Participant Individuals:
Senior personnel(s) : Joseph J Bozell; Sheldon May; Charlie Oldham
Graduate student(s) : Paul Kabiru; Veronica De-Silva
Post-doc(s) : Jianguo Zhang
Graduate student(s) : Kamika Felder
CoPrincipal Investigator(s) : Paul L Houston
Graduate student(s) : Rajalaxmi Dash

Partner Organizations:

Other collaborators:

Dr. J. Bozell was at NREL but at the initiation of this project but has since moved this past year to University of Tennessee, Department - Forestry, Wildlife and Fisheries (2506 Jacob Drive, Knoxville, TN 37996-4570). This relocation delayed his contribution, these issues have been addressed and this fiscal year he has been fully involved in his research efforts.

Activities and findings:

Findings:

This program was focused at the chemistry that could be employed to convert biomass biopolymers to bio-based materials and biofuels. In our initial studies, we published several articles highlighting the opportunity that the integrated biorefinery could provide society to address its needs for sustainable development. As highlighted in the Science article by Ragauskas et al. (The Path Forward for Biofuels and Biomaterials. Science (2006), 311(5760), 484-489) an integrated biorefinery is a manufacturing facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass. It seeks to fully utilize all components of biomass to make a range of foods, fuels, chemicals, feeds, materials, heat and power in proportions that maximizes economic return.

Key findings that were developed in this program:

Chemical reaction conditions were determined that permit the development of a new nanostructure of cellulose, nanocellulose balls ranging in size from ~ 350 – 80 nm could be readily synthesized. This new structure of cellulose is currently being examined as a novel material for the delivery of drugs and material applications.

The derivatization of cellulose whiskers (i.e., ~10 nm in diameter and 300 nm length) by periodate oxidation followed by sulfonation yielded a unique green water absorbent. The enhanced water absorption properties were approximately two-fold better that what can be accomplished with mm-based natural fibers. These benefits were attributed to the structural dimensions of the whiskers.

The application of a high-charge of periodate to cellulose followed by
sulfonation was shown to provide a new water soluble form of cellulose that has not been previously reported. Given the commercial applications of water soluble cellulose (i.e. CMC derivatives) we anticipate innovative future applications of this material as a unique green chemical to modify the viscosity of aqueous solutions.

Crosslinking of cellulose whiskers with poly(methyl vinyl ether-co-maleic acid) and poly(ethylene glycol) was shown to yield a novel hydrogel that could absorb +800% of its weight in water. This breakthrough in nanocellulose is notably on several accounts as we have shown that crosslinking cellulose whiskers address a key challenge of self-association of whiskers. Aggregation of whiskers has been a serious challenge in whisker composite manufacturing. This advancement addresses this issue. The novel hydrogel properties provides a new methodology for the production water absorbing films that may have application in the health care and food packaging fields.

An efficient experimental methodology, based on part, on NMR was developed to screen microorganisms for their ability to degrade lignin to green diesel precursors. Three strains of Roseobacter were shown to be viable on an organ-solv lignin resource which provides a novel biorefining pathway for lignin

Molecular Operating Environment modeling was used to create a general tool for determining enantiospecificity for Candida antarctica Lipase B (CALB) and used this computational tool to synthesize novel compounds via Michael addition reactions, that can further be used as monomers in polymerization reactions via enzymatic and non-enzymatic catalyzed reactions.

Developed a quantitative HPLC methodology to monitor metabolite concentrations and reaction time courses during the in vitro enzymatic synthesis of poly-( )-hydroxybutyric acid. These experimental results were then used to develop the first metabolic model of in vitro PHB biosynthesis. All previous modeling studies of PHB production have been designed to analyze in vivo biosyntheses, typically in bacteria but also in yeast and plants. Furthermore, previous simulations were not based on quantitative analyses of the concentrations of the PHB pathway metabolites: acetyl-CoA, CoA, acetoacetyl-CoA, and 8-hydroxybutyryl-CoA. Our results can therefore serve as a basis for further development of the potential of in vitro enzymatic syntheses of polyesters as a compliment to the more widely used approach of in vivo production of these polymers.

Last Set of Publications and Presentations

Publications

Published


Under Review

Preparation of microwave assisted polymer grafted softwood kraft pulp

In Preparation

Poly(methyl vinyl ether-co-maleic acid) – polyethylene glycol nanocomposites crosslinked in-situ with cellulose nanowhiskers. by Goetz, L.; Foston, M.; Mathew, A.J.; Oksman, K.; Ragauskas, A.J.

Synthesis of 2,3-disulfonated derivative of cellulosic beads by Rajalaxmi, D.; Jiang, N.; Gelbaum L.; Ragauskas, A.J.

Presentations:
Ragauskas/GA Tech

Forest Biorefinery: Time to Be. Polysaccharides as a Source of Advanced Materials Conference, Abo Academia, Turku, Finland (Sept, 2009)

Forest BioRefinery Accomplishments, Latvian State Institute of Wood Chemistry (June, 2009)


US Forest Biofinery, Lithuanian Energy Institute, Kaunas, Lithuanian (June, 2009)

Recent Development in US Forest Biorefinery, Kaunas University of Technology, Lithuanian (June, 2009)

Transformational Forest BioRefinery Technologies, 10th Baltic Economic Forum, Riga, Latvia (June, 2009)

Recent Developments in US Forest Biorefinery, Novel Products and Fuels from Forest Trees Seminar Series, Umeå Plant Science Centre, Umeå University, Sweden (May, 2009).

The Biorefinery Concept: Opportunities, Challenges and Innovation, Chalmers University of Technology (May, 2009).

US Perspective on Biorefinery, Forest Products Industry Research College (FPIRC), Royal Institute of Technology, Stockholm (Jan., 2009)

Transformational Forest Biorefinerries Opportunities and Challenges, KETJU (Sustainable Products and Production Conference, Finnish Academy Science (Feb., 2009).

Engineering The Next Generation of LignoCellulosic Fibers, Colloquium Wood Valorisation Epinal, France (Jan., 2009)

Forest Biorefineries Opportunities, Challenges and Innovation, Colloquium Wood Valorisation Epinal, France (Jan., 2009)

Wood Chemistry In The Biorefinery, Booregard, Sarpsborg, Norway (Dec., 2008)

Putting Chemistry Back Into The Biorefinery, Imperial College London, London (Dec., 2008)


J. Bozell/ University Tennessee

Biomass as the New Carbon. Conversion of Renewable Feedstocks into Chemicals and Materials, University of Maine (May, 2008)

New Opportunities for Lignin Conversion in the Biorefinery, International Humic Substances Society - Moscow, Russia (Sept., 2008)

Biomass as the New Carbon, Eastern Kentucky University (Nov., 2008)


Structural Modification of Lignin Using Biochemical Dihydroxylation -Pathways for the Production of Biofuel, Sanyal, N.; Bozell, J.J. SE Regional ACS meeting (SERMACS), Nashville, TN (Nov., 2008)

Biomass as a Source of Renewable Carbon - New Approaches to Chemicals and Fuels, Eastern Kentucky University (Nov., 2008).

Biomass as a Source of Renewable. University of Missouri (Jan., 2009).

Training and Development:

Students and post doctoral research fellows have been educated in fundamental concepts of the integrated biorefinery, sustainable green chemistry of cellulosics and enzymatic synthesis of biopolymers. In addition, the presentations and publications have broadened the impact of our studies to industry representatives and the general public.

The CIBER web site has been public the last year providing general information to students, researchers and the general public at http://ciber.chemistry.gatech.edu and web-site this is linked with addition information at Ragauskas research web site at:
http://www.ipst.gatech.edu/faculty_new/faculty_bios/ragauskas/ragauskas_tech_reviews.html.
This site provides a series of stand-alone technical web-presentations that summarize the fundamental chemistry of biomass resources and their chemistry for conversion to biomaterials and biofuels.

