Oklahoma NSF EPSCoR Bioenergy Research and Education

Genomic Analysis of the Lignocellulotic Anaerobic Fungus Orpinomyces C1A

MB Couger, Audra Liggenstoffer
Mostafa Elshahed

Oklahoma State University

STUDENT #1

STUDENT #1

Objectives

- Efficient conversion of recalcitrant Non-Feed Stock biomass, such as switchgrass, currently is a major obstacle preventing the actualization of Bio-fuels as a widely used energy source.
- Identification of additional enzymes capable of lignocellulose degradation from relevant organisms would provide a greater toolset for bio-fuel production.
- Genomic Analysis of the lignocellolosic anaerobic fungal genus Neocallamastix could identify such enzymes.

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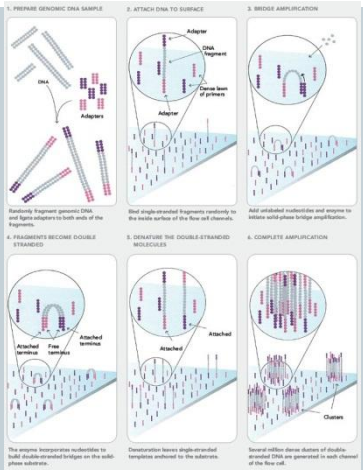
Methods

Genomic Sequence Generation

Genome/Transcriptome Sequence Assembly

cDNA Alignment Gene Calling Functional Annotation

illumina®

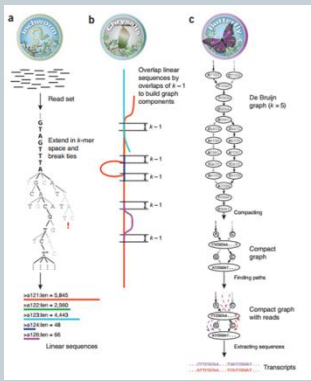


Velvet

EMBL-EBI

Trinity

Inchworm Chrysalis Butterfly



Genome Training set Creation using PASA cDNA Alignment

- Alignment of cDNA to create accurate training set
- Specific training of any Ab Ininto Models used

Generation of Consensus Models from Multiple Lines of Evidence

- Model input includes Ab Initio predictions and alignment data
- Models are integrated using Evidence Based Modeler

Functional Annotation by PFAM and Homology analysis

- Domains are identified using PFAM database
- First Hit Blast is Conducted to assign most relevant homologous function

STUDENT #1

Results

Assembly/Annotation Statistics	Value
Total Reads	146 Million Pairs
Total Amounf of Bases	29.2GB
Total Assembled Bases	100MB
n50	1600bp
n90	520bp
AT content Range	84-94%
At Content Average	87%
Total Amount of Bases	176 Million
Total Assembled Bases	35GB
Transcripts Assemblies > 300BP	25935
Transcripts with BLASTX evalue < e-5	10449
Average Length of Blastx Transcripts	1075bp
Number of Consensus Models	14564
Average Length of Model with top hit of e-5 or less	349 AA

PFAM Domain e-4 or less	Number of Unquie Models
Glycoside Hydrolase Models	392
CBM PFAM Models	588
Carboxylesterase PFAM Models	83
Pectin Metabolism	40
Polysaccharide De-Acetlyase	59
Sugar and Other Transporters	65

GH	Number In C1A	CAZy Group Substrate Specificities
GH10	47	HemiCellulose
GH11	44	HemiCellulose
GH5	38	Mixed Cellulose/HemiCellulose
GH43	36	HemiCellulose
GH6	30	Cellulose
GH48	26	Cellulose/Chitanse
GH3	24	HemiCellulose
GH13	21	Othe Sugar Mixed
GH45	20	Cellulose
GH9	18	Cellulose/ Other Sugar mixed
GH31	17	Other Sugars Mixed
GH16	12	Other Sugars Mixed
GH18	11	Other Sugars Mixed
GH1	9	Cellulose/ Other Sugar mixed
GH26	7	HemiCellulose
GH25	5	Lysozyme
GH39	4	HemiCellulose/Other Sugar Mixed
GH47	4	HemiCellulose
GH53	4	HemiCellulose
GH67	3	HemiCellulose
GH8	3	Mixed
GH32	2	Mixed
GH38	2	HemiCellulose
GH57	2	Other Sugars Mixed

STUDENT #1

Conclusions

- Sequencing and analysis of the anaerobic fungus Orpinomyces C1A has yielded a large amount of functionally relevant Bio-Fuel Gene candidates
- Expansion of Endoxylanase/Hemicellulase, Proccesive Cellbiohydrolase (Exocellulase), and Expansin-like Endoglucanases (Cellulase K) differentiate the CAZynome of Orpinomyces from other sequenced organisms currently annotated in the CAZy database.
- Further analysis of gene candidates that contain relevant domains could reveal potentially novel functional groups/classes of Lignocellulose enzymes.

Development of Lowland Inbreds and Upland-Lowland Hybrids in Switchgrass

Laxman Adhikari and Yanqi Wu

Department of Plant and Soil Sciences, Oklahoma State University

Stillwater, Ok 74078.

STUDENT #2

STUDENT #2

Objectives:

- To develop inbreds of lowland switchgrass
- To develop hybrids between upland and lowland switchgrass genotypes
- To examine hybrid switchgrass plants for male sterility

STUDENT #2

Materials and Methods:

- The S2 progenies of lowland genotypes, NL94/85 and SL93/34 are being bagged to produce selfed seeds in a greenhouse.
- Seven upland and lowland plant pairs were made in relatively isolated environments for crossing to produce upland-lowland hybrids.
- Male fertility of first generation (S1) selfed progenies of upland-lowland hybrids will be tested and the sterile genotypes will be used to develop cytoplasmic-genic male sterile lines.

STUDENT #2

Materials and Methods Contd.

Fig . 1: Selfing of lowland switchgrass

Fig . 2: Self pollinated (SL93/34) lowland switchgrass genotype seedlings

Fig . 3: Crossing between lowland and upland switchgrass

STUDENT #2

Expected Results

- Third generation (S3) of two lowland plants: NL94/85 and SL93/34 will be developed.
- Selfed progenies of upland-lowland hybrids with male sterile genes will be identified.
- Genotypes of each selfed and crossed progenies will be identified using simple sequence repeat markers.

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Characterization of a grass mutant with decreased cell wall cross-linking

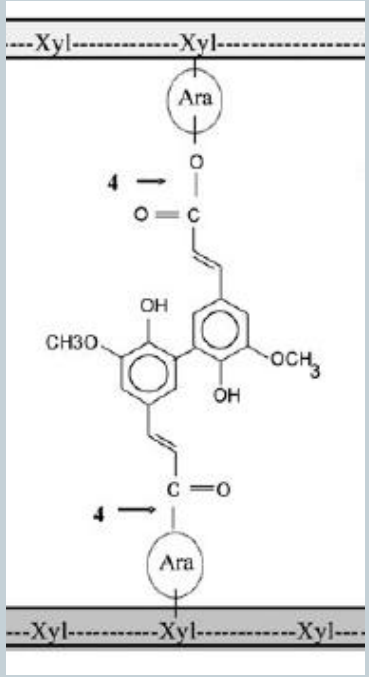
Fan Lin, Laura Bartley
University of Oklahoma

STUDENT #3

STUDENT #3

Characterization of OsAT15-D1 mutant

- Study the function of *Oryza sativa* acyltranferase 15 (OsAT15), a member of subclade ii of a CoA acyltransferase family.
- Confirm decreased ferulic acid incorporation in hemicellulose in T-2 progeny
- Effect of cell wall modification on plant growth and biofuel production



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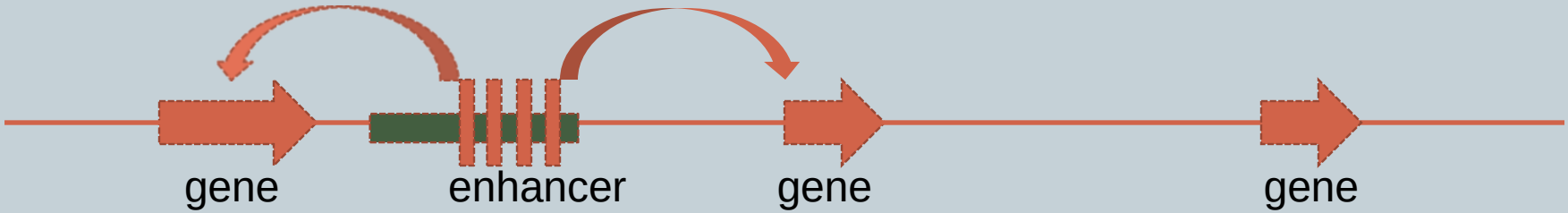
Reverse Genetic Study

T-DNA tagged activation mutant



PCR based genotyping

Confirm increased gene expression in mutant by quantitative reverse transcription PCR (RT-qPCR)

Cell wall preparation-> alkali lysis -> ferulic acid extraction->High-Performance Liquid Chromatography (HPLC)

