	463,975 (88.24)	261,779 (68.15)	729,300 (80.15)	347,291 (81.52)
Archaea (%)	63 (0.012)	24 (0.006)	88 (0.010)	44 (0.010)
Eukarya (%)	549 (0.104)	1,897 (0.494)	2,443 (0.268)	1,656 (0.389)
Viruses (%)	473 (0.090)	11,571 (3.012)	12,123 (1.332)	1,624 (0.381)
Unclassified (%)	39 (0.007)	31 (0.008)	73 (0.008)	43 (0.010)
No hits (%)	60,742 (11.55)	108,824 (28.33)	165,940 (18.24)	75,359 (17.69)

Composition of the snail microbial community analyzed







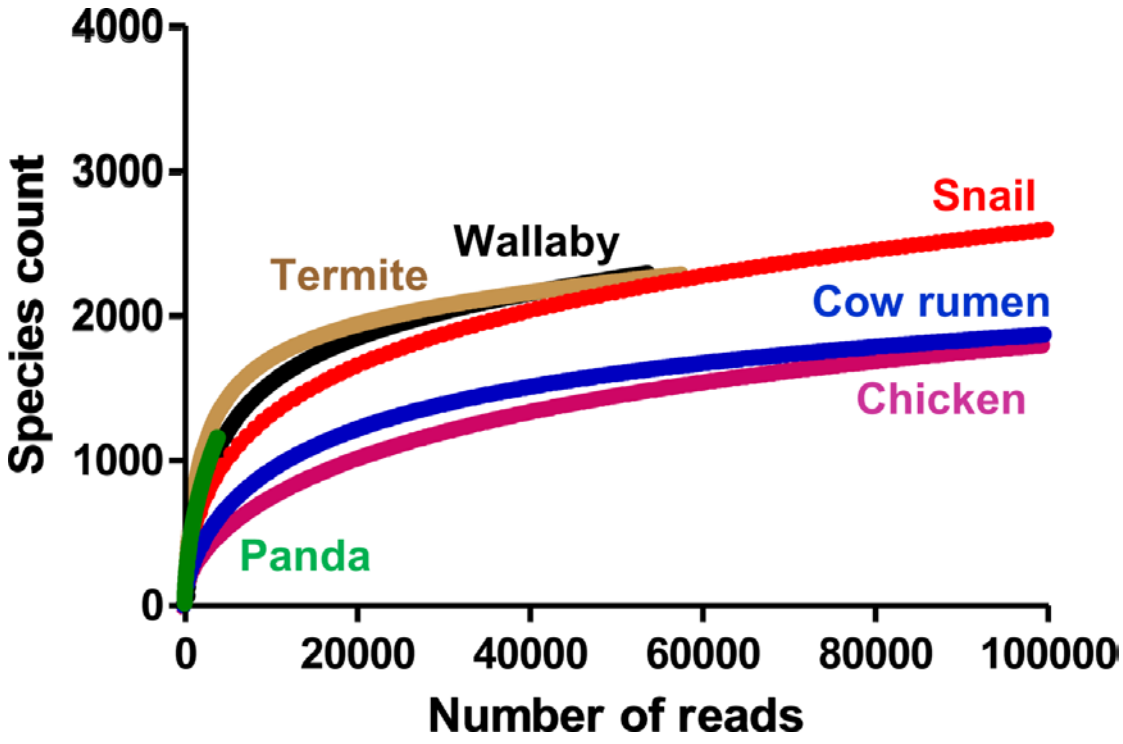


To best of our knowledge this is the first report on the detection of *Archaea*, *Fungi*, and *Viruses* within the crop of a snail. Comparing to other metagenomes, the snail microbiota has high species richness

(A) Phylogenetic diversity of metagenome sequences computed by MEGAN based on a BLASTX using an e-value cut-off of 1e-5 comparison for filtered (red) and non-filtered (blue) sequences. The size of the circles is scaled logarithmically to represent the number of reads assigned to each taxon. (B) Bacteria, (C) Archaea, and (D) Eukaryota.