As part of our out-reach program Ragauskas has been interviewed by several public news organizations to comment on the future development of the biorefinery and cellulosic ethanol including Georgia GP TV. In addition, the research results acquired by Lee Goetz (GA Tech chemistry graduate student) during her visit to Professor K. Oksman at Lulea University of Technology in the Division of Manufacturing and Design of Bionanocomposites Skelleftea, Sweden (Note: funded by Center for Innovative Biomaterial Education and Research Supplemental IREE Program) has been accepted for published in Carbohydrate Polymers, 'A novel nanocomposite film prepared from crosslinked cellulosic whiskers,' by L. Goetz, A. Mathew, K. Oksman, P. Gatenholm, and A.J. Ragauskas. ((2009), 78(2), 357-360)

Professor Bozell/University of Tennessee
Dr. Nibedita Sanyal participated in this project as a postdoctoral research associate, and was in charge of incorporating the biochemical procedures into our laboratory regimen. As part of her tenure in our group, Nibedita was able to participate in the following training and mentoring activities:

1. We established a collaboration with Dr. Alison Buchan and Ms. Mary Hadden of the UT Department of Microbiology. Alison and Mary provided one-on-one hands-on training in new microbiological techniques necessary to carry out the research program. This training allowed Nibedita to expand her knowledge base beyond organic chemistry, and played a role in her securing of a new position in a biochemical synthesis laboratory in St. Louis.

2. Nibedita was a participant in our regular research seminar series, and had opportunities to receive feedback on her presentation style, content of seminar and interpretation of her work. This public experience was supplemented with regular weekly meetings to discuss not only research, but how to position herself successfully for the job market, insight with regard to expectations in a new laboratory, and how to move from postdoctoral work into a permanent research job.

3. Nibedita also had opportunity for three public presentations at regional
and national ACS meetings. In preparation for the meetings, our group routinely holds dry runs, as well as providing feedback on the content and style of the presentation. This experience is supplemented with one-on-one discussions prior to the meetings.

4. Our group holds an annual lab for one of the department's classes that involves hands-on preparation of biodiesel. Nibedita has worked directly with these students providing instruction and mentoring during the procedures.

5. We have frequent visitors to our lab from industry and academia. Nibedita has routinely been asked to prepare and present brief summaries of her work to these groups.

Professor Ragauskas/Georgia Institute of Technology

Drs. N. Jiang and J. Zhang were post doctoral research fellows supported by this program. During their productive research studies they were also involved in several professional development activities including:

1. They managed program safety programs for the Ragauskas team, reviewing safety procedures, developing Standardized Operating Procedures and helping orientate new students and visitors studying in Ragauskas research program.

2. The Institute continues to manage an industrial forest products consortium program which hosts industry review sessions in the spring and fall semesters. Drs. Jiang and Zhang presented research updates to these workshops and discussed future research opportunities while supported by this program.

3. Ragauskas' research team holds monthly group meetings to review group research accomplishments and relevant literature developments. The two postdocs hosted several of these meetings, guided discussions and provided critical data analysis insight.

4. Dr Zhang presented the results of his investigations at the 231st ACS National Meeting, Atlanta, GA, (March, 2006), titled 'The synthesis of carbohydrates in ionic liquids'

5. During their tenure on this program Drs. Jiang and Zhang also assisted graduate students in their research studies, aided in proposal development, reviewed manuscripts for several journals and reviewed student manuscripts.

Outreach Activities:

Technical outreach has been accomplished through three publicly accessible websites that summarize a host of information regarding biomass and its conversion to value-added materials. Please see:

- http://ciber.chemistry.gatech.edu
- http://www.ipst.gatech.edu/faculty_new/faculty_bios/ragauskas/ragauskas_tech_reviews.html
- http://www.ipst.gatech.edu/faculty_new/faculty_bios/ragauskas/ragauskas_posters.html
- http://www.ipst.gatech.edu/faculty_new/faculty_bios/ragauskas/ragauskas_tech_reviews.html

CIBER Project Presentations

- Ragauskas/GA Tech
- Forest Biorefinery: Time to Be. Polysaccharides as a Source of Advanced Materials Conference, Abo Academia, Turku, Finland (Sept, 2009)
- Forest BioRefinery Accomplishments, Latvian State Institute of Wood Chemistry (June, 2009)
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- Putting Chemistry Back Into The Biorefinery, Imperial College London, London (Dec., 2008)

J. Bozell/ University Tennessee
- Biomass as the New Carbon. Conversion of Renewable Feedstocks into Chemicals and Materials, University of Maine (May, 2008) (Previously unreported)
- New Opportunity for Lignin Conversion in the BioRefinery, International Humic Substances Society - Moscow, Russia (Sept., 2008)
- Biomass as the New Carbon, Eastern Kentucky University (Nov., 2008) (Previously unreported)
- Structural Modification of Lignin Using Biochemical Dihydroxylation Pathways for the Production of Biofuel, Sanyal, N.; Bozell, J.J. SE Regional ACS meeting (SERMACS), Nashville, TN (Nov., 2008)
- Biomass as a Source of Renewable. University of Missouri (Jan., 2009).
- Developing the new lignocellulosic energy age. 235th ACS National Meeting, New Orleans, LA, United States (April, 2008)
- Tying cellulose whiskers together. 235th ACS National Meeting, New Orleans, LA, United States (April, 2008)
- The 30th Symposium on Biotechnology for Fuels and Chemicals, New Orleans, LA (May, 2008)
- NSF PFI Workshop, Arlington, Virginia (April, 2008)
- US Perspective on BioRefinery, STFI-Packforsk/KTH (Jan., 2008)
- Forest BioRefinery At the Cross Roads of Science, Engineering and Innovation, Mid Sweden University (Jan., 2008)
- Lignocellulose Chemistry: Lonesome BioPolymer to Key BioFuel Resource. School of Polymer, Textile and Fiber Engineering, GT (Jan., 2008)
- Seeing Fibers in the Pulp Forest, Lectures at the Leading Edge, Department of Chemical Engineering and Applied Chemistry, University of Toronto (Oct., 2007)
- Over View of Cellulosic Material Research. Industry Days IPST@GT (Nov., 2007)
- Forest Biorefineries: Pulp-Lumber-Biofuels Third Leg of Forest Products Industry, TAPPI Gulf Coast Conference, Auburn University (Oct., 2007)
- The New Lignocellulosic Age, Luleå University of Technology, Skellefteå Sweden (Sept., 2007)
Journal Publications:


Book(s) of other one-time publications(s):

Other Specific Products:

2nd web site summarizing select program accomplishments

http://ipstgatech.edu/faculty_new/faculty_bios/ragauskas/student_presentations/index.html
Internet Dissemination:

http://ciber.chemistry.gatech.edu

A website has been created to provide general and scientific information on biomass sources, biomaterials, polymers, and enzymes. The site includes a continuously expanding searchable database of literature abstracts covering the areas mentioned above. A glossary of definitions is included to help those readers who are not familiar with the area of biomaterials. This website is designed to be useful to high school readers as well as scientists in the field.

The CIBER website design has a navigation pane on the left side with major topics that expand as the links are followed. A search application appears in the upper right corner that allows the viewer to quickly search the entire website for a specific term or phrase. There is an option to print or email the viewed page and breadcrumbs appear in the upper bar to allow the viewer to trace their path through the website. These features are seen on every page of the website containing content.

A Teacher’s Resource page has been added in order to provide teachers of all levels (K-12) with an easily accessible compilation of websites containing numerous activities, lesson plans, and laboratory experiments for use in teaching biomaterials and biomass sources and conversion. The websites included in this list are reputable sources of facts and information on the topics and include the U.S. Dept. of Energy and the Energy Information Administration. Linked to this page is a list of downloadable PDFs of classroom activities and laboratory experiments aimed towards high school teachers.

The CIBER website is constantly being updated and is serving as a tool to introduce the area of biomass conversion to the public and to focus attention on the major problems that still remain to be solved by researchers in this area. As seen from the most recent 6-month statistics, generated by Awstats Website, the site is being used by an average of 243 different users each month and is generating an average of 26,865 hits per month. Navigations to the website have been referred by search engines such as Google, Yahoo and Bing.

Contributions:

Contributions within Discipline:

Our Science article 'The Path Forward for Biofuels and Biomaterials'(2006), 311(5760), 484-489 has been noted by Essential Science Indicators as a highly cited paper in the Special Topic of biofuels.

In light of Ragauskas expertise and accomplishments in the Modern Cellulosic Biorefinery, funded in part by this NSF program, he was awarded the Fulbright Distinguished Chair in Alternative Energy Technology at Chalmers.