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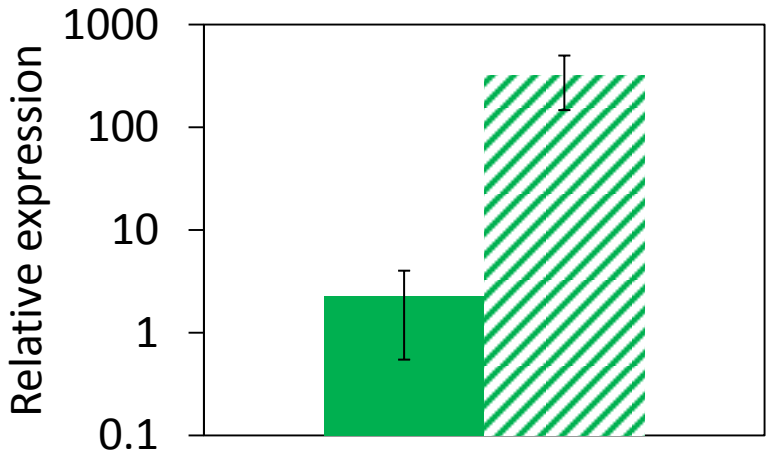
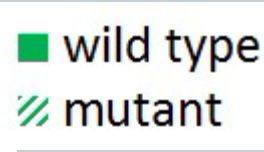
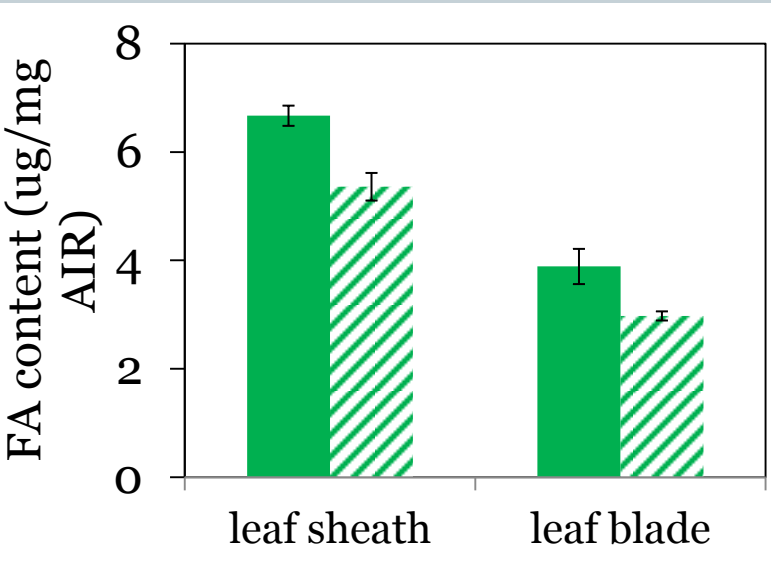
Phenotype of OsAT15 D1-T2 progeny







Adult plants of T2 progeny

STUDENT #3

Conclusions

- OsAT15, the target gene, is up-regulated for 100 fold in mutant plant
- The mutant has a ~20% decrease of ester-linked ferulic acid in relative to near isogenic wild type
- Decreased ferulic acid content does not affect plant growth.

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MULTILOCUS "DNA BARCODES" FOR IDENTIFICATION OF SWITCHGRASS RUST POPULATIONS.

Gabriela Orquera, Kihyuck Choi,
Carla D. Garzon, Stephen M. Marek

Oklahoma State University
Stillwater, Oklahoma, USA

STUDENT #4

STUDENT #4

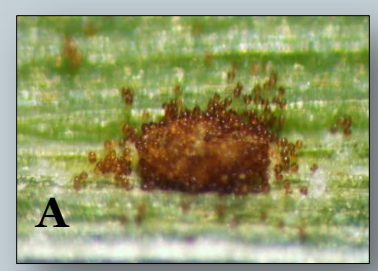
Objectives

- Develop “DNA barcodes” (phylogenetic loci) to identify the fungus or fungi causing switchgrass rust.
  - Internal transcribed spacers of ribosomal DNA (ITS-rDNA)
  - Translation elongation factor-1 α (TEF1a)
  - β-tubulin (bTub)
  - Mitochondrial cytochrome b (cytb)
- Obtain high quality sequences of individual barcode alleles

STUDENT #4

Methods

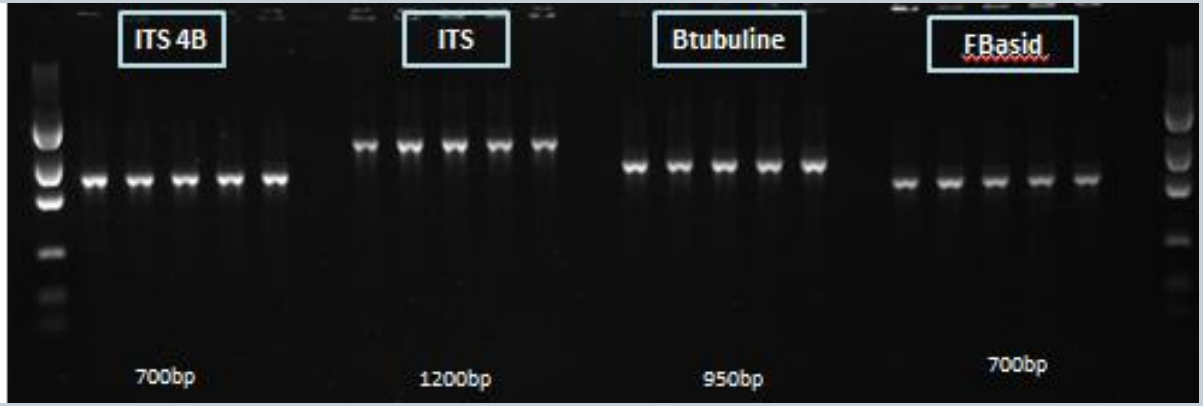
Handling Spores



Select single pustule

Microvaccum and spray

Pustules develop in 7 to 14 days



PCR of DNA barcodes → Sequencing

- **Samples**: Switchgrass rust spores were collected in Oklahoma and Virginia, and subcultured .
- **Molecular methods**: Variability of barcodes in spores disrupts direct PCR and sequencing.

Troubleshooting:

- Approach 1: From each collection, subclone and sequence ≥5 barcode products using TA cloning.
- Approach 2: Whole genome amplification (WGA) of DNA from single rust spores WGA, then amplify and sequence, directly.

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Results

ITS-rDNA

- Consensus 99% identical with *Puccinia emaculata*
- High allelic variability (polymorphism): 37 SNPs and 8 indels
- 1,731 rust taxa with ITS-rDNA in NCBI

TEF1a

- Moderate polymorphism: 7 SNPs and 1 indel
- 60 rust taxa with TEF1a in NCBI

bTub

- Low polymorphism: 2 SNPs
- 180 rust taxa with bTub in NCBI

cytb

- Monomorphic, short (340 bp)
- 20 rust taxa with cytb in NCBI
- Did not distinguish *P. emaculata* from other rust fungi

- Subcloning improved sequences of individual alleles
- WGA from single spores generated working PCR templates, which are expected to be homozygous

STUDENT #4

Conclusions

- Rust fungus barcodes are relatively few in GenBank, compared to other Fungi.
- The ITS-rDNA is the best represented rust barcode in GenBank with over 2,110 accessions (~1,700 spp. from class Pucciniomycetes, rust fungi).
- ITS-rDNA is the best barcode for rust fungi, but challenging to directly amplify and sequence, due to polymorphisms
- Using ITS-rDNA, phylogenetically, *P. emaculata* groups with *P. sorghi*, *P. andropogonis*, and *P. asparagi*.
- WGA of DNA from single spores may improve direct sequencing and eliminate the need for subcloning.

INVESTIGATING CELL WALL CHANGES DURING GRASS LATERAL ROOT EMERGENCE

David Ponder and Laura E. Bartley

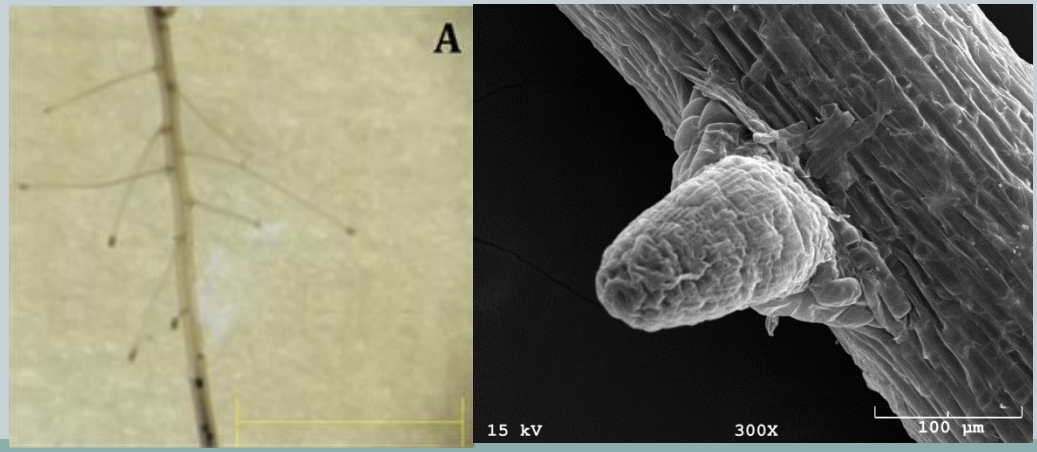
Department of Botany and Microbiology, University of Oklahoma

STUDENT #5

STUDENT #5

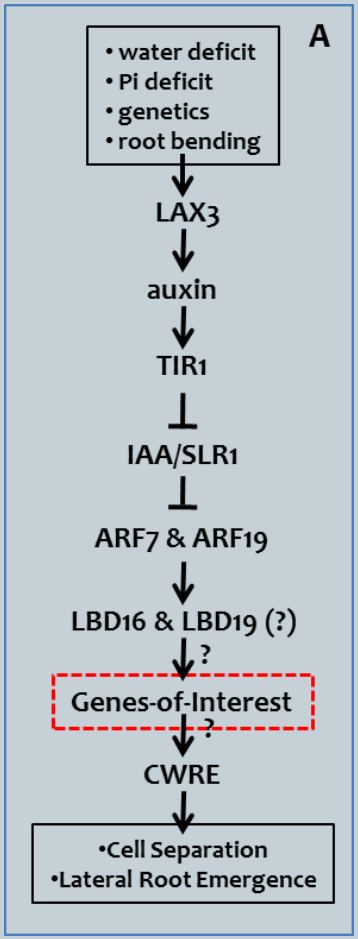
Research Goals

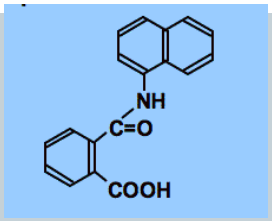
- Determining changes to the cell wall chemical composition that occur during lateral root (LR) emergence in using bulk cell wall analysis
- Identifying the regulatory genes that control the cell wall changes during lateral root emergence using gene expression analysis