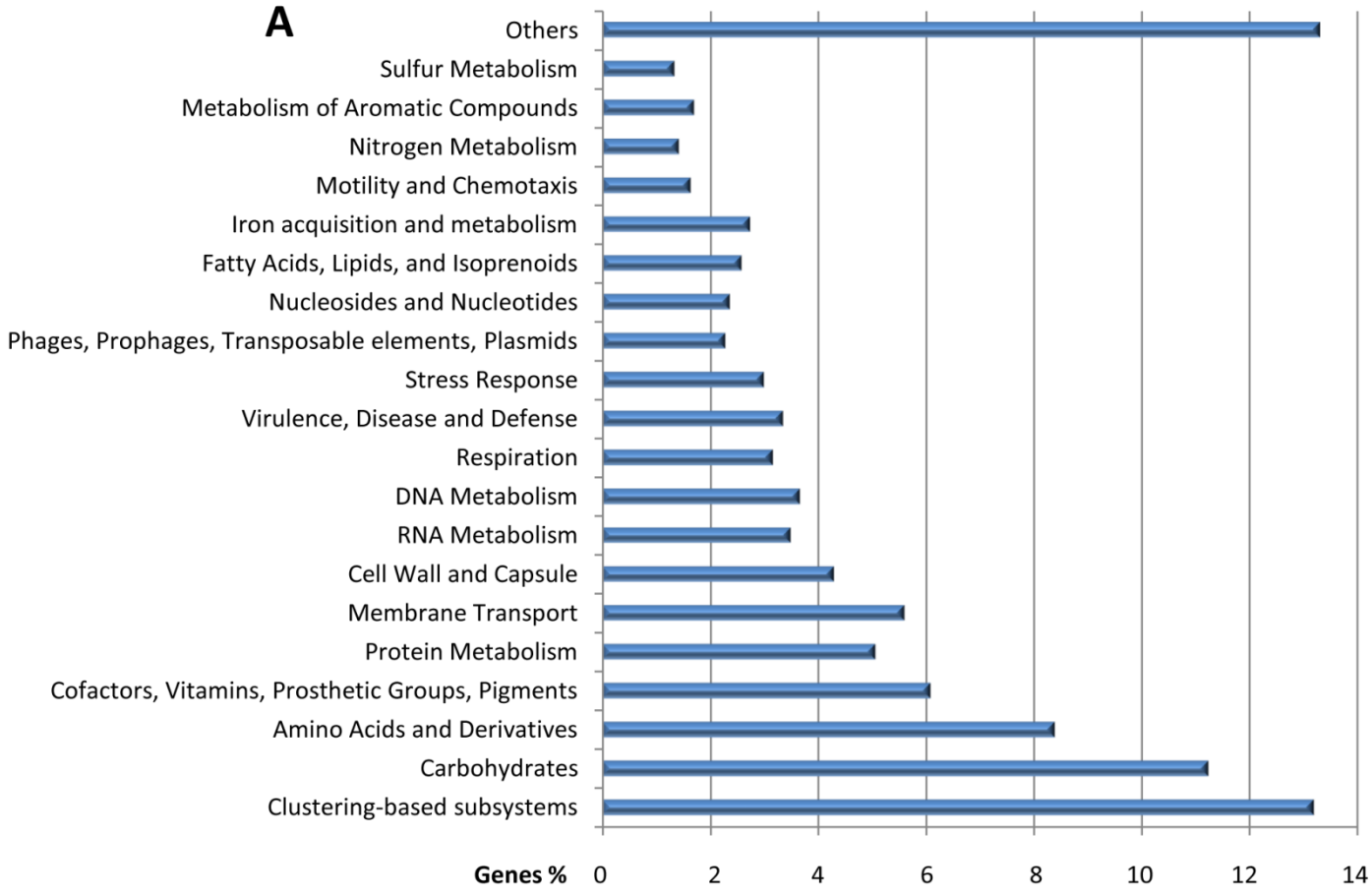
Species richness

Compared with other metagenomes, the snail microbiota has high species richness, similar to the wallaby foregut and P3 luminal fluid from termite microbiomes



Rarefaction curve of annotated species richness. The curves represent the average number of different species annotations for subsamples of the complete dataset calculated in MGRAST.

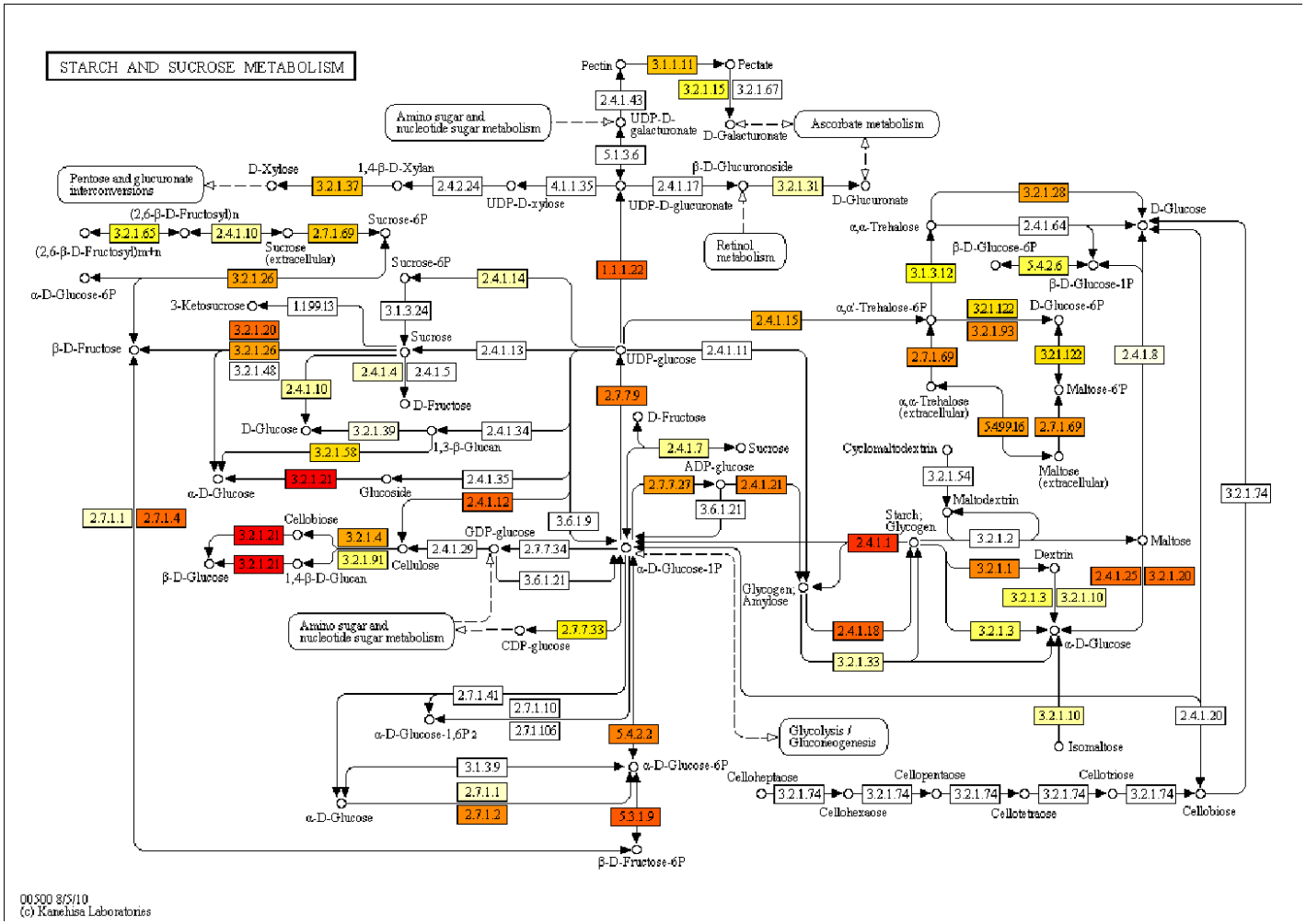
Functional metagenome analysis



Subsystems-based annotation (SEED) analysis of the snail crop microbiome.
A) Percent of genes assigned by MG-RAST to each SEED subsystem.