Contributions Within Discipline

This program was focused at the chemistry that could be employed to convert biomass biopolymers to bio-based materials and biofuels. In our initial studies, we published several articles highlighting the opportunity that the integrated biorefinery could provide society to address its needs for sustainable development. As highlighted in the Science article by Ragauskas et al. (The Path Forward for Biofuels and Biomaterials. Science
an integrated biorefinery is a manufacturing facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass. It seeks to fully utilize all components of biomass to make a range of foods, fuels, chemicals, feeds, materials, heat and power in proportions that maximizes economic return.

Key findings that were developed in this program:

• Chemical reaction conditions were determined that permit the development of a new nanostructure of cellulose, nanocellulose. Balls ranging in size from ~350 to 80 nm could be readily synthesized. This new structure of cellulose is currently being examined as a novel material for the delivery of drugs and materials applications.

• The derivatization of cellulose whiskers (i.e., ~10 nm in diameter and 300 nm length) by periodate oxidation followed by sulfonation yielded a unique green water absorbent. The enhanced water absorption properties were approximately two-fold better than what can be accomplished with mm-based natural fibers. These benefits were attributed to the structural dimensions of the whiskers.

• The application of a high-charge of periodate to cellulose followed by sulfonation was shown to provide a new water soluble form of cellulose that has not been previously reported. Given the commercial applications of water soluble cellulose (i.e., CMC derivatives) we anticipate innovative future applications of this material as a unique green chemical to modify the viscosity of aqueous solutions.

• Crosslinking of cellulose whiskers with poly(methyl vinyl ether-co-maleic acid) and poly(ethylene glycol) was shown to yield a novel hydrogel that could absorb ~800% of its weight in water. This breakthrough in nanocellulose is notably on several accounts as we have shown that crosslinking cellulose whiskers address a key challenge of self-association of whiskers. Aggregation of whiskers has been a serious challenge in whisker composite manufacturing. This advancement addresses this issue. The novel hydrogel properties provide a new methodology for the production water absorbing films that may have application in the health care and food packaging fields.

• An efficient experimental methodology, based on part, on NMR was developed to screen microorganisms for their ability to degrade lignin to green diesel precursors. Three strains of Roseobacter were shown to be viable on an organ-solv lignin resource which

• Molecular Operating Environment modeling was used to create a general tool for determining enantiospecificity for Candida antarctica Lipase B (CALB) and used this computational tool to synthesize novel compounds via Michael addition reactions, that can further be used as monomers in polymerization reactions via enzymatic and non-enzymatic catalyzed reactions.

• Developed a quantitative HPLC methodology to monitor metabolite concentrations and reaction time courses during the in vitro enzymatic synthesis of poly-(fD)-hydroxybutyric acid. These experimental results were then used to develop the first metabolic model of in vitro PHB biosynthesis. All previous modeling studies of PHB production have been designed to analyze in vivo biosyntheses, typically in bacteria but also in yeast and plants. Furthermore, previous simulations were not based on quantitative analyses of the concentrations of the PHB pathway metabolites: acetyl-CoA, CoA, acetoacetyl-CoA, and \( \beta \)-hydroxybutyryl-CoA. Our results can therefore serve as a basis for further development of the potential of in vitro enzymatic synthesis of polyesters as a compliment to the more widely used approach of in vivo production of these polymers.

Contributions to Other Disciplines:

Ragauskas is working with Bach Energy and C2-Biofuels to help commercialize the conversion of biomass to biofuels.

Ragauskas has been invited to present overview of these studies to several other disciplines including:

1. Developing the new lignocellulosic energy age. 235th ACS National Meeting, New Orleans, LA (April, 2008)
Our studies have been cited well within our field of study and their impact on the biorefinery has had a broad, multidisciplinary impact. The Science article by Ragauskas et al. titled 'The path forward for biofuels and biomaterials' (2006), 311(5760), 484-489 is noted by Essential Science Indicators as a highly cited paper in the Special Topic of biofuels. It has been cited 390 times in a host of different fields including plant science, engineering, biochemistry, biotechnology and climate change. Listed below are a few select publications that have cited this publication and are representative of the impact of our studies in a variety of different disciplines from nationally and internationally institutions:

Switchgrass (Panicum virgatum) possesses a divergent family of cinnamoyl CoA reductases with distinct biochemical properties. Escamilla-Trevino, Luis L.; Shen, Hui; Uppalapati, Srinivasa Rao; Ray, Tui; Tang, Yuhong; Hernandez, Timothy; Yin, Yanbin; Xu, Ying; Dixon, Richard A. Samuel Roberts Noble Foundation, New Phytologist (2010), 185(1), 143-155.

Genetic resources for maize cell wall biology. Penning, Bryan W.; Hunter, Charles T.; III; Tayengwa, Reuben; Eveland, Andrea L.; Dugard, Christopher K.; Olek, Anna T.; Vermerris, Wilfred; Koch, Karen E.; McCarty, Donald R.; Davis, Mark F.; Thomas, Steven R.; McCann, Maureen C.; Carpita, Nicholas C. Purdue University, Plant Physiology (2009), 151(4), 1703-1728.

Life Cycle Emissions and Cost of Producing Electricity from Coal, Natural Gas, and Wood Pellets in Ontario, Canada. Zhang, Yimin; McKechnie, Jon; Cormier, Lynne; Lyman, Robert; Mabee, Warren; Ogino, Akitumi; MacLean, Heather L. Department of Civil Engineering and School of Public Policy and governance, University of Toronto, Environmental Science & Technology (2010), 44(1), 538-544. Tuercke, T.; Panic, S.; Loebbecke, S.


Synergy in the hybrid thermochemical-biological processes for liquid fuel production. Agrawal, Rakesh; Singh, Navneet R.; Ribeiro, Fabio H.; Delgass, W. Nicholas; Perkis, David F.; Tyner, Wallace E. School of Chemical Engineering and Energy Center, Purdue University, Computers & Chemical Engineering (2009), 33(12), 2012-2017.
Contributions to Education and Human Resources:

4. US Perspective on Biorefinery, STFI-Packforsk/KTH (Jan., 2008).
5. Forest BioRefinery At the Cross Roads of Science, Engineering and Innovation, Mid Sweden University (Jan., 2008).
6. The New Lignocellulosic Age, Luleå University of Technology, Skellefteå Sweden (Sept., 2007).
11. Seeing Fibers in the Pulp Forest, Lectures at the Leading Edge, Department of Chemical Engineering and Applied Chemistry, University of Toronto (Oct., 2007)
13. BioEnergy Science Center: DOE Bioenergy Research Center, IPST@GT (Sept., 2007)

This program continues to have a strong element of human resource development including:
• We have trained several students and technicians in the concepts of sustainable biomass chemistry that are directly involved in this project.

Research outcomes have been shared with a consortium of forest products representatives at annual spring program review meetings held at the Institute of Paper Science and Technology

Ragauskas has took several GA Tech graduate students, an academic professional, and a high school student to Portugal to exchange issues of sustainability with students, faculty and industrialists in Portugal. The travelers included: Dr. Arthur J. Ragauskas, Ms. Tina Dreaden, Dr. Cameron Tyson, Mr. Matyas Kosa, Mr. Gregory Marr, Ms. Kathy Woody. Mr. Anthony Baldridge and Ms. Christina Young. This team visited the following institutions in Portugal:
• Universidade da Beiria Interior
• University of Aveiro
• RAIZ; Research Institute of Forestry and Paper
• Portucel Soporcel group
• University of Coimbra

At each site the participant’s seminars and discussed issues of sustainability. An overview of the GT presentations can be found at:

http://ipst.gatech.edu/faculty_new/faculty_bios/ragauskas/student_presentations/index.html

Representative letters of review of the overall Portugal sustainability trip are presented below:

Participant 1: As part of an NSF exchange program, Georgia Tech students were given the opportunity to travel to Portugal to discuss current research and education within the areas of biomaterials and fuels under the Center for Innovative Biomaterial Education and Research (CIBER). I was fortunate to be chosen for this trip and throughout the course of the entire journey, I found myself surprised at the amount of research being done on renewable materials, especially trees. Renewable sources of energy and materials provide something that is coveted by all, sustainability.
Throughout the course of the trip, one of the major themes of all presentations was the notion of sustainability. At about the midpoint of the trip, the group visited a paper mill on the outskirts of Aveiro. In Portugal, the paper industry is a central part of the country’s economy. Within this paper mill the bark that is removed from the tree trunks is used to produce electricity for the plant, yet with their engineering, the plant facility is able to generate 120 MW of energy but only requires 80 MW to function. This excess energy is placed into the power grid and powers 5000 nearby homes. What is fantastic about this energy is that it is first off renewable and second off sustainable through the paper industry and tree-farming industry.