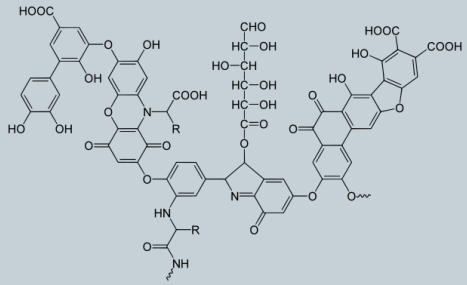


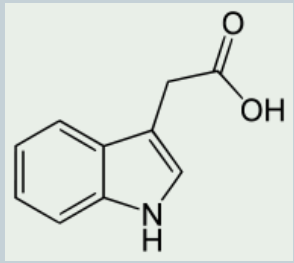
STUDENT #5

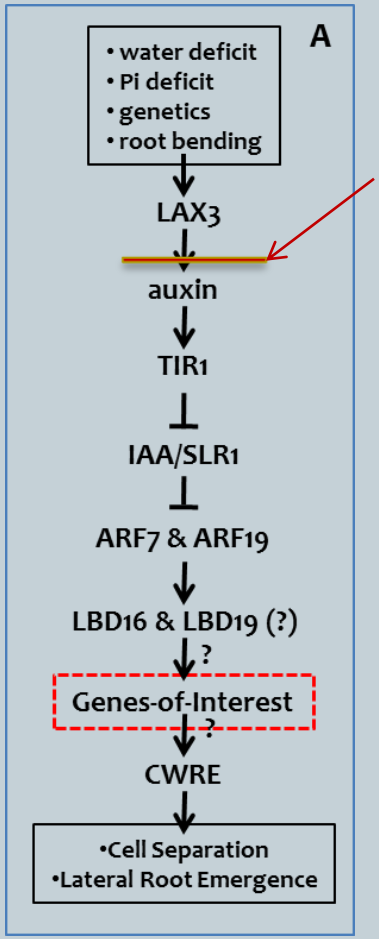
Synchronous lateral root emergence system









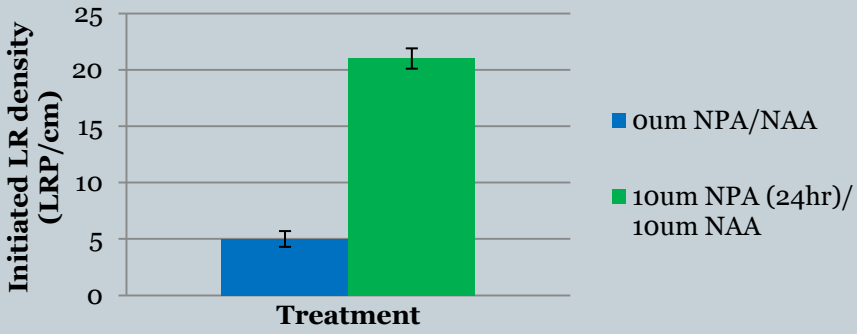


- By using the synchronous LR emergence system the amount of cell wall material undergoing changes related to LR emergence can be maximized
- This system also greatly enhances our ability to locate emerging LRs with microscopy

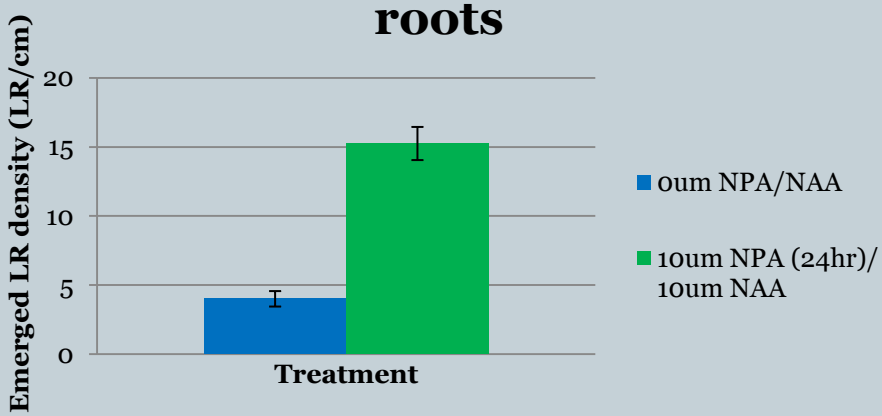
STUDENT #5

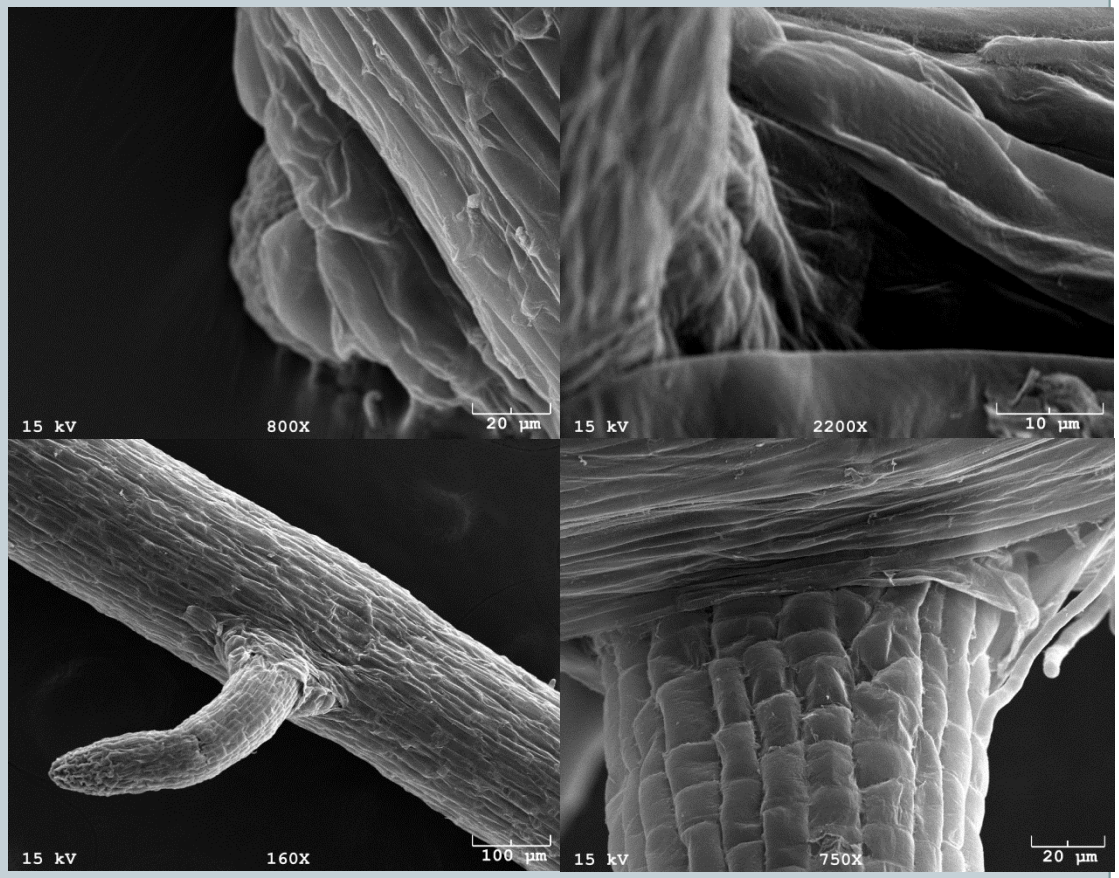
Synchronous LR emergence system improves LR density and aids in microscopic analysis of LR emergence

Treatment improves the number of initiated LRs



Treatment also improves the density of emerged lateral roots





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Conclusions and future plans

- Treatment with auxin inhibitors and exogenous auxin can synchronize LR emergence and improve the density of emerged LRs
- Lateral root emergence involves cell wall loosening, the regulators of which could be used to improve feedstock deconstruction for biofuel production
- Harvest synchronized cell wall material for bulk analysis and genetic material for gene expression analysis

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Gene networks associated with tillering trait in switchgrass

Xin Zeng, Yixing Wang, Yanqi Wu and Ramamurthy Mahalingam

STUDENT #6

STUDENT #6

Objectives

- Objective 1: Assess the extent of transcriptome difference in switchgrass lines with contrasting phenotypes for the tiller number trait using microarray technology.

- Objective 2: Identify gene ontologies unique to high and low tillering lines or between buds and nodal tissues.

- Objective 3: Identify key regulatory factors and signaling genes that are differentially expressed between the high and low tillering lines.

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Methods

Stems from high and low tillering lines were collected from field.

Nodes and buds were carefully sectioned with a scalpel.

RNA was isolated from these tissues.

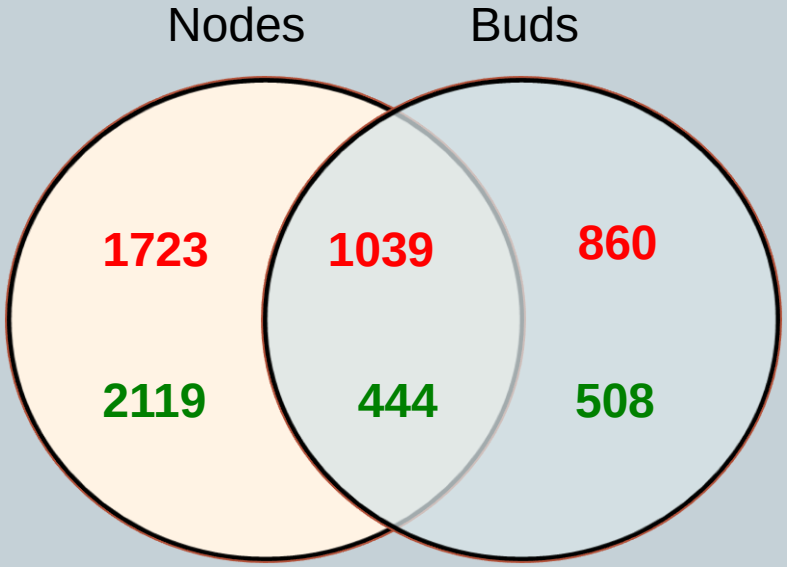
Switchgrass Affymetrix genechips were used for conducting microarray hybridizations.

Data analysis was conducted using the R statistical Package.