Functional metagenome analysis

A detailed analysis on the carbohydrate metabolic pathways revealed a significant amount (~7.5%) of putative genes belonging to the subgroup “starch and sucrose metabolism”, including sequences coding for critical enzymes that cover all steps of cellulose deconstruction.



Inventory of putative glycoside hydrolases (GHs), identified in the snail crop microbiome.

Glycoside Hydrolases (GHs): hydrolysis and/or rearrangement of glycosidic bonds					
CAZy family	Pfam family	Know activities (resumo CAZy website)	Reads	NR	Source Organisms
GH2	PF00703	β-Galactosidase, β-mannosidase, β-glucuronidase, others	100	66	49 Bacteroidetes (49%)
GH4	PF02056	α-Glucosidase, α-galactosidase, α-glucuronidase, others	79	56	58 Proteobacteria (73%)
GH5	PF00150	Chitosanase, β-mannosidase, cellulase, endo-β-1,4-xylanase, others	40	36	21 Proteobacteria (53%)
GH6	PF01341	Endoglucanase, cellobiohydrolase	4	4	4 Actinobacteria
GH8	PF01270	Chitosanase, cellulase, licheninase, endo-1,4-β-xylanase, others	79	46	73 Proteobacteria (92%)
GH9	PF00759	Endoglucanase, cellobiohydrolase, β-glucosidase, others	17	15	12 Proteobacteria (71%)
GH10	PF00331	Endo-1,4-β-xylanase, endo-1,3-β-xylanase	33	25	15 Bacteroidetes (45%)
GH11	PF00457	Xylanase	1	1	1 Proteobacteria
GH15	PF00723	Glucoamylase, glucodextranase, α,α-trehalase	59	23	55 Proteobacteria (93%)
GH16	PF00722	Endo-1,3-β-glucanase, licheninase, β-agarase, xyloglucanase, others	12	12	4 Proteobacteria (33%); 4 Bacteroidetes (33%)
GH17	PF00332	Glucan endo-1,3-β-glucosidase, glucan 1,3-β-glucosidase, licheninase, others	28	2	28 Proteobacteria (100%)
GH18	PF00704	Chitinase, endo-β-N-acetylglucosaminidase, xylanase inhibitor; concanavalin B, others	99	50	73 Proteobacteria (74%)
GH19	PF00182	Chitinase, lysozyme	59	34	44 Proteobacteria (75%)
GH20	PF00728	β-Hexosaminidase, lacto-N-biosidase, β-1,6-N-acetylglucosaminidase, others	142	108	67 Bacteroidetes (47%)
GH24	PF00959	Lysozyme	127	56	82 Proteobacteria (64%)
GH25	PF01183	Lysozyme	16	16	8 Proteobacteria (50%)
GH26	PF02156	β-Mannanase, β-1,3-xylanase	12	11	9 Bacteroidetes (75%)
GH27	PF02065	α-Galactosidase, α-N-acetylgalactosaminidase, β-L-arabinopyranosidase, others	52	32	19 Proteobacteria (36%)
GH28	PF00295	Polygalacturonase, rhamnogalacturonase, others	101	69	55 Proteobacteria (54%)
GH29	PF01120	α-L-Fucosidase	94	70	61 Bacteroidetes (65%)
GH30	PF02055	Glucosylceramidase, β-1,6-glucanase, β-xylosidase, β-fucosidase, others	21	18	10 Proteobacteria (48%)
GH31	PF01055	α-Glucosidase, α-xylosidase, α-glucan lyase, others	231	125	166 Proteobacteria (72%)
GH32	PF00251	Invertase, endo-inulinase, endo-levanase, exo-inulinase, others	118	68	88 Proteobacteria (75%)
GH33	PF02012	Sialidase or neuraminidase, others	0	6	
GH35	PF01301	β-Galactosidase, exo-β-glucosaminidase	38	32	29 Bacteroidetes (76%)
GH37	PF01204	α,α-Trehalase	104	55	95 Proteobacteria (91%)

We found a wide diversity of GH catalytic modules: 2,534 EGTs belonging to 53 different GH families, which indicates an abundance of potential polysaccharide and plant cell wall biomass-degrading enzymes

Carbohydrate-Binding Modules (CBMs) : adhesion to carbohydrates

Carbohydrate-Binding Modules (CBMs) : adhesion to carbohydrates

CBM2	PF00553	CBM_2	Chitin or xylan	2	100% Proteobacteria	3
CBM4/9/16/22†	PF02018	CBM_4_9	Cellulose (found only in xylanases), Xylan, glucomannan, β-1,3-glucan, β-1,3-1,4-glucan, β-1,6-glucan, amorphous cellulose	19	42% Proteobacteria	12
CBM5/12†	PF02839	CBM_5_12	Chitin	21	57% Proteobacteria	15
CBM6	PF03422	CBM_6	Amorphous cellulose, β-1,4-xylan, β-1,3-glucan, β-1,4-glucan, others	14	43% Proteobacteria; 43% Bacteroidetes	13
CBM13	PF00652	Ricin_B_lectin	Xylan, mannose, galactose residues, others	10	60% Actinobacteria	13
CBM20	PF00686	CBM_20	Granular starch, cyclodextrins	11	73% Proteobacteria	3
CBM32	PF00754	F5_F8_type_C	Galactose, lactose, polygalacturonic acid, LacNAc	72	39% Bacteroidetes	37
CBM33	PF03067	Chitin_bind_3	Chitin, chitosan	54	94% Proteobacteria	22
CBM34	PF02903	Alpha-amylase_N	Granular starch	6	100% Proteobacteria	3
CBM50	PF01476	LysM	Chitopentaose	239	82% Proteobacteria	152
CBM51	PF08305	NPCBM	Galactose	10	80% Proteobacteria	5
Total				**458**		**278**