An additional push of researchers in the United States as well as Portugal is the production of biomaterials. During the trip, there was multiple times where biomaterials were highlighted. One of the more significant ones is the use of cellulose derived materials to make a polymer similar to rubber but also is reversible thus giving tires that could be derived from renewable, biological resources that are recyclable. The study is in its infancy but shows great promise. Additional studies to note included exhaustive research on the composition of plant fibers, with an emphasis on lignin, and how to change the fibers content to maximize various industrial processes. During the course of this trip multiple presentations were given on this topic but all presentations shared common goals of sustainability and feasibility. This notion manifests itself in that changing the composition of plant fibers could remove the harsh pretreatment step for papermaking and biorefining thus making the overall process more sustainable.

To close, I will say that this trip was a success. The exchange students on the trip had great opportunity to see what Portugal is doing in terms of sustainability, as well as groups within the United States, and the trip provided an excellent overall view of the global advancement within this important field. It is my hopes that all the research I saw will become a reality and help lead to greener, more sustainable industries to help power the economies and communities of the future.

Participant 2: Visions of energy independence, economic security, and ecological perpetuation drive education and research in the fields of biomaterials and biofuels throughout the Western European country of Portugal. From the university perspective, chemical engineers, biotechnologists, chemists, and materials science researchers work together towards a comprehension of the chemistry of lignocellulosics, efficient conversion to new biomaterials, as well as extraction and conversion of hemicelluloses. With far-reaching aims to utilize natural products as alternative energy sources, the scientific complexity of such research benefits from study on a multi-disciplinary level. From a research and development standpoint, RAIZ, The Forest and Paper Research Institute, works in collaboration with several Portuguese universities to increase efficiency of paper production from eucalyptus, as well as develop region-specific forestry solutions to minimize raw material costs and the resultant environmental impacts. Subsequently, the technology and knowledge acquired is passed along to the industry through networking system. Thus, university education and research overlaps with industrial development and production to understand and promote sustainability in an exceptional manner in the coastal country of Portugal.

Participant 3: My trip to Portugal will positively impact my career as a high school science teacher in the following ways: (1) I have a better understanding of the energy demands of the world. (2) I am better equipped to discuss some abstract concepts in the field of science. (3) I am more knowledgeable of attitudes toward education and can put my students in a better position to compete globally.

I certainly have a better understanding of the energy demands of the United States and the rest of the world. As the world’s population increases by more than 2.6 billion people over the next 50 years, energy demands will surely increase. If society does not seek out alternative energy sources that are sustainable, carbon dioxide levels will increase. The U.S. currently uses ethanol, but at relatively low percentages. The push is on for the use of biomass, specifically cellulosic biomass as a viable energy source. This cellulosic biomass could decrease the carbon footprint by as much as 86%. Furthermore, political pressure to decrease U.S. foreign oil dependence is increasing. The American people dislike depending on unstable sectors in the world for energy. The potential for bio-fuels...
to replace foreign oil dependence seems possible and the current government is making this research a priority. New technology brings new employment prospects, which are also welcome in today’s weak economy.

At the various Portuguese universities we visited, I listened to graduate students and professors discuss some of the current research being conducted in the fields of Chemistry, Biotechnology, Chemical Engineering, and Paper Science. These people have a profound passion for the work they are conducting. The opportunity for Portuguese and U.S. scholars to come together to discuss, argue, and collaborate was very positive. While it is clear that many aspects of the science of sustainable projects are yet unanswered, much progress has also already been made. I listened to scholars recognize the financial implications of their work as well as the environmental. I was also able to see how some of the differences in paper research are very specific to the region of the world. For example, Southern Pine and Eucalyptus are different in structure and present different challenges. Nevertheless, researchers from both sides of the Atlantic were able to find common threads in their work.

At RAIZ, I was able to see firsthand, the goals of a company seeking to educate Portuguese farmers on responsible farming practices for a truly sustainable resource for economic prosperity. I was excited to see a partnership exits between RAIZ and several of the prominent universities in the country. This business partnership with education reflects the same goals established at American colleges and universities.

Besides looking at energy demands, the trip afforded me the opportunity to hear experts in specific areas of science. These areas are naturally abstract and have been sources of weakness in my own teaching. Having heard specific examples of applied science will enable me to relate these stories to my own students. The application of such concepts will make it easier to build relevance in the minds of the students. Some specific concepts explored include the following: spectroscopy, electric fields, nanotechnologies, gas sensors, phosphorescence, and tensile strength. Some areas of science are newly developing and fail to make the pages of high school science textbooks. Regardless, Advanced Placement students have questions concerning these concepts. As a teacher I value any information that makes me more capable of teaching.

Finally, the trip afforded me the opportunity to converse with graduate students and professors from Europe and the United States. We, as educators in the United States, are always being told of the need for our students to compete globally in the ever-flattening world market. Admittedly, it is difficult to know what that means if you have never traveled abroad. My understanding concerning foreign cultures, and specifically, their attitudes toward education was very limited prior to this trip.

My discussions with individuals on this trip also let me see what level of work is required of students at the next level, after high school. This knowledge helps me to set the bar even higher for my own students. I feel a duty to the students and their future universities to prepare them for the work they will continue when their time arrives.

The results of this project have been incorporated into a graduate course that Ragauskas has presented in the School of Chemistry Biochemistry at Georgia Institute of Technology titled 'Biorenewable Polymers.'

The course is directed at reviewing advanced chemical principles of biorenewable polymers including polysaccharides and lignin with a special emphasis on chemical derivatization, reactivity, and conformational analysis. Students are introduced to current concepts in conformational analysis of carbohydrates, chemical derivatization of carbohydrates and lignin, and the conversion of these biopolymers into biocomposites and biofuels.

Course Objectives:

To provide an advanced understanding of the chemical properties of carbohydrates and lignin with a special emphasis on wood polysaccharides.

To understand the basic principles of conformational analysis of carbohydrates and the application of these concepts to plant polysaccharides such as starch, cellulose, and xylan.

The research outcomes have been reported by Ragauskas in numerous public
outlets and has been cited by more than 450 news outlets. He has been interviewed by numerous news organizations including Fox, NPR, BBC, ABC, New York Times, Canadian Broadcasting Organization, Discovery, Associated Press, and Research Horizons. The outcome of these research and educational outreach efforts have also been summarized at:

http://ipst.gatech.edu/faculty_new/faculty_bios/ragauskas/student_presentations/index.html
http://ciber.chemistry.gatech.edu/

In addition, M. Kosa, a student that went on the NSF sponsored Portugal sustainability trip wrote an editor article titled 'Sustainability in Wood-Using Sectors: A Student Perspective' in TAPPI Journal (September 2009).

Contributions to Resources for Science and Technology:

The PI and Co-PI's have developed a series of reference data-sheets summarizing the chemical constituents of a variety of biomass resources and their conversion technologies to biofuels and biomaterials. This information is summarized at:

http://www.ipst.gatech.edu/faculty_new/faculty_bios/ragauskas/ragauskas_tech_reviews.html

Special Note

Special Note: In light of Ragauskas expertise and accomplishments in the Modern Cellulosic Biorefinery, funded in part by this NSF program, he was awarded the Fulbright Distinguished Chair in Alternative Energy Technology at Chalmers. The award is one of the largest in the 60-year history of the Fulbright Program. Funding support for the Distinguished Chair is being provided to the Fulbright Commission for Educational Exchange between the United States and Sweden by Marianne och Marcus Wallenbergs Stiftelse. The award in Alternative Energy Technology has been created as part of the U.S. Embassy's One Big Thing initiative, fostering alternative energy cooperation between the U.S. and Sweden.