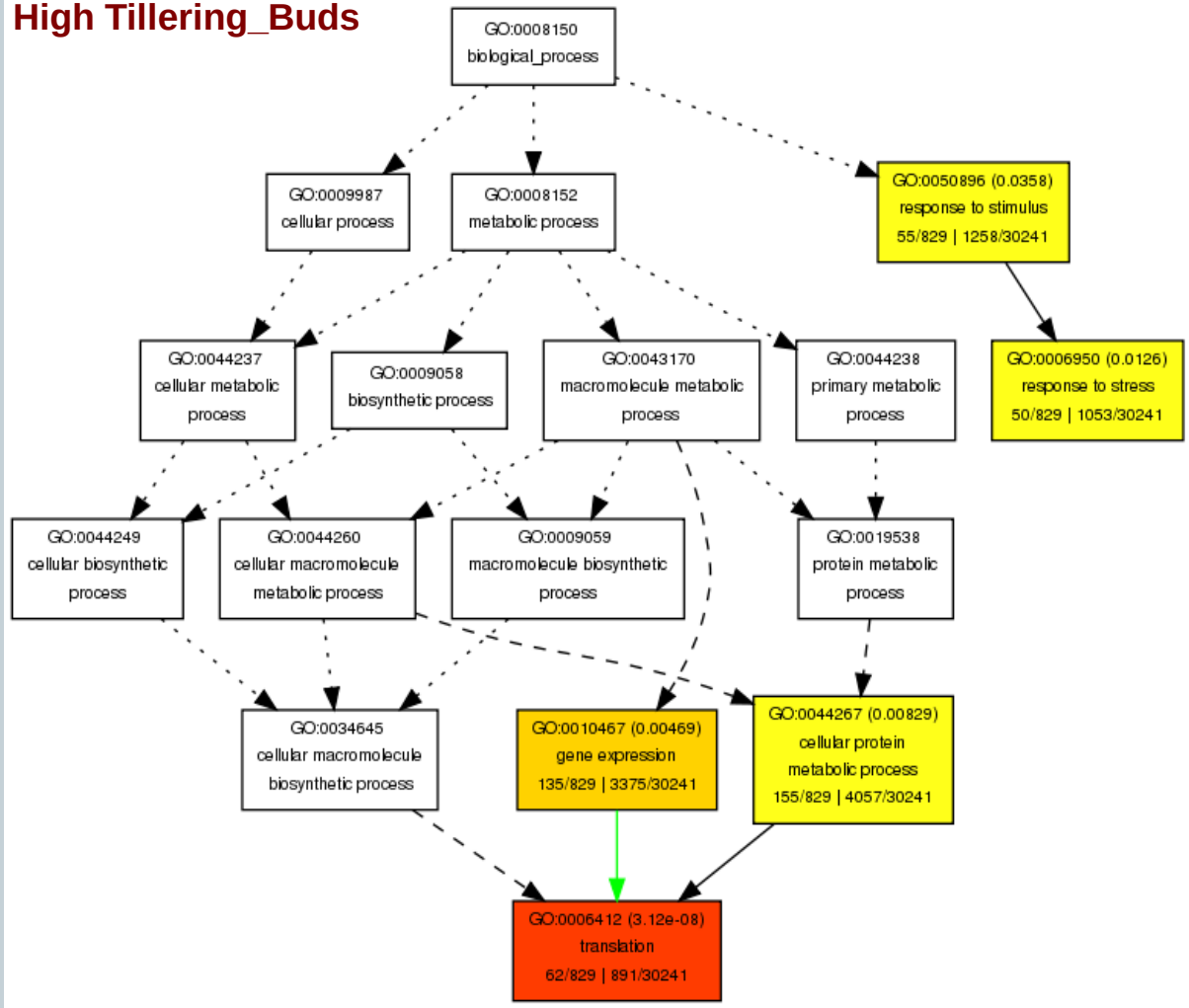
STUDENT #6

Results

	LOW	HIGH
BUDS	8413	9169
NODES	7996	7877



High Tillering_Buds



STUDENT #6

Conclusions

- Conclusion 1: Switchgrass Affymetrix genechips are useful for assessing transcriptome variation.
- Conclusion 2: Differential gene expression between tissues (bud versus node) from a single line is 3-fold more than differential expression between different lines (high versus low tillering) for a given tissue.
- Conclusion 3: GOs specific for high or low tillering line were not found. Gene ontologies associated with translation are significant among genes up regulated in buds while GO for cell death was important in node tissues.

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Identification of Regulatory Genes in the Phenylpropanoid Biosynthesis Pathway by Network Analysis of Rice

Kangmei Zhao, Prasenjit Saha, Laura E.Bartley
Department of Botany and Microbiology
University of Oklahoma

STUDENT #7

U.S. DEPARTMENT OF ENERGY

STUDENT #7

Objective:
Identify Regulators of Grass Cell Wall Cross-linking

Protein Kinase

Transcription Factors

Transcription Factors

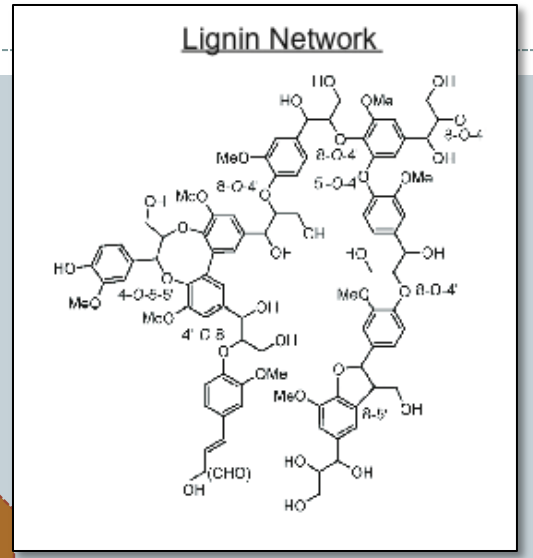
Phenylpropanoid Biosynthesis

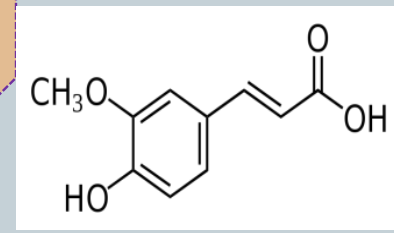
Lignin

Cell Wall Recalcitrance

CoA Aclytranferase

Ferulic Acid





STUDENT #7

Methods: Mine Rice Networks for Novel Cell Wall Regulators

Input: CoA Acyltranferase

Rice Oligonucleotide Array Database

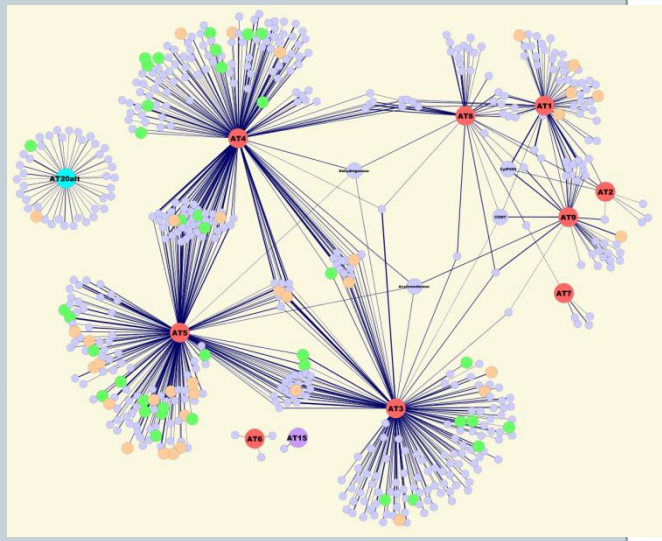
RiceArray: (part of ROAD) Coexpression Net, P.C. Ronald Cao et al. in press *Rice*

RiceNet (part of PlaNet) Coexpression Net, S. Persson Mutwil et al. (2011) *Pl. Cell*

RiceNet
Probabilistic Functional Gene Network of *Oryza sativa*

RiceNet: Homology-Influenced Functional Net I. Lee & P.C. Ronald Lee *et al.* (2011) *PNAS*

Output 1: Example 1-Step (i.e., Level 1Neighborhood)



Output 2: 2-Step Networks (not shown, stats below)

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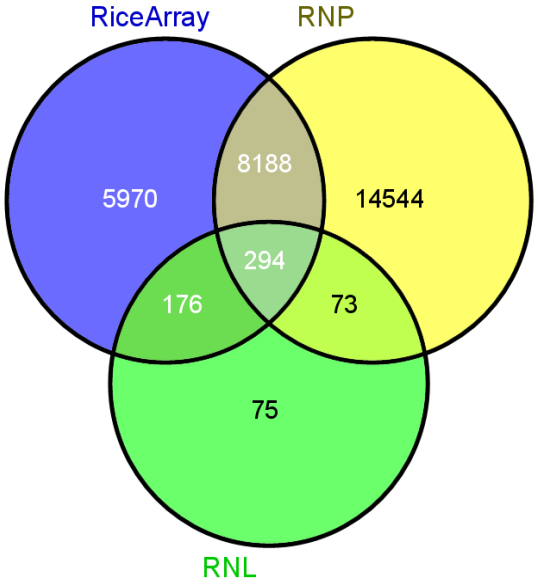
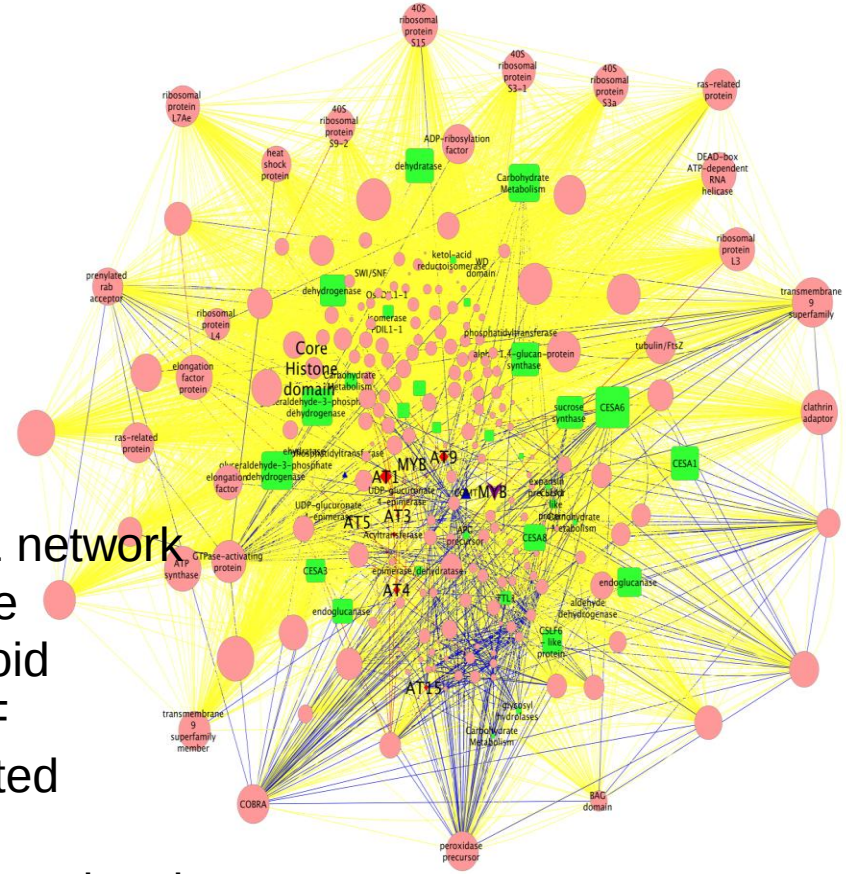
Gene Network for Rice CoA Acyltransferase Genes