We also detected a total of 458 EGTs from 11 families of non-catalytic carbohydrate-binding modules (CBMs), which may promote the interaction of an enzyme with its target substrate, thereby increasing the efficiency of catalysis. This abundance reflects a high capacity to metabolize and degrade diverse lignocellulosic substrates.

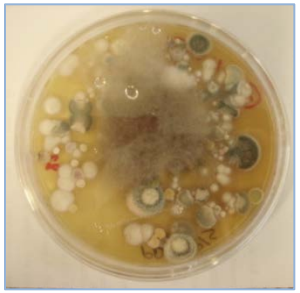
GlycosylTransferases (GTs): formation of glycosidic bonds

CAZy family	Pfam family	Know activities (resumo CAZy website)	Reads (R)	(NR)	Source Organisms
GT1	PF00201	UDP-glucuronosyltransferase, 2-hydroxyacylsphingosine, 1-β-galactosyltransferase, others	7	5	4 Proteobacteria (57%)
GT7	PF02709	Lactose synthase, N-acetyllactosamine synthase, others	1	1	Unclassified
GT8	PF01501	Lipopolysaccharide α-1,3-galactosyltransferase, lipopolysaccharide glucosyltransferase 1, others	40	30	30 Proteobacteria (75%)
GT9	PF01075	Lipopolysaccharide N-acetylglucosaminyltransferase, heptosyltransferase	267	157	243 Proteobacteria (91%)
GT10	PF00852	Galactoside α-1,3/1,4-L-fucosyltransferase, others	4	2	3 Unclassified (75%); 1 Bacteroidetes
GT11	PF01531	GDP-L-Fuc: Galactoside α-1,2-L-fucosyltransferase, others	3	2	2 Bacteroidetes
GT17	PF04724	β-1,4-Mannosyl-glycoprotein β-1,4-N-acetylglucosaminyltransferase	1	1	1 Proteobacteria
GT19	PF02684	Lipid-A-disaccharide synthase	158	64	145 Proteobacteria (92%)
GT20	PF00982	α,α-Trehalose-phosphate synthase	97	62	78 Proteobacteria (80%)
GT22	PF03901	Dol-P-Man α-mannosyltransferase	2	1	1 Proteobacteria; 1 Unclassified
GT25	PF01755	β-1,3-Glucosyltransferase, β-1,2-glucosyltransferase, β-1,2-galactosyltransferase, others	11	11	9 Proteobacteria (82%)
GT26	PF03808	UDP-ManNAcA: β-N-Acetyl mannosaminuronyltransferase, UDP-Glc: β-1,4-glucosyltransferase, others	57	41	54 Proteobacteria (95%)
GT28	PF04101	1,2-diacylglycerol 3-β-galactosyltransferase, 1,2-diacylglycerol 3-β-glucosyltransferase, others	96	45	88 Proteobacteria (92%)
GT29	PF00777	Sialyltransferase, β-galactoside, α-2,6-sialyltransferase, others	0	1	
GT34	PF05637	UDP-Gal: galactomannan α-1,6-galactosyltransferase, α-1,2-galactosyltransferase, others	0	1	
GT35	PF00343	Glycogen or starch phosphorylase	397	133	375 Proteobacteria (95%)
GT39	PF02366	Dol-P-Man: protein α-mannosyltransferase	72	22	70 Proteobacteria (97%)
GT43	PF03360	B-Glucuronyltransferase, UDP-Xyl: xylan β-1,4-xylosyltransferase	1	1	1 Unclassified
GT48	PF02364	1,3-β-Glucan synthase	1	1	1 Ascomycota
GT51	PF00912	Murein polymerase	405	198	358 Proteobacteria (88%)
GT66	PF02516	Dol-PP-α-Oligosaccharide: protein β-oligosaccharyltransferase	23	3	22 Proteobacteria (96%)
Total			**1643**	**782**	

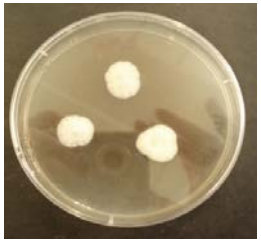
GlycosylTransferases: the enzymes that catalyse the transfer of sugar moieties from activated donor molecules to specific acceptor molecules, forming glycosidic bonds