We have currently developed several informative web resources that are posted at:

• http://ciber.chemistry.gatech.edu
• http://www.ipst.gatech.edu/blog/index.php/category/performance-for-innovationciber-exchange/
• http://www.ipst.gatech.edu/faculty_new/faculty_bios/ragauskas/ragauskas_tech_reviews.html
• http://www.ipst.gatech.edu/faculty_new/faculty_bios/ragauskas/student_presentations/index.html
• http://www.ipst.gatech.edu/faculty_new/faculty_bios/ragauskas/student_presentations/index.html
• http://www.ipst.gatech.edu/faculty_new/faculty_bios/ragauskas/ragauskas_postsers.html
• http://www.ipst.gatech.edu/faculty_new/faculty_bios/ragauskas/ragauskas_tech_reviews.html

A series of seminars to technical staff in the forest products industry and policy managers we have begun to educate interested participants in alternative applications of lignocellulosics beyond the traditional pulp and paper applications. To this end Ragauskas was invited to discuss this subject at several workshops and conferences including:

• Forest Biorefinery: Time to Be. Polysaccharides as a Source of Advanced Materials Conference, Abo Academia, Turku, Finland (Sept, 2009)
• Forest BioRefinery Accomplishments, Latvian State Institute of Wood Chemistry (June, 2009)
• Alternative Energy in the US and State-of-the-Art Biological Conversion Technologies to Bioethanol, Lithuanian Confederation of Industrialists, Vilnius, Lithuanian (June, 2009)
• US Forest Biofinery, Lithuanian Energy Institute, Kaunas, Lithuanian (June, 2009)
• Recent Development in US Forest Biorefinery, Kaunas University of Technology, Lithuanian (June, 2009)
• Transformational Forest BioRefinery Technologies, 10th Baltic Economic Forum, Riga, Latvia (June, 2009)
Recent Developments in US Forest Biorefinery, Novel Products and Fuels from Forest Trees Seminar Series, Umeå Plant Science Centre, Umeå University, Sweden (May, 2009).

- The Biorefinery Concept: Opportunities, Challenges and Innovation, Chalmers University of Technology (May, 2009).
- US Perspective on Biorefinery, Forest Products Industry Research College (FPIC), Royal Institute of Technology, Stockholm (Jan., 2009)
- Transformational Forest Biorefineries Opportunities and Challenges, KETJU (Sustainable Products and Production Conference, Finnish Academy Science (Feb., 2009).
- Engineering The Next Generation of LignoCellulosic Fibers, Colloquium Wood Valorisation Epinal, France (Jan., 2009)
- Forest Biorefineries: Opportunities, Challenges and Innovation, Colloquium Wood Valorisation Epinal, France (Jan., 2009).
- Wood Chemistry In The Biorefinery, Booregard, Sarpsborg, Norway (Dec., 2008)
- Putting Chemistry Back Into The Biorefinery, Imperial College London, London (Dec., 2008)
- US Perspective on Biorefinery, STFI-Packforsk/KTH (Jan., 2008).
- Forest Biorefinery At the Cross Roads of Science, Engineering and Innovation, Mid Sweden University (Jan., 2008).
- The New Lignocellulosic Age, Luleå University of Technology, Skellefteå Sweden (Sept., 2007).
- Over View of Cellulosic Material Research. Industry Days IPST@GT (Nov., 2007)
- Seeing Fibers in the Pulp Forest, Lectures at the Leading Edge, Department of Chemical Engineering and Applied Chemistry, University of Toronto (Oct., 2007)
- BioEnergy Science Center: DOE Bioenergy Research Center, IPST@GT (Sept., 2007)

In addition, Professor J. Bozell (University of Tennessee) shared his research experience and views on the conversion of lignin from biomass, in part, at the following technical presentations:

- Biomass as the New Carbon. Conversion of Renewable Feedstocks into Chemicals and Materials, University of Maine (May, 2008)
- New Opportunities for Lignin Conversion in the Biorefinery, International Humic Substances Society - Moscow, Russia (Sept., 2008)
- Biomass as the New Carbon, Eastern Kentucky University (Nov., 2008)

- Structural Modification of Lignin Using Biochemical Dihydroxylation ~Pathways for the Production of Biofuel, Sanyal, N.; Bozell, J.J. SE Regional ACS meeting (SERMACS), Nashville, TN (Nov., 2008)
- Biomass as a Source of Renewable. University of Missouri (Jan., 2009).

Contributions Beyond Science and Engineering:
The results of this program have been broadly disseminated and this is reflected by citations in public news organizations, public presentations in North and South America and Europe. In addition, the PI has had an active public outreach effort via the web.

The research outcomes have been reported by Ragauskas in numerous public outlets and has been cited by more than 450 news outlets. He has been interviewed by numerous news organizations including Fox, NPR, BBC, ABC, New York Times, Canadian Broadcasting Organization, Discovery, Associated Press, and Research Horizons. The outcome of these research and educational outreach efforts have also been summarized at:

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http://ciber.chemistry.gatech.edu/

Conference Proceedings:

Categories for which nothing is reported:
Participats: Partner organizations
Research and Education Activities
Products: Book or other one-time publication
Conference Proceedings

We welcome comments on this system
Final Research Report  
Center for Innovative Biomaterial Education and Research (CIBER)

Professor Bozell/ the University of Tennessee Forest Products Center: The Center for Innovative Biomaterial Education and Research (CIBER) was established to develop a comprehensive, far-reaching, multi-institutional center focused on developing innovative biomaterials from renewable biomass resources. Part of CIBER’s mission involved training of professionals in fundamental research activities related to developing new materials derived from wood biopolymers. The specific goal of the portion of the project carried out at the University of Tennessee was to develop new conversion technologies aimed at utilizing lignin as a chemical feedstock. In order to achieve this goal we examined biochemical dihydroxylation of lignin models to 1) disrupt the aromaticity present in lignin polymer, 2) activate it towards further chemical transformation, 3) introduce new reactive sites, 4) minimize or eliminate the impact of lignin’s structural heterogeneity on selective conversion processes and 5) prepare an “activated” lignin useful as a starting material for biobased chemicals and fuels. This report summarizes work carried out at the Biomass Chemistry Laboratories of the University of Tennessee Forest Products Center, directed by Professor Joseph J. Bozell, a co-PI on the CIBER proposal. This program and the results of this work were central to securing a new DOE Energy Frontier Research Center award in 2009 by Bozell.

Biochemical dihydroxylation of lignin and lignin models:

Background - the impact of lignin as a biorefinery process stream
The biorefinery is a valuable unifying concept for the conversion of agricultural and silvicultural feedstocks to chemicals and fuels. There is no doubt that lignin will readily available from the biorefinery, as it is the second most abundant carbon source in nature. Materials of particular interest to the CIBER program, e.g., woody biomass, contains 20-25% lignin by weight, and ongoing biorefinery development is defining biomass separation technology that will make lignin an easily accessible process stream. For example, several processes for production of EtOH from lignocellulosic feedstocks generate a large amount of lignin through acidic pretreatment of biomass. Alternatively, solvent-based pretreatments selectively convert biomass into separate cellulose, hemicellulose, and lignin fractions. The kraft process used for making cellulose pulp consumes over 100x10⁶ metric tons/yr of wood, generating a correspondingly large amount of lignin.

For each of these biomass pretreatments, the cellulose and hemicellulose streams are allocated to production of fuel or chemicals. However, no general higher value use for lignin from any of these sources has been developed. In each case, the lignin can be burned as fuel, but this is the least effective and lowest value ($0.03 – 0.04/lb) use for an abundant and renewable carbon source. The ability to convert lignin to value added compounds selectively and in high yield would be an important step forward in the use of biomass as a chemical feedstock, and a key improvement for integrated biorefinery development. However, several features inherent to lignin combine to make it difficult to use as a chemical feedstock, and relegate it to low value applications:
A primary difficulty is its high degree of structural heterogeneity. Treatment of lignin with a single chemical reagent can result in a number of different reactions at different sites in the biopolymer because of the wide variety of substructural units present. Therefore, chemical transformations of lignin tend to give mixtures of materials. Higher value products may be components of these mixtures, but their isolation is normally difficult.

A second important difficulty is the variance in lignin structure as a function of external factors, such as the source of the lignin and conditions of its isolation. These factors can have a profound effect on the nature of the substructural units present. Depending on the isolation technique employed, lignin from a single biomass source can exhibit a range of different structures, making prediction of chemical reactivity difficult.

Finally, the highly aromatic nature of the lignin polymer introduces a significant amount of stability into its structure. Although processes able to selectively disrupt this aromatic nature would be highly valuable, such transformations generally require harsh conditions, resulting in nonselective transformations.