Rice Networks Seeded with Acyltransferases Overlap Significantly and Identify 294 Common Genes



hypergeometric
$p < 1X10^{-15}$



RiceArray
RNP
RNL
Curves = in >1 network
Acyltransferase
Phenylpropanoid
2° Cell Wall TF
Cell Wall-Related
Other
Node Size Proportional to Degree
Visualized with Cytoscape

STUDENT #7

Conclusions and Next Step Network Analysis

- Rice network analysis is consistent with a role for the acyltransferases in cell wall modification.
- There are two transcription factors in the network that we are now functionally validating.
- We are working on refining our network analysis to develop a single ranked list of candidate regulatory genes by combining the three networks in to a single network using a general linearized model and other methods.

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Biochar and Biochar-derived Activated Carbon as Catalysts for Syngas Tar Removal

Pushpak Bhandari, Dr. Ajay Kumar, Dr. Danielle Bellmer, and Dr. Raymond Huhnke

Biosystems & Agricultural Engineering, Oklahoma State University

STUDENT #8

STUDENT #8

Objectives

- Synthesis of activated carbon catalysts using gasifier biochar to improve surface properties
- Evaluation of activated carbon and biochar as catalysts for removal of toluene (model tar) from syngas

STUDENT #8

Methods

- Catalyst Synthesis:
  - KOH impregnated biochar
  - Ultrasonication for 30 mins.
  - Carbonization at 600 °C
- Evaluation to Remove Tars:
  - Fixed bed reactor
  - Temperatures: 700 °C, 800 °C
  - Steam to Carbon ratio = 2

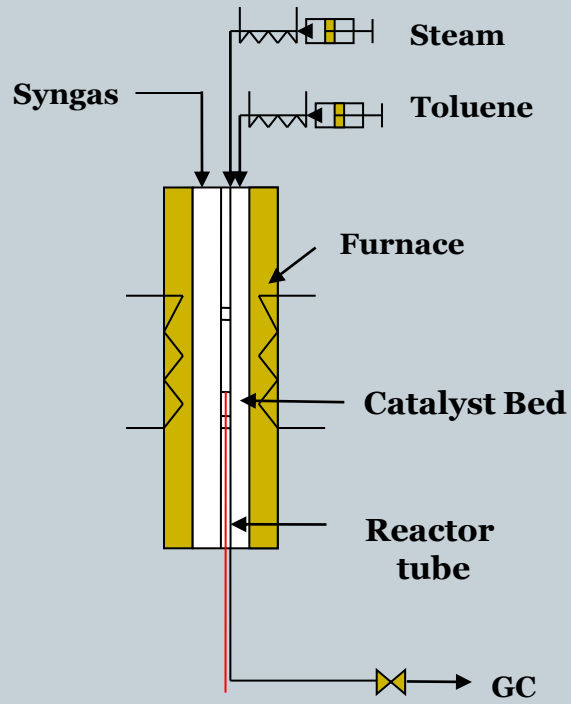

Fig. 1: Process schematic

STUDENT #8

Results

Table 1: Surface and pore characteristics for biochar and activated carbon

Surface Property	Biochar	Activated Carbon
Surface Area (m^2/g)	2.1	900.05
Pore Volume (cc/g)	0.024	0.45
Pore Diameter (A°)	15.5	19.33

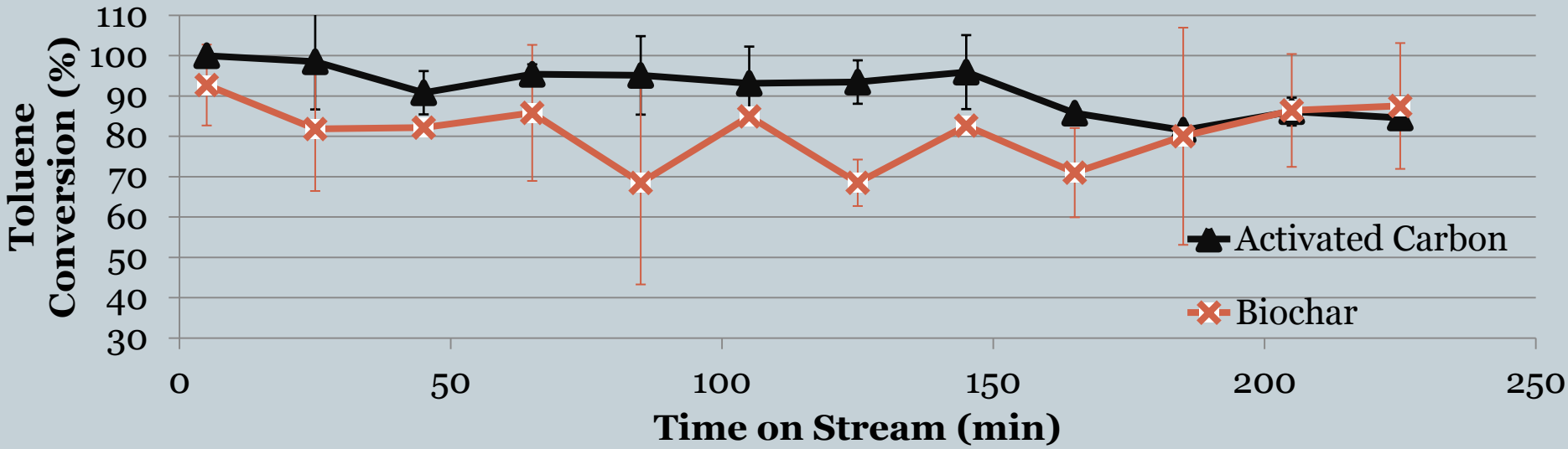

Fig. 2: Conversion of toluene vs. time on stream for catalysts at 800 °C.

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Conclusions

- Activated carbon synthesized from biochar had high surface area (~900 m^2/g) and large pore volume (0.45 cc/g)
- Both, activated carbon and biochar catalysts effectively removed toluene
- Activated carbon showed higher toluene conversion (91.6%) as compared to biochar (81%) at 800 °C.

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Solubility of major producer gas tar compounds in water

Prakash R. Bhoi, Research Engineer;
Krushna N. Patil, Assistant Researcher;
Ajay Kumar, Assistant Professor;
Raymond L. Huhnke, Professor

Biosystems & Agricultural Engineering Department
Oklahoma State University

STUDENT #9

STUDENT #9

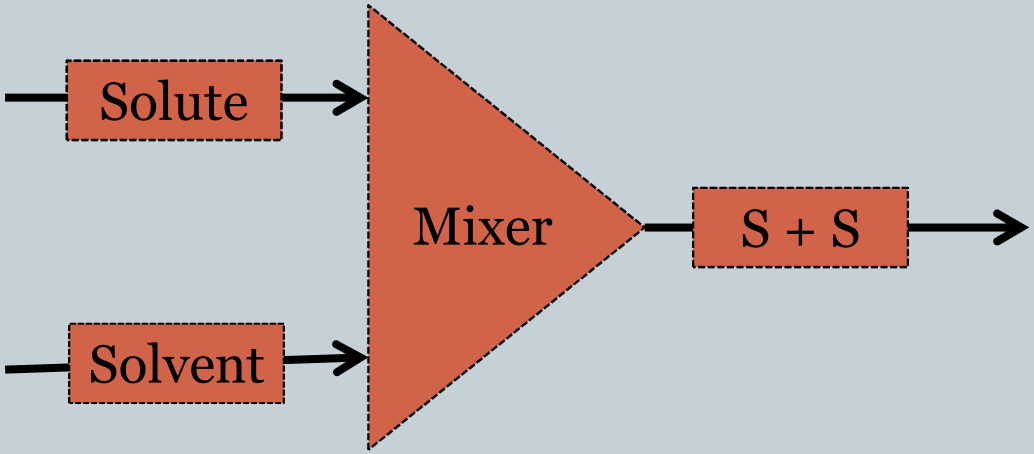
Objectives

- Develop a solubility model in Aspen Plus™ .
- Evaluate the solubility of major producer gas tar compounds in water.