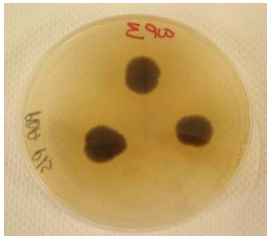
Fungal Isolation

Aerobic
30°C
PDA
P/S 100 U/L



Mix culture





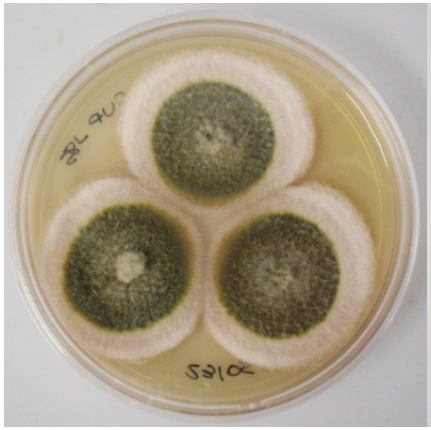
Pure Culture

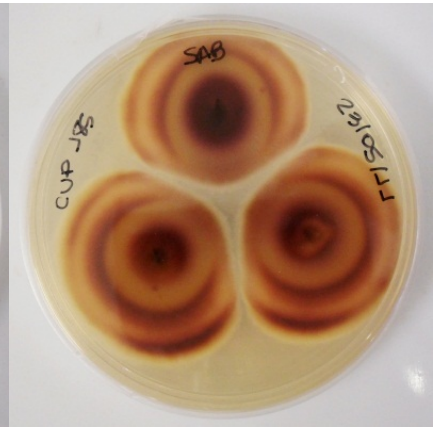
Isolation

- Identification
  - Clássica: Morfológica e Bioquímica
  - Molecular: 18S, 5.8S-ITS e sequenciamento
- Cellulolytic activity
  - CMC, Bagasse
  - Isolation
  - Characterization
  - Clonning