Although lignin is a structurally heterogeneous material, all lignins retain a continuous network of aromatic rings as a common structural feature. It is therefore the most reasonable location to focus efforts for selective chemical conversion. Developing chemistry that transforms a large portion of the aromatic ring network to a single structure would be a significant step toward using lignin as a chemical feedstock, but such transformations are normally scarce. Biochemical degradation of lignin suggests a method to focus reactivity on this common structural feature. Such processes, both aerobic and anaerobic, are well recognized in bacteria, and a variety of metabolic pathways are available for the conversion of aromatics into intermediates consumed by the tricarboxylic acid cycle.

Biochemical degradation of lignin suggests a method to focus reactivity on this common structural feature. Such processes, both aerobic and anaerobic, are well recognized in bacteria, and a variety of metabolic pathways are available for the conversion of aromatics into intermediates consumed by the tricarboxylic acid cycle. Aerobic systems based on dioxygenases frequently rely on the general mechanism shown in Figure I.1.

![Figure I.1. General Mechanism for Aerobic Biochemical Degradation of Aromatics](image-url)
Dioxygenase systems activate the normally stable aromatic ring (1) by converting it to cis-dihydrodiol 2. This initial dihydroxylation step proceeds by transfer of electrons from NADH to the aromatic substrate via a multicomponent flavoprotein reductase, ferredoxin, and oxygenase system. In wild-type organisms, intermediate 2 undergoes a further rearomatization reaction via cis-diol dehydrogenase, giving catechol 3. Compound 3 undergoes cleavage either between the newly introduced -OH groups (intradiol cleavage) to give dicarboxylic acid 4, or adjacent to the C-3 -OH group (extradiol cleavage), giving intermediate 5. Both compounds 4 and 5 can be further degraded to CO₂ and H₂O. The dioxygenase systems leading to 2 have been classified in terms of the arene originally used as the carbon source, i.e., benzene, naphthalene, toluene, benzoic acid, and chlorobenzene dioxygenases are among those that have been identified. The structures of some of the enzyme systems have also been solved.

In 1970 Gibson reported that a Pseudomonas mutant (a variety of bacteria found widely in soils) contained toluene dioxygenase (TDO), but lacked cis-diol dehydrogenase, thus allowing a selective accumulation of the toluene 1,2-cis-arenediol (Figure I.2).

The organic chemistry community largely ignored this transformation until the late 1980s when work by Hudlicky and others demonstrated its utility for the synthesis of a wide range of natural products. Biochemical dihydroxylation has since proven to be a powerful transformation for the selective conversion of aromatic compounds. Several features of the transformation are important:

- The process overcomes the significant stabilization energy of the aromatic ring (approximately 36 kcal/mol for benzene) under mild physiological conditions. This is probably the most powerful aspect of the biochemical transformation since such a reaction has few analogs in conventional organic chemistry. Selective disruption of lignin’s aromaticity will play a central role in enhancing its utility as a chemical feedstock.

- The reactivity of the remaining double bonds is dramatically changed, making them subject to a much wider range of chemical conversions.

- The reaction introduces two OH groups to the molecular structure that could be sites for further functionalization. The OH groups are also introduced stereo- and enantiospecifically, with a regioselectivity that is predictable.

- The conversion has implications for green chemical production, as the biochemical reactions are carried out in aqueous solution, and lead to valuable synthetic intermediates using significantly fewer steps and reagents than traditional chemical routes.
The activity of these organisms is very broad, and upon induction of dioxygenase activity, a wide range of aromatic substrates containing diverse functionality undergoes dihydroxylation. Further, each of these dioxygenases exhibits a range of selectivities as a function of substrate size, with some optimized for monoarenes and others optimized for polyaromatics containing as many as five ring systems. Improvement of conversion efficiency has been realized by expressing TDO in the recombinant E. coli JM109 (pDTG601). The process has also been adapted for large-scale operation, and several dihydroxyarenes are now commercially available from this transformation. Reaction with electron rich arenes, representative of the electronic nature of lignin-like materials, has been reported.

Our intent in this program is to use this biochemical dihydroxylation as a means to selectively convert lignin’s structure and activate it toward further chemical transformations. Biochemical dihydroxylation will address each of the features described earlier that make lignin difficult to use as a chemical feedstock: 1) the impact of lignin’s structural heterogeneity will be minimized or eliminated by limiting reaction to the aromatic ring network; 2) it will be possible to use lignin from all sources, regardless of variances in structure as a function of source or isolation method, and 3) disruption of the aromatic network will occur under mild conditions, resulting in the predictable and selective introduction of new reactive functional groups. Figure I.3 describes some of the potential structural changes, using a common lignin subunit as an example.

![Figure I.3. Structural changes resulting from biochemical dihydroxylation of lignin](image)

Given the nature of lignin as a polyaromatic material, it is interesting that literature reports of lignin’s interaction with Pseudomonas or related organisms are rare. Such reactions have been reported only in the context of complete, nonselective breakdown of the lignin polymer as part of waste stream treatment processes from pulp and paper operations. A very large body of work has developed around laccase and peroxidase enzymes and their reaction with lignin, but again, the intent is to develop biochemical processes for the conversion of lignin into small, easily removed fragments. This project addresses a well-recognized need for the biorefinery – development of new conversion technologies tailored to use lignin as a chemical feedstock. To accomplish this goal, we have examined a biochemical process that could minimize or eliminate the normally detrimental impact of lignin’s structural heterogeneity on selective conversion processes. Biochemical dihydroxylation will be used to focus reactivity on the single unifying structural feature of the lignin polymer: its network of aromatic rings. The resulting material will be an “activated” lignin – useable directly as a chemical product, or of improved utility in downstream chemical transformations as a result of the introduction of new reactive sites. Although this project’s focus was an understanding of the fundamental aspects of the transformation, success will ultimately lead to wider use of biorefinery lignin as a valuable source of high value chemical materials.

**Results summary – lignin model dihydroxylations**
Biochemical dihydroxylation successfully and selectively reacts with compounds that model lignin (Figure I.4).

The models were chosen to compare the effect of different electronic features of the aromatic ring (para-chloro vs para-methyl; electron-poor vs electron-rich) and the presence of a lignin side chain surrogate (para-propyl). In each case, oxidation occurred, affording dihydroxylated material with high selectivity. NMR proved valuable in identification of the corresponding dihydroxylated compounds, which were characterized by symmetrical doublets at 4 and 6 ppm for methine and diene hydrogens respectively. Additional evidence was provided by HRMS data. The absolute, isolated yields are fair to good. However, the very high selectivity in each case suggests that the oxidation can be driven to completion with appropriate modification of the reaction conditions via an increase in enzyme production, longer reaction times, or better control of the biochemical conditions used for growth of the organism. Selectivities in these reactions were typically >90%, with unconverted starting material being the only side product. Evaluation of the reactions suggests that the electron-poor para-chloroanisole underwent oxidation with lower conversion than the electron-rich para-methylanisole. Para-propylanisole also exhibited high selectivity but low conversion, possibly as a result of the steric effects of the propyl side chain. Higher yields in case of electron rich para-methylanisole is consistent with the expected tendency of more electron rich substrates to undergo oxidation. In addition, the dihydroxylated products are somewhat sensitive. Upon standing, water loss can occur, particularly in the presence of even small amounts of acid, inducing rearomatization and formation of the two isomeric phenols. NMR clearly revealed formation of these materials along with the presence of a distinctive phenolic odor from the reaction product. Mass spectral data further confirmed this transformation. The dihydroxylation compound from oxidation of para-chloroanisole could be seen to give a strong fragment ion resulting from the loss of water.

**Reaction protocols**
Under sterile conditions, colonies of *E. coli* JM109 (pDTG601), provided by Rebecca Parales (UC-Davis) were grown on plates consisting of bactotryptone (10 g/L), yeast extract (5 g/L),
NaCl (5 g/L), agar (30 g/L), and ampicillin. *E. coli* JM 109 (pDTG601) were streaked onto the plate and incubated at 35°C for 18 h. A sample was taken from the plate and streaked onto a second plate and incubated for an additional 24 h. A single colony was taken from the second plate and placed in a sterile plastic culture tube containing 3 ml of LB medium consisting of tryptone (10 g/L), yeast extract (5 g/L), NaCl (5 g/L) and ampicillin (200 μg/mL). The capped preculture was grown at 35°C on an orbital shaker (180 rpm) for 6 h. A 10 μl sample of the preculture was added to 500 ml of LB medium containing 250 μl of aqueous ampicillin solution (100 mg/ml) in a 2l Fernbach or Erlenmeyer flask and was incubated at 31°C on an orbital shaker (150 rpm) for 15 h. IPTG (10 mg/L) was added to induce protein synthesis via a sterile filter, and the cells grown for an additional 5 h at 35°C on an orbital shaker (200 rpm). The resulting solution was generally a blue-gray color, due to conversion of indole (produced by the organism by consuming a small amount of bactotryptone from the culture medium) to indigo by the enzyme (Figure I.5).