STUDENT #9

Methods

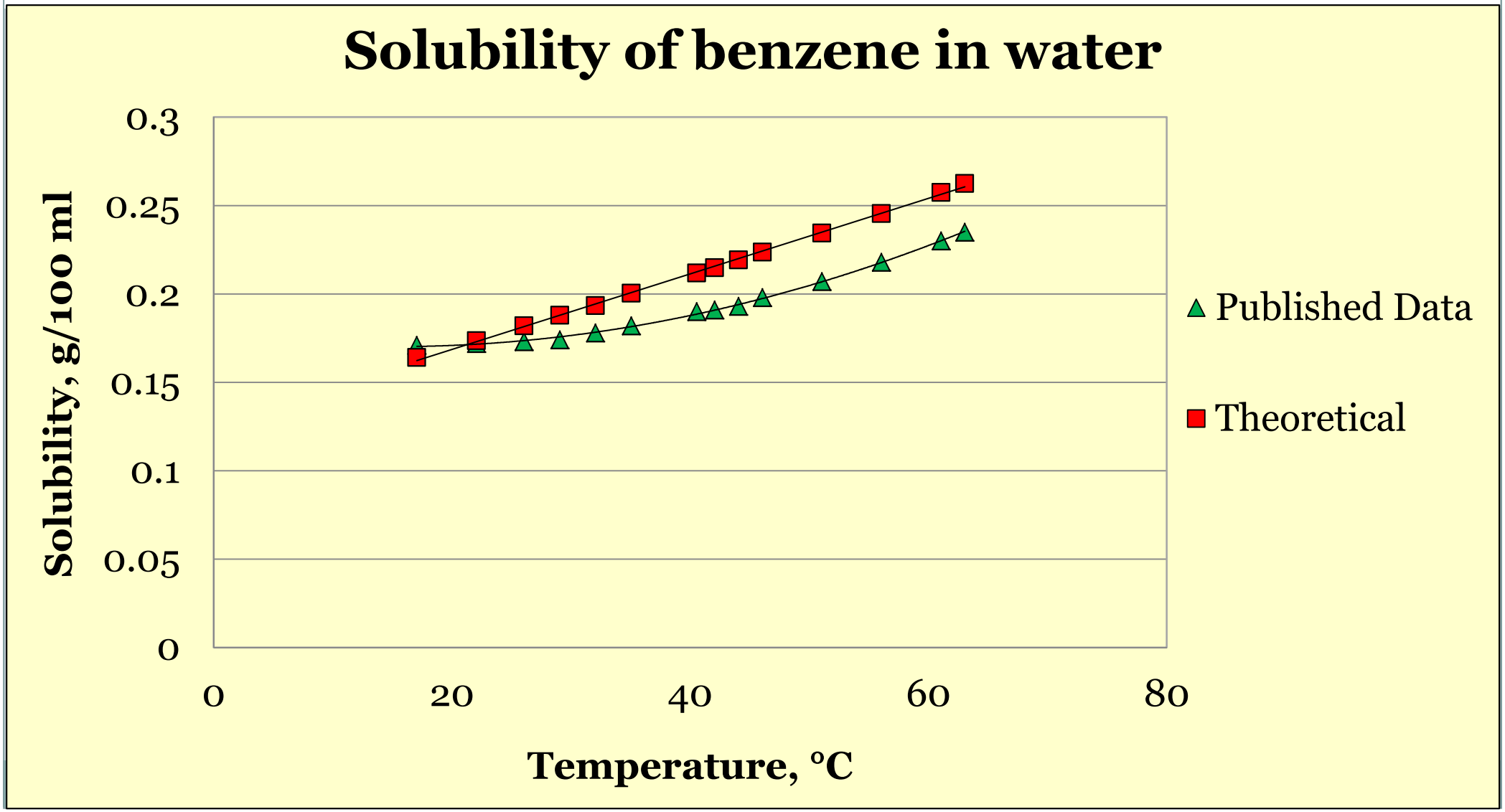
- Activity coefficient model
  - Non-random two-liquid (NRTL)
    - Predict binary coefficients
    - Activity coefficients



- Solubility Model
  - Aspen Plus V7.0 (1981-2008 , Aspen Technologies, Inc.)

STUDENT #9

Results



STUDENT #9

Conclusions

- Model study results are in close agreement with the published data.
- Aspen model predicted a weak solubility for all tar compounds in water.
- Water temperature has a significant effect on the solubility data.

Oklahoma NSF EPSCoR Bioenergy Research and Education

INFLUENCE OF RUTHENIUM TITANIA CATALYST PRETREATMENT CONDITIONS ON THE UPGRADING OF BIOMASS FAST-PYROLYSIS OIL VAPORS

Taiwo Omotoso
Steven Crossley
Daniel Resasco
Richard Mallinson

School of Chemical, Biological and Materials Engineering
University of Oklahoma

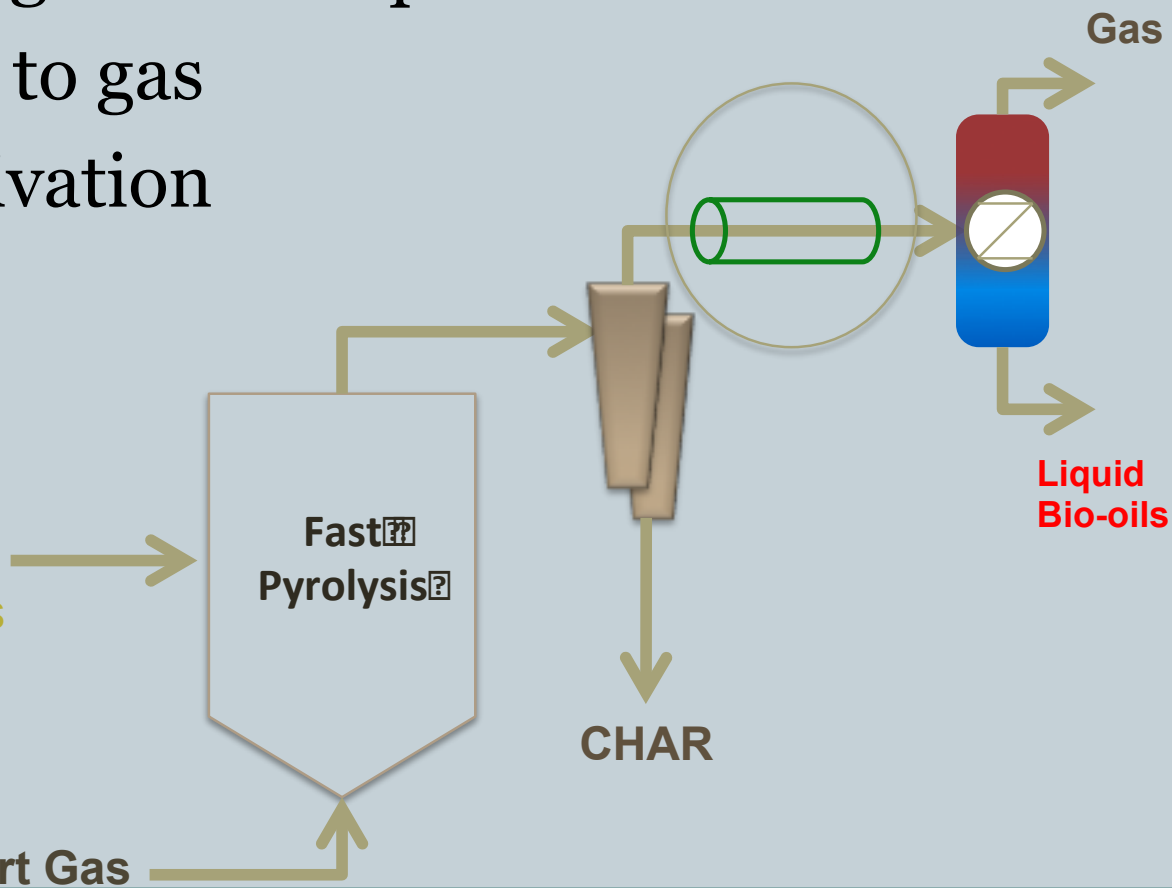
STUDENT #10

STUDENT #10

Objectives

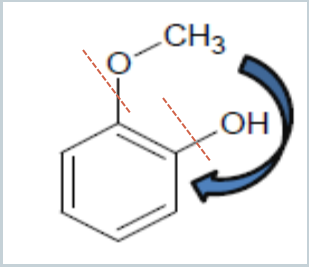
- Catalytically remove oxygen from liquid bio oil
- Minimize loss of carbon to gas
- Minimize catalyst deactivation

BIOMASS
Switch grass
Sawdust, Pine
Torrefied-biomass



STUDENT #10

Methods



→ Model compound, Guaiacol

5wt% Ru/TiO_2

CALCINATION PRETREATMENT

- Calcine under air at 400 °C
- Calcine under air at 500 °C

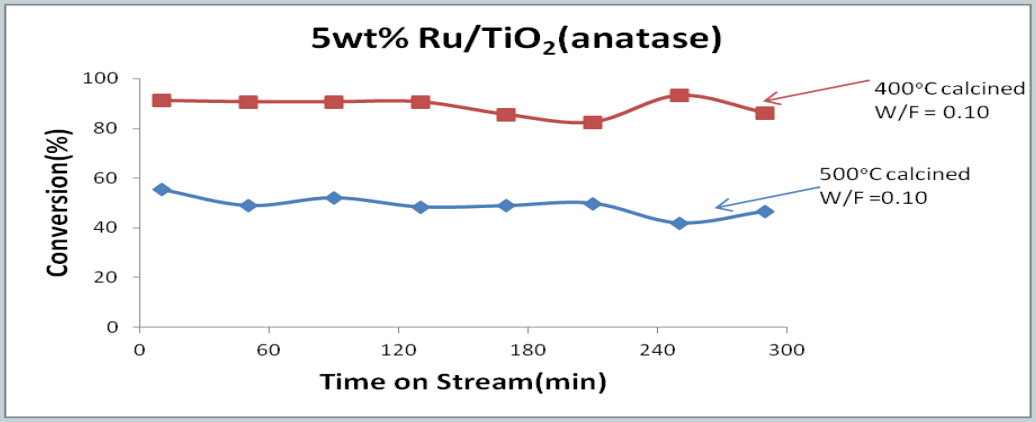
TITANIA SURFACE AREA EFFECT

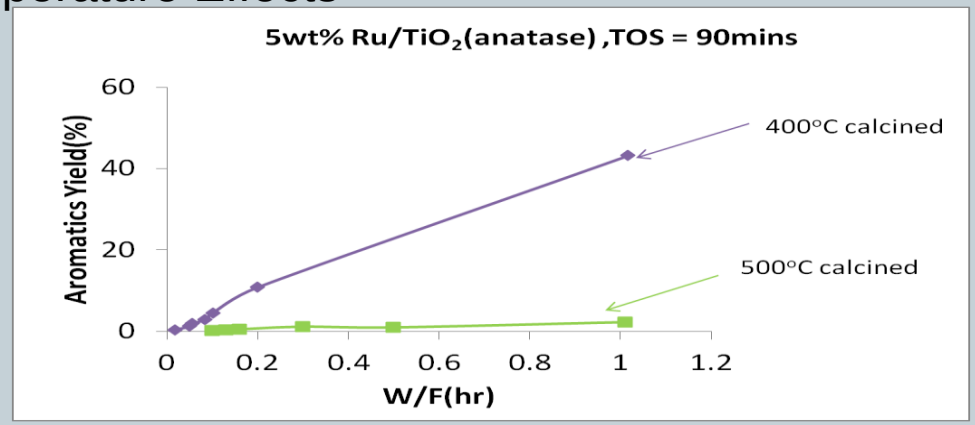
- High surface area pure anatase $160 m^2/g$
- Low surface area P25(anatase/rutile) $50 m^2/g$