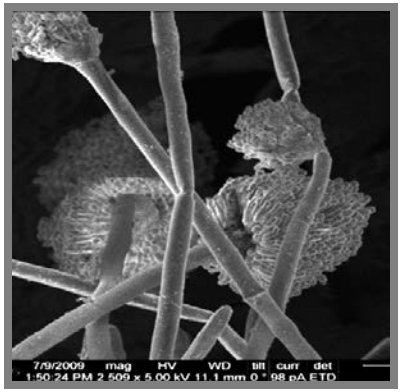
Classical Identification

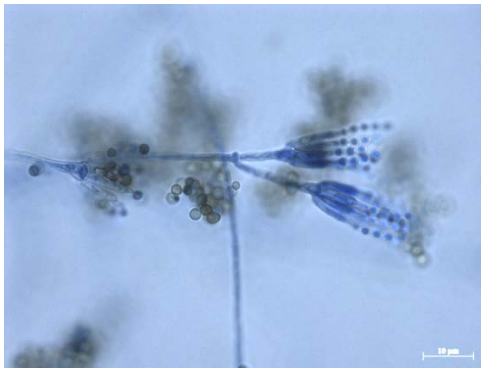
Culture

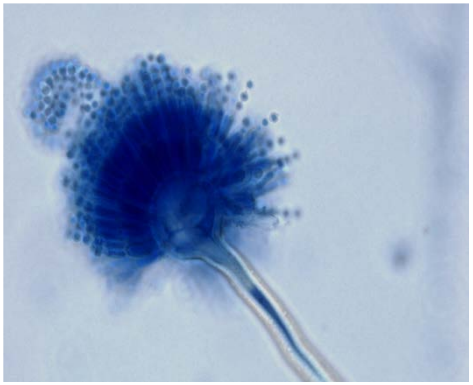




Microscopy







TERMITS

Cornitermes cumulans

12 isolates

Syntermes spp

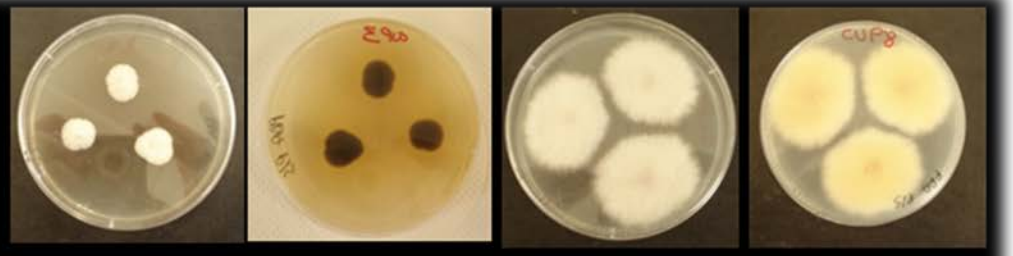
31 isolates

Nasutitermes spp

4 isolates

Cornitermes silvestrii

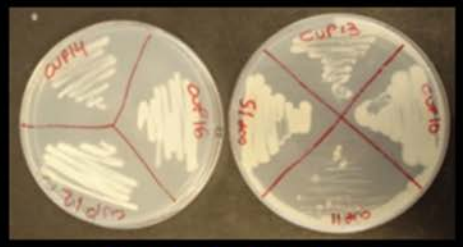
2 isolates