**Figure I.5.** Typical appearance of shake flask transformations (L: immediately prior to induction; center: after completion of induction; R: isolated cell pellet prior to resuspension)

Growth curves for the organism in preculture and initial scaleup are shown in Figure I.6. The supernatant was separated from the cells by centrifugation at 7000 rpm for 15 min. The supernatant was decanted and the cell pellet resuspended in 100 mL of 0.1 M phosphate buffer consisting of KH₂PO₄ (6.8 g L⁻¹) and K₂HPO₄ (8.7 g L⁻¹). The aromatic substrates (800 mg L⁻¹) were added as a neat liquid dropwise and the reaction stirred for 4 hours at 35°C. Product formation was monitored by thin-layer chromatography (silica gel, 1:1 hexane-ethyl acetate). The pH of the culture medium was adjusted to 8.5 with 6M NaOH, and a cell pellet was obtained by centrifugation at 7000 rpm at 4°C for 15 min.
The supernatant liquid was thoroughly extracted with acid-free ethyl acetate prepared by prior stirring with saturated Na₂CO₃. The extract was dried over MgSO₄, filtered, and the solvent removed under reduced pressure. The crude material was purified by flash chromatography (silica gel deactivated with 10% distilled water) immediately after concentration of the solvent in order to minimize the decomposition of the diene diols. Alternatively, the culture mixture could be extracted directly without prior centrifugation. In addition, literature procedures used for isolation of the dihydroxylated materials as intermediates in longer synthetic schemes generally call for extraction using base washed EtOAc, to remove trace amounts of acid that induce rearomatization of the substrate. We normally employed this procedure, but found that product could also be isolated in some cases without prior treatment of the extractant with base. Typical spectral data are as follows:

**3-chloro-6-methoxycyclohex-4-ene-1,2-diol**

$^1$H NMR(CDCl₃): $\delta$ 3.66 (s, 3H, O-CH₃), 4.29 (d, 1H, J₂,₃=6Hz, H₂), 4.42 (d, 1H, J₃,₂= 6Hz, H₃), 4.91 (d, 1H, J₅,₆=6.8 Hz, H₅), 6.10 (d, 1H, J₆,₅=6.8 Hz, H₆). $^{13}$C (CDCl₃): $\delta$ 56.69 (OCH₃), 70.30 (C2), 71.70 (C3), 91.84 (C6), 122.60 (C5), 126.17(C4) 166.50 (C1). IR (neat, cm⁻¹): 3416, 2914, 2838 (sh). HRMS (EI) calcd for C₇H₉ClO₃, [(M-H₂O) + H⁺] calcd 159.0208, found 159.0219.

**3-methoxy-6-methylcyclohex-4-ene-1,2-diol**
1HNMR (CDCl3): δ 1.87 (s, 3H), 3.63 (s, 3H, O-CH3), 4.12 (d, 1H, J2, 3=5.2Hz, H2), 4.42 (d, 1H, J3, 2= 5.6 Hz, H3), 4.91 (d, 1H, J5, 6=6 Hz, H5), 6.10 (d, 1H, J6, 5=6 Hz, H6).

We also examined alternative oxidation methods, to include the use of stored, frozen cell pellets. This approach was undertaken to allow preparation of larger amounts of dioxygenase enzyme in a single run. The stored, frozen cell pellets were initially sonicated to lyse the cells and release the enzyme into the medium. Biochemical dihydroxylation of para-chloroanisole was performed using frozen cells with and without sonication. For comparison, reaction was also carried out with fresh sonicated cells. Yields of the dihydroxylated compound obtained were comparable using 1) fresh sonicated cells, 2) fresh nonsonicated cells, 3) frozen sonicated cells and 4) frozen nonsonicated cells. For practical convenience, method 4 was chosen to carry out dihydroxylation reactions on other substrates.

In order to estimate toluene dioxygenase activity we examined a spectrophotometric assay for toluene dioxygenase using indole as substrate in presence of Fe³⁺ and NADH. Literature reports show production of a soluble yellow dye during aerobic incubations with indole and NADH. Accumulation of this yellow dye (indoxyl) corresponds to a linear increase in absorbance at 400 nm. The rate of increase in absorbance can be shown to be a specific measure of toluene dioxygenase activity. We have attempted to perform the assay following a literature procedure²⁸ with cell extracts from E. coli JM109 culture using indole as substrate in presence of NADH. Currently we are in the process of validating the procedure for future assays. This spectrophotometric assay is very sensitive and would enable us to estimate and compare the enzyme activity used for each substrate.

Initial reaction scaleup
Shake flask culture is convenient, but limited as to the amount of substrate that can be converted in a single reaction sequence. Accordingly, we also examined scaleup of the procedure using a New Brunswick 1.5 liter fermentor, using Hudlicky’s exceptionally detailed procedure.²⁹ The procedure is similar to the shake flask approach, except the medium is changed from LB to a mineral salts broth. The dissolved oxygen content and pH are crucial components during fermentation, and are controlled automatically by the fermentor. In addition, growth of the organism is controlled by the rate of glucose addition, which is determined by the level of dissolved oxygen in the fermentor. Upon completion of the fermentation, the dihydroxylated product is isolated using a continuous extractor and base washed EtOAc. Unfortunately, and despite numerous attempts, we realized only moderate success in the use of the fermentor for the production of larger quantities of dihydroxylated material. The greatest source of variability was control of oxygen content in the reactor, which we believe has a significant effect on the transformation of the substrate. Over a series of runs, we observed significant variability in yields and conversions using both para-chloroanisole and toluene as substrates. The variability in toluene conversion is surprising as it is a standard substrate in many biochemical dihydroxylation procedures. For these reasons, our upcoming work will be carried out by returning to shake flask systems. Once that protocol and the associated isolation procedures are fully understood, we will return to larger scale fermentations and develop a procedure that is not limited by the variables in fermentor operation.

Results summary – identification of new sources of dioxygenases
Part of our effort included preliminary efforts to locate and identify novel dioxygenases from alternate sources. In collaboration with Professor Alison Buchan of the UT Microbiology department, we carried out preliminary investigation of members of the *Roseobacter* family of organisms for their ability to metabolize lignin. *Roseobacter* are frequently found in high biomass producers such as cordgrass growing in salt marshes. *Roseobacter* are heterotrophic marine bacteria that compose about 30% of the total prokaryotic organisms found in coastal salt marshes in the southeastern United States, and unlike many other marine lineages, are readily cultured in the laboratory. *Roseobacter* possesses the genes for the degradation of plant aromatic compounds. Buchan’s group has demonstrated that *Roseobacter* organisms will grow on aromatic substrates, have carried out gene mapping on these systems, and have expressed some of the genes in *E. coli*. These systems metabolize aromatics via the -ketoadipate pathway, and demonstrate that aromatics similar to those studied in this project may undergo the desired oxidation reaction. The *E. coli* organisms that we employed offer greater selectivity for production of isolable organic products through their use of a modified enzyme system. The organisms tested were collected from several sources, chosen primarily to represent locations where significant levels of biomass consumption were expected to occur. These included organisms collected from pulp mill effluent and areas where indigenous grasses are undergoing decay, possibly metabolizing lignin as a carbon source. The lignin samples are summarized in Table I.1, and the organisms and their source are shown in Table I.2. All lignin samples were obtained by heating the wood source with a 16:34:50 mixture of methylisobutyl ketone, ethanol and water in the presence of a H$_2$SO$_4$ catalyst.