STUDENT #10

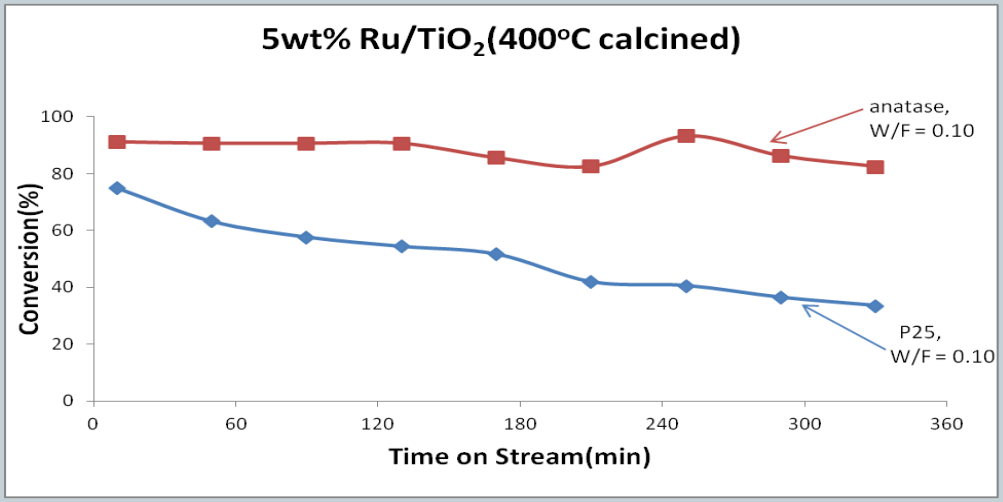
Results

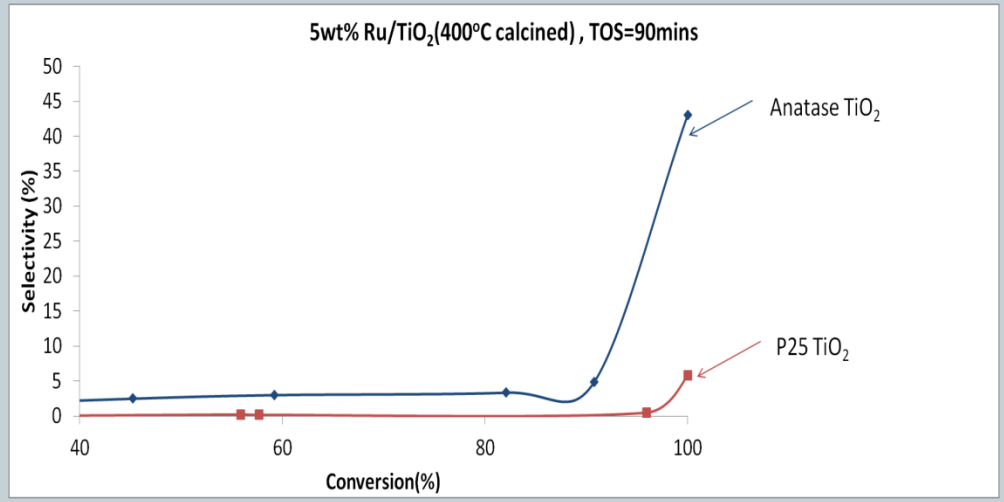
Calcination Temperature Effects





TiO_2 Surface Area Effects





STUDENT #10

Conclusions

- Ru/TiO_2 is an active and stable catalyst for deoxygenation of model phenolic compounds under atmospheric pressure of hydrogen.
- Calcination temperature plays an important role in the activity and selectivity of the Ru/TiO_2 catalyst for catalytic upgrading.
- The nature of the TiO_2 support also has an effect on the stability of the catalyst and product selectivity.

Oklahoma NSF EPSCoR Bioenergy Research and Education

Effect of steam injection location on syngas generated from fluidized-bed gasification of switchgrass

Ashokkumar M Sharma, Research Engineer
Dr. Ajay Kumar, Assistant Professor
Dr. Raymond L. Huhnke, Professor
Biosystems and Agricultural Engineering Department,
Oklahoma State University

STUDENT #11

STUDENT #11

Objectives

- To investigate effect of steam injection location on following:
  - Syngas yield and composition,
  - Syngas tar and particulates contents, and
  - Conversion efficiencies.

STUDENT #11

Methods

- Materials:
  - Air and steam
  - Chopped switchgrass
  - Silica sand

Fig.1 – Materials

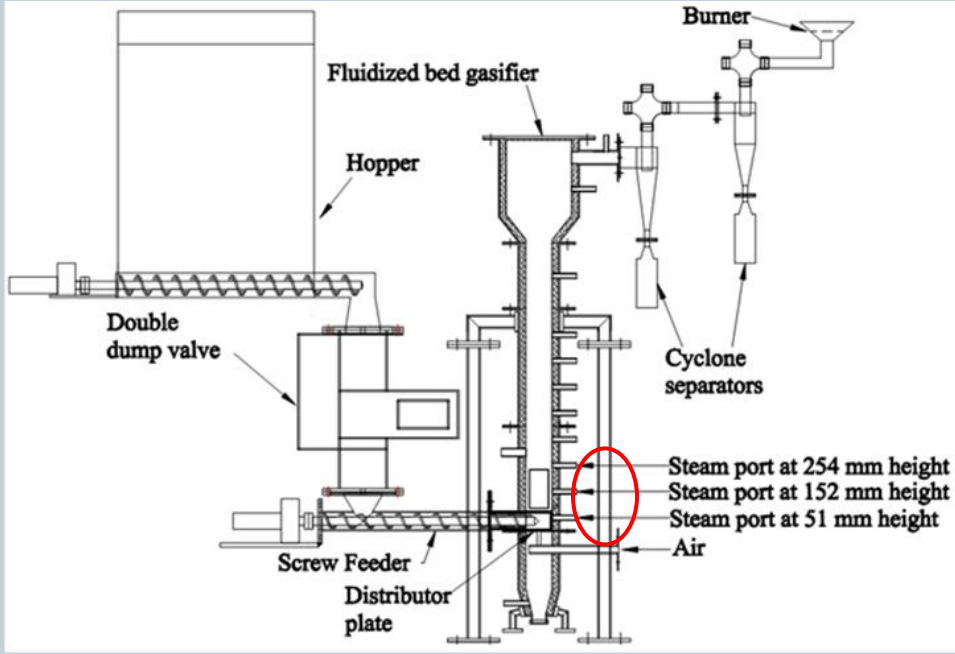


Fig.2 – Test setup

- Operating conditions:

Steam injection location	51 mm	152 mm	254 mm
Steam-to-biomass ratio (SBR)	0.1	0.2	0.3

STUDENT #11

Results

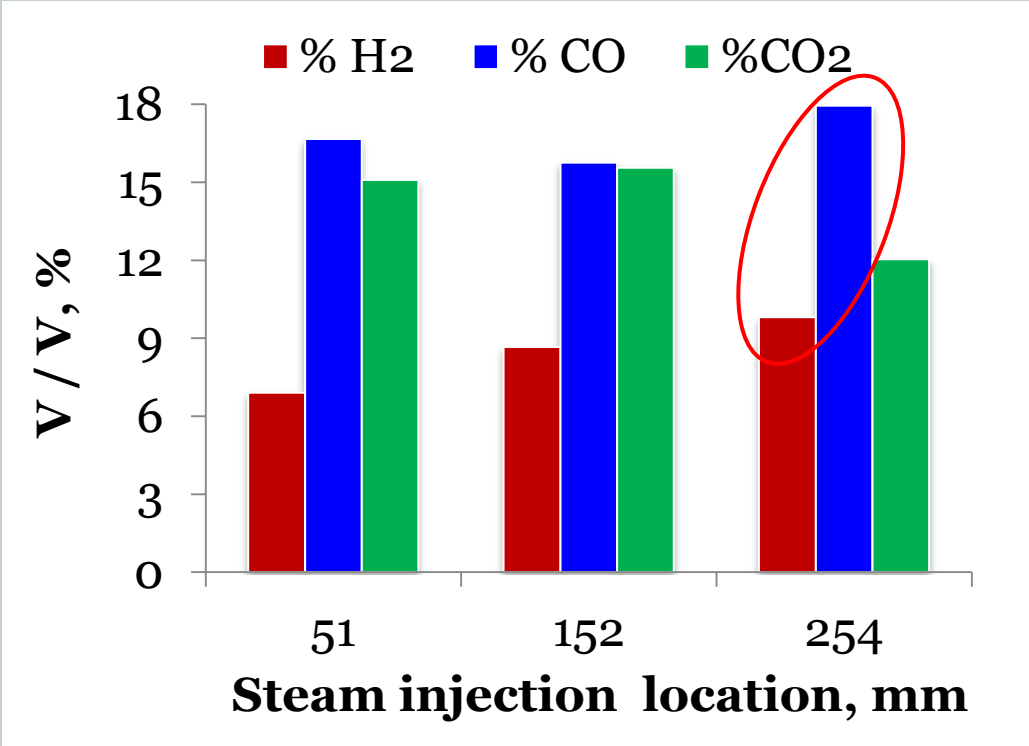


Fig.3 - H_2, CO and CO_2 at three steam injection locations (SBR=0.1)

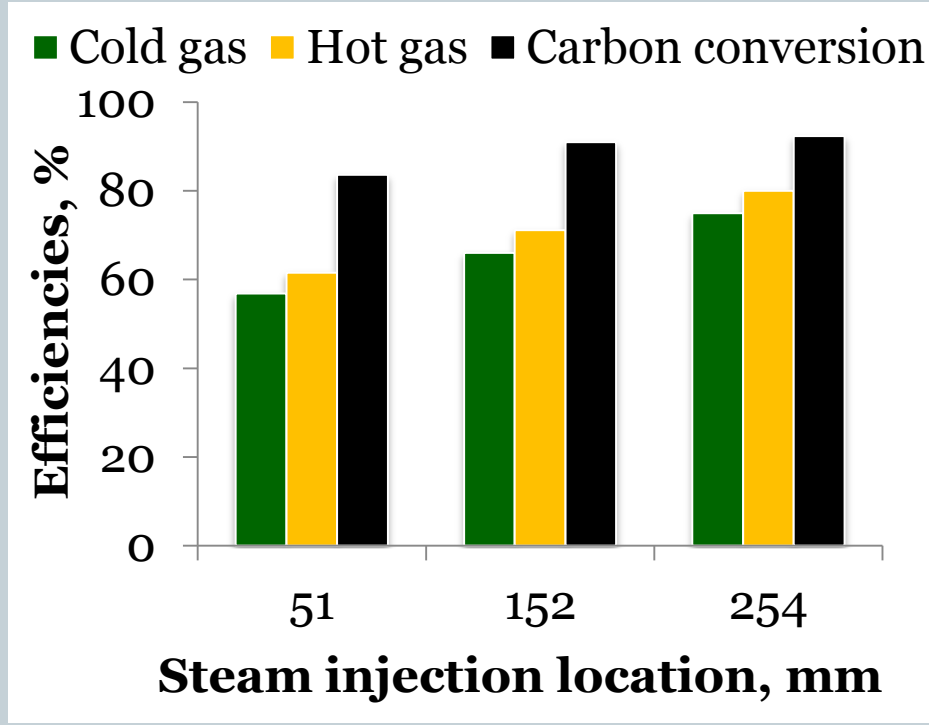


Fig.4 - Gasifier efficiencies at three steam injection locations (SBR=0.1)

STUDENT #11

Conclusions

- Steam injection location showed significant influence ($p < 0.05$) on CO content, and cold gas and hot gas efficiencies.
- Maximum values were observed at following conditions.