CUP 3

CUP 8

CUP 28



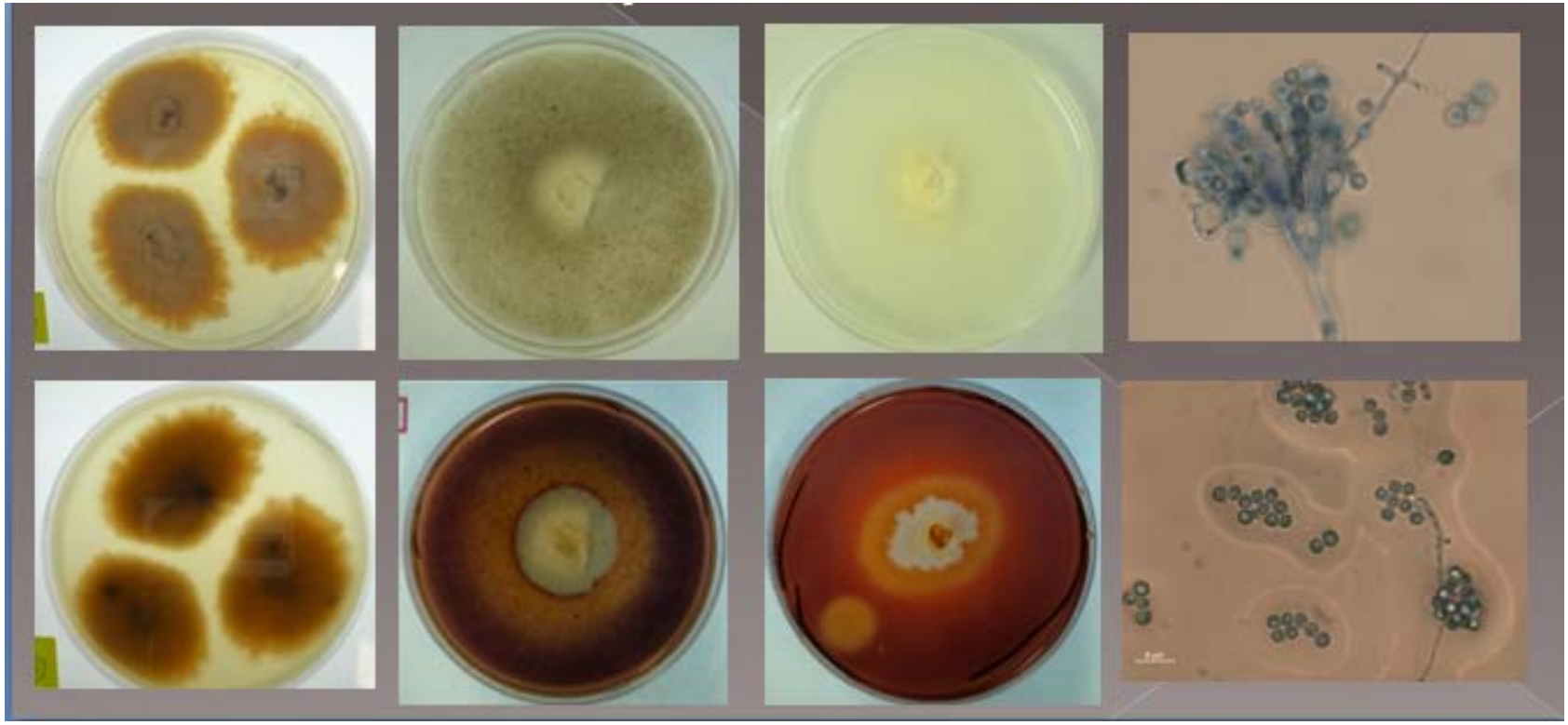
CUP 10-CUP 16

CUP26

CUP23

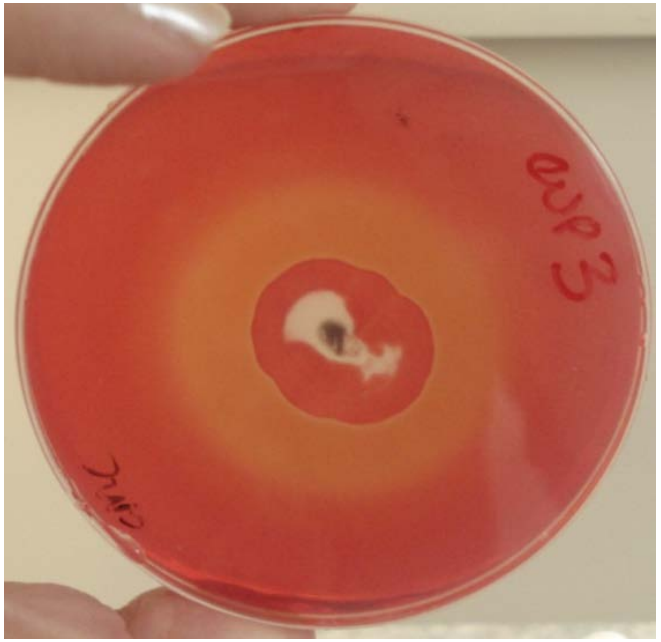
Achatina fulica

- 60 isolates



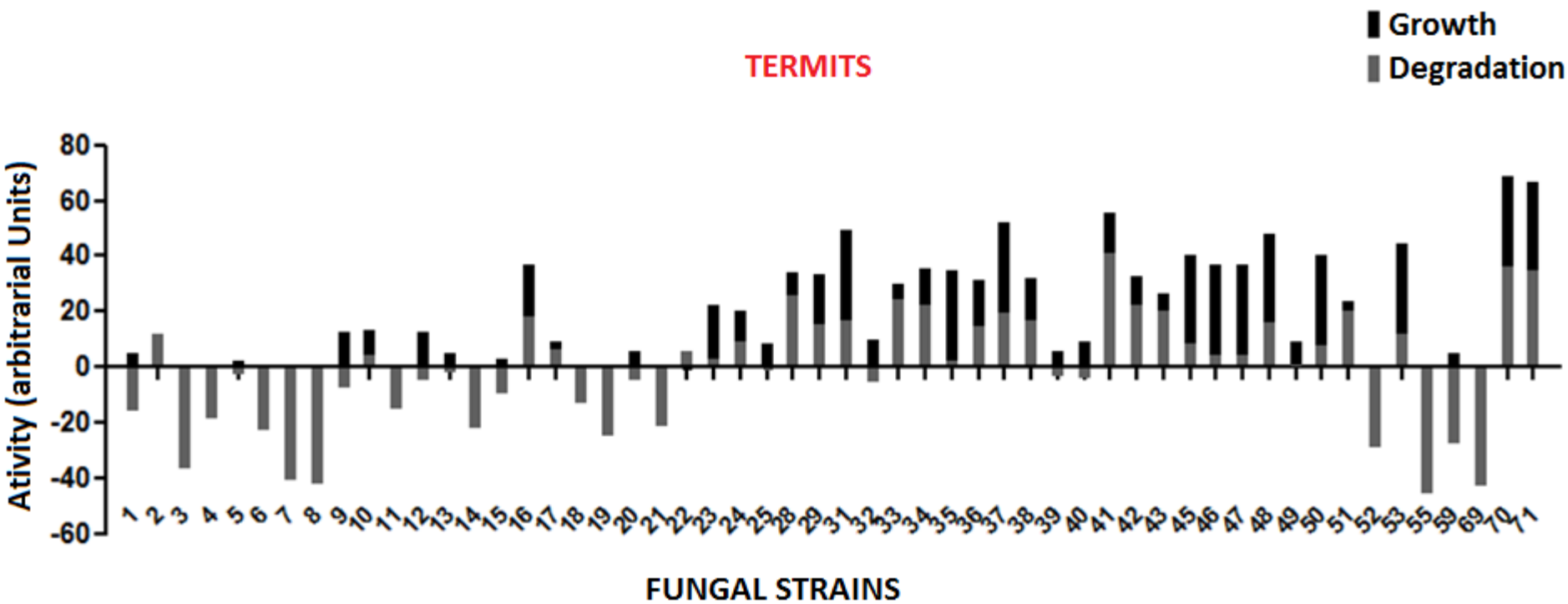
Screening of cellulolytic fungi

Congo Red

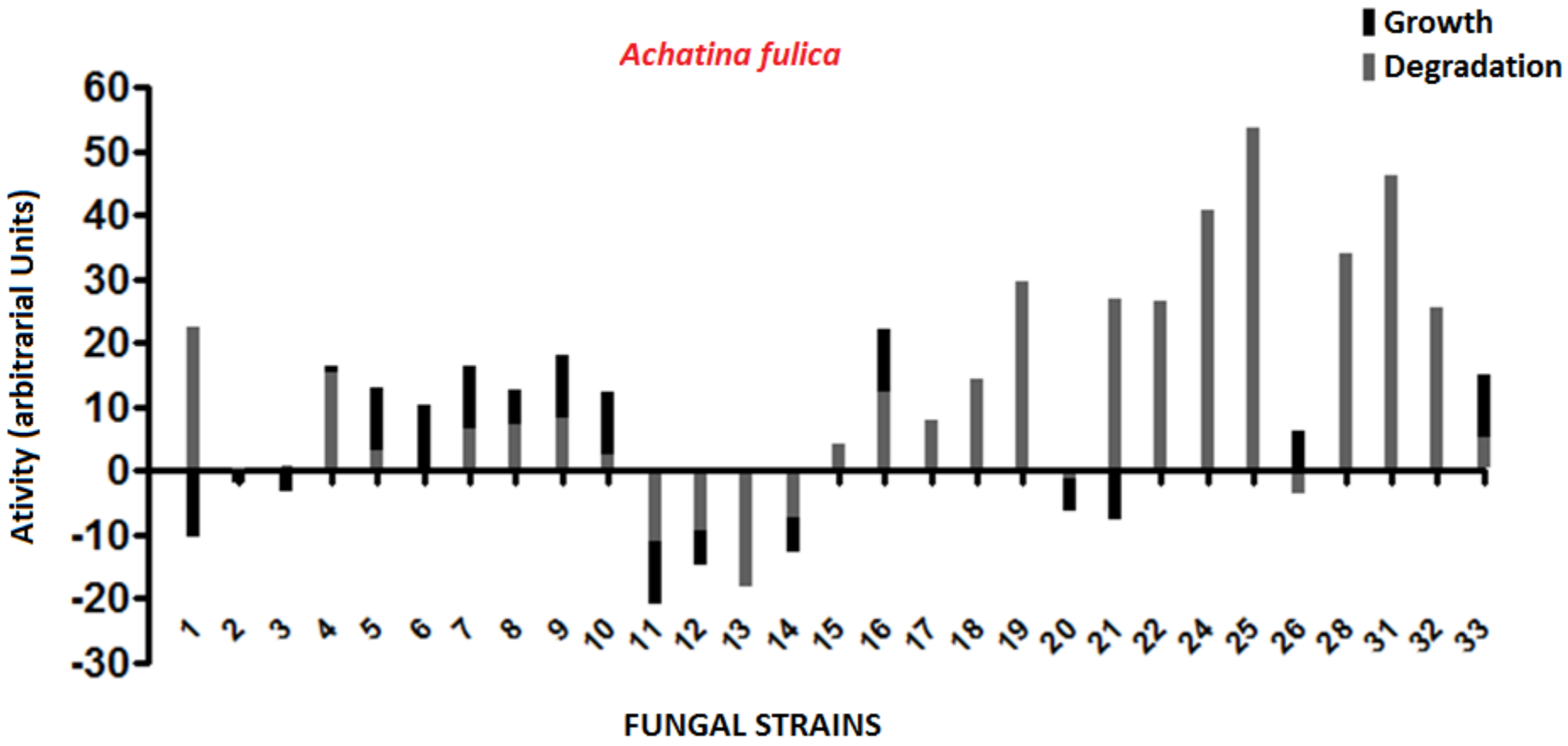


Different Carbon sources, including Carboxyl methyl Cellulose (CMC) and Sugar cane Bagasse