Variable	Maximum	Location	SBR
H_2	9.8%	254 mm	0.1
CO	17.9%	254 mm	0.1
Cold gas efficiency	75%	254 mm	0.1
Hot gas efficiency	80%	254 mm	0.1
Carbon conversion efficiency	98%	254 mm	0.3

Oklahoma NSF EPSCoR Bioenergy Research and Education

Hydrophobic zeolites for biofuel upgrading reactions at the liquid-liquid interface in water/oil emulsions

Paula A. Zapata, Jimmy Faria, M. Pilar Ruiz, Rolf E. Jentoft, Daniel E. Resasco

University of Oklahoma, Norman, OK

STUDENT #12

STUDENT #12

Objectives

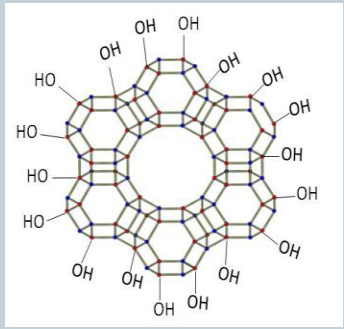
- Synthesize zeolites that would be stable under the effect of liquid hot water and catalyze reactions of bio oil model compounds.

- Develop a catalytic biphasic system that will be used as model to treat the lignin derived fraction and the light products from fast pyrolysis of biomass producing molecules that will be in the range of fuel.

STUDENT #12

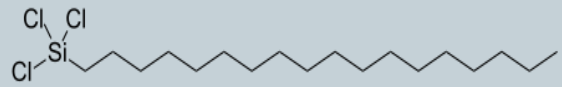
Methods

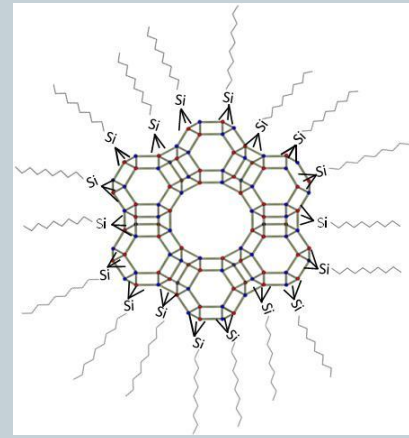
Zeolite Functionalization



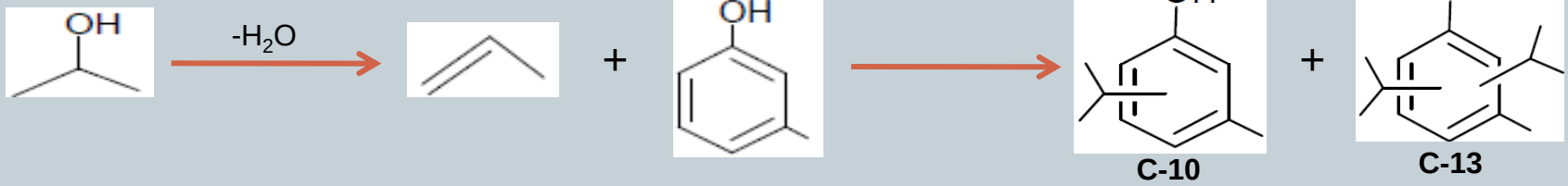
Si/Al: 30

+





Reaction

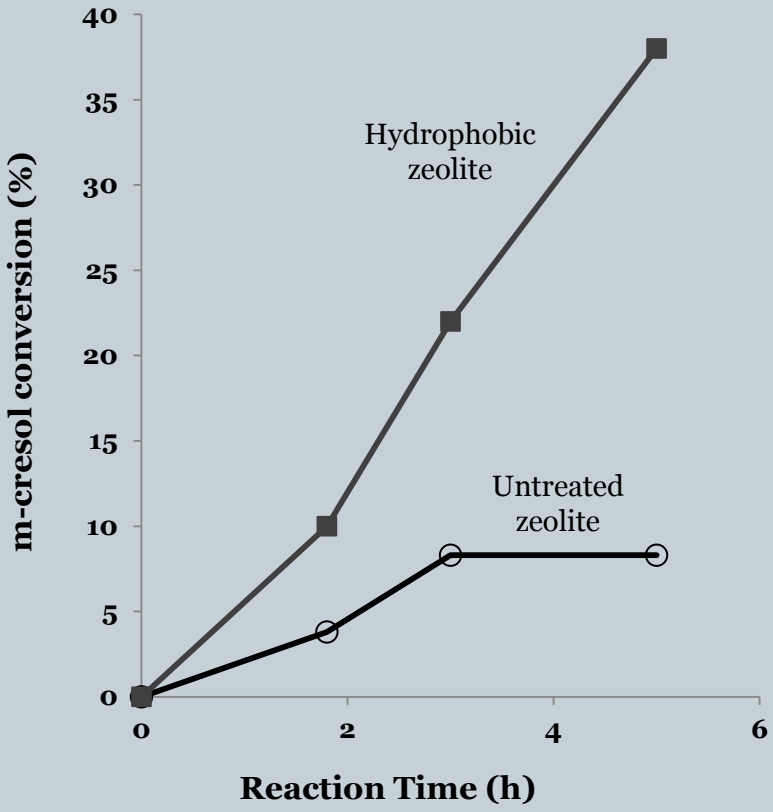


Conditions: 200°C, 400 psig He. Feed: isopropanol/m-cresol molar ratio:3; total molar concentration: 2M.

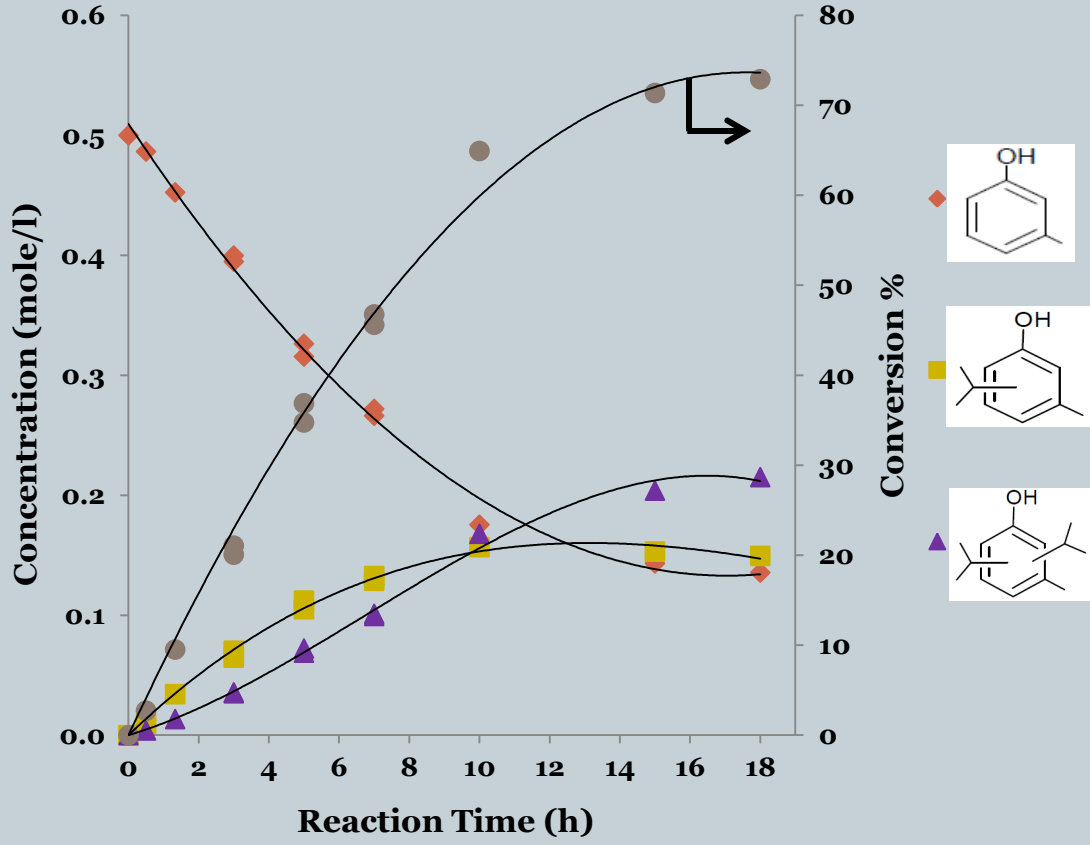
STUDENT #12

Results

Effect of the hydrophobization



m-cresol alkylation with isopropanol over hydrophobic zeolite



STUDENT #12

Conclusions

- Functionalization of the external surface of HY zeolite with hydrophobic octadecyltrichloro silane, gives it the ability to catalyze reactions in hot aqueous media, as is necessary for the refining of biomass pyrolysis oil (bio-oil).

- Modification of the external surface stabilizes the zeolite against losses of crystallinity, greatly enhances the catalytic activity, regenerability, and reusability in liquid biphasic systems.