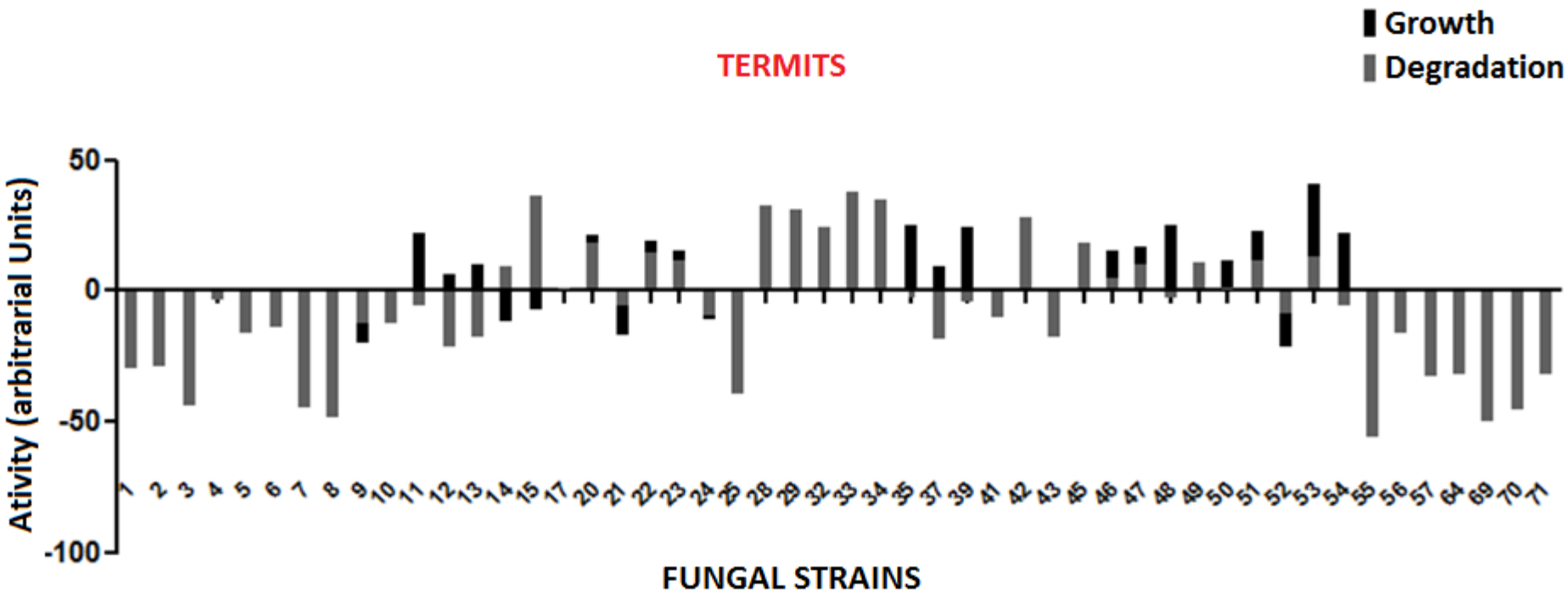
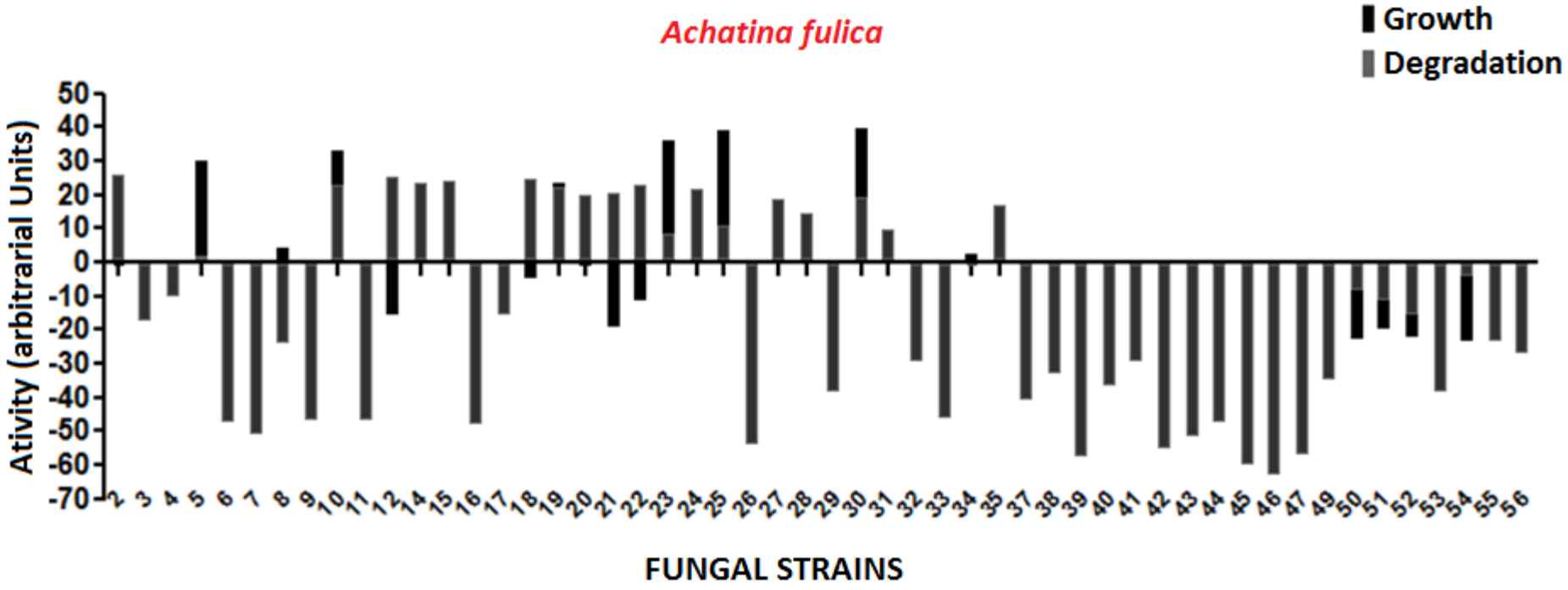
Cellulolytic Activity in CMC media



Cellulolytic Activity in CMC media



Cellulolytic Activity in Bagasse media



Cellulolytic Activity in Bagasse media