### Table I.1. *Roseobacter* sources investigated

<table>
<thead>
<tr>
<th>Strain</th>
<th>Isolation site</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Phaeobacter</em> sp. Y4I</td>
<td>HMW pulp mill effluent (Georgia coast)</td>
</tr>
<tr>
<td><em>Phaeobacter</em> sp. Y3F</td>
<td>HMW pulp mill effluent (Georgia coast)</td>
</tr>
<tr>
<td><em>Sagitulla stellata</em> E-37*</td>
<td>HMW pulp mill effluent (Georgia coast)</td>
</tr>
<tr>
<td><em>Citricella</em> sp. SE45</td>
<td>Decaying salt marsh grass (Georgia coast)</td>
</tr>
<tr>
<td><em>Silicibacter</em> lacuscaerulensis (S.lac)</td>
<td>Geothermal lake (Iceland)</td>
</tr>
<tr>
<td><em>Silicibacter pomeroyi</em> DSS-3</td>
<td>Georgia coastal seawater</td>
</tr>
<tr>
<td><em>Silicibacter</em> sp. TM1040</td>
<td>Dinoflagellate culture (Chesapeake Bay)</td>
</tr>
<tr>
<td><em>Sulfitobacter</em> sp. EE-36</td>
<td>Georgia coastal seawater</td>
</tr>
</tbody>
</table>

### Table I.2. Lignin sources investigated

<table>
<thead>
<tr>
<th>Wood type</th>
<th>Heating Time (min)</th>
<th>Temperature (˚C)</th>
<th>H$_2$SO$_4$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Southern mixed hardwood</td>
<td>56</td>
<td>140</td>
<td>0.175</td>
</tr>
<tr>
<td>Southern mixed hardwood</td>
<td>56</td>
<td>140</td>
<td>0.05</td>
</tr>
<tr>
<td>Southern mixed hardwood</td>
<td>225</td>
<td>125</td>
<td>0.19</td>
</tr>
<tr>
<td>Southern mixed hardwood</td>
<td>375</td>
<td>122</td>
<td>0.1</td>
</tr>
<tr>
<td>Mixed oak</td>
<td>56</td>
<td>140</td>
<td>0.2</td>
</tr>
<tr>
<td>Mixed oak</td>
<td>56</td>
<td>160</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Lignin consumption tests were carried out by mixing lignin with 100 µL inocula (~10^7 cells/ml) of a *Roseobacter* strain, in the presence of 50 mg of the lignin substrate, mixed in 10 ml of carbon-free minimal media, and compared against controls with no added strain. The samples were visibly monitored for growth and optical density of suspensions spectrophotometrically. After 1 month, the samples were streaked on YTSS agar plates to determine viability. All samples retained their viability over at least one month. In some samples, a visible color change to light brown was observed. The results of the tests are shown in Figure I.7. Three strains, EE-36, E-37 and SE-45, were seen to have higher O.D. than the controls suggesting that they may be using organosolv lignin as their primary carbon source.

We have initiated examination of these lignin samples using 2D HMQC NMR. The lignin samples were prepared by filtering the cultures and air-drying the residue. NMR measurements were prepared by dissolving about 100 mg of treated lignin in 750 µl of d_6-DMSO. The spectra were collected on a Varian 400MR instrument using a 1.5 second delay between pulses, a proton-carbon coupling constant of 147 Hz, 1024 increments and 16 scans/increment. Typical run times were about 16 hours/sample.

Figures I.8 and 9 show a series of spectral overlays of the first group of samples. Each of the treated lignin samples was compared to a typical aspen organosolv lignin and a control sample that had not been exposed to the *Roseobacter* source. For the most part, the samples exhibit few differences from the control or the isolated aspen lignin. However, treated sample K1a contains a group of unique cross peaks not observed in the other samples at (^1H/^13C) 5.82/129.06, 5.13/64.80, 5.10/94.50, 5.00/93.36, 4.09/62.66, 3.93/63.66, and 3.62/67.82 ppm. This initial result suggests that new structures are present as part of the lignin samples. However, identification of these peaks has not been completed. The next stage of the research effort will continue examination of the NMR results to determine the source of the new NMR signals. Identification of any structural changes will be important to future stages of the project, as the substructural profile offered by any source of biorefinery lignin will be crucial to the types of transformations that are possible in the production of chemicals or fuels. Control of these processes may also lead to methodology able to tailor lignin structure for the desired product portfolio of the biorefinery.
Figure I.7. - Results of *Roseobacter* growth in the presence of organosolv lignin
Figure I.8. Expansion of the aromatic region of organosolv lignins treated and untreated with *Roseobacter* species (red - control; yellow - sample K1a; green - sample K2a; light blue - sample K3a; dark blue - sample K4a; magenta – isolated aspen organosolv lignin
Future research directions
The funding from NSF has allowed us to directly address one of the goals of the CIBER effort – development of new technology applicable for the conversion of lignin to chemicals or fuels. Work now starting in our laboratory will examine incorporation of this methodology as part of a process for converting lignin to liquid fuels. Recent publications have described the catalytic formation of alkanes and hydrogen through aqueous phase reforming of sorbitol. Reforming offers a potentially powerful method for the conversion of renewables to hydrocarbons under relatively mild conditions (Figure I.10), offering a key link between the existing petrochemical industry and the growing biorefinery industry. Alkane production results from an initial reforming reaction (C-C bond cleavage) in aqueous solution, and formation of H$_2$ via water-gas shift processes. The process can be used simply as a means to generate hydrogen from the starting renewable feedstock, or alternatively, alkanes can be generated as a coproduct through repeated dehydration and reduction cycles, using the H$_2$ produced from the initial water-gas shift. Despite the number of parallel reaction pathways that occur, this process exhibits good
selectivity for the production of hexane. Such work could help link the biorefinery with the petrochemical infrastructure it will likely emulate, and could help transition the petrochemical industry to a wider use of renewable feedstocks by demonstrating the utility of highly oxygenated feedstocks as raw materials.

![Diagram of aqueous phase reforming of carbohydrates](image)

**Figure I.10.** Aqueous phase reforming of carbohydrates

However, the largest alkane currently available from the conversion of sorbitol or other readily available biomass carbohydrates is hexane, a material with little value as a fuel because of its volatility. Methods for the conversion of carbohydrates to longer, fuel grade (C7 – C15) alkanes have been reported, but require a considerably more complex reaction system consisting of an additional aldol condensation step and use of a specialized four-phase reactor.31

Changing lignin’s normal reactivity offers a solution to these limitations and will greatly increase its value and importance within the biorefinery. Biochemical conversion products, such as those in Figure I.11, could be considered as “activated” lignins. Disruption of the aromaticity renders the resulting material subject to transformations unavailable to the starting lignin. As a result of biochemical dihydroxylation and an increase in the reactivity of the remaining double bonds, the lignin fragment in Figure I.11 would possess an overall reactivity much closer to that of sorbitol, making it subject to the hydrogenation and hydrogenolysis (reforming) reactions. Even if a first generation biorefinery made nothing more than liquid fuel, the ability to incorporate lignin could increase the fuel output by as much as 20 – 25%.

![Diagram of conversion of lignin products to alkanes via catalytic reforming](image)

**Figure I.11.** Conversion of lignin products to alkanes via catalytic reforming

**Professor S. May/Georgia Institute of Technology:**
**Construction of a Predictive Template for Lipase Catalysis**
Using literature and modeling data, we created a general tool for determining enantiospecificity for *Candida antarctica* Lipase B (CALB). When performing novel enzymatic polymerizations, the most important step is choosing an enzyme that will accept all the monomers involved and
will catalyze the desired reaction. This step not only decides what functional groups the monomer must contain, but also determines if any stereospecific or enantiospecific preferences will need to be considered when designing the polymerization. CALB, due to its broad range of substrates and its ability to form different types of bonds, was chosen as the enzyme of interest (1-5).32,33,34,35,36

It is one of the few lipases that accept secondary alcohols preferentially.37,38,39,40,41,42,43,44 This characteristic allows CALB to resolve α-hydroxy carboxylic acids. Adam et al.45 reported that CALB exhibited the best results out of several lipases for the kinetic resolution of α-hydroxy valeric acid. The S-enantiomer was shown to be the preferred enantiomer, having an enantiomeric excess of 80% at 55% conversion, while the (R)-enantiomer was left unreacted with an enantiomeric excess of 99%. The authors reported results from kinetic analysis, but neglected to give any structural reason for the difference between the preferred (S)-enantiomer and the unreactive (R)-enantiomer.

Modeling software, Molecular Operating Environment (MOE), was used to model the binding of the two enantiomers in the enzyme active site. To begin the docking both enantiomers were built individually and energy minimized. The molecules were then docked into an energy-minimized structure derived from the crystal structure of the enzyme with the ligand, n-hexylphosphonate ethyl ester bound in the enzyme’s active site.46 The results from the docking simulation were ranked by a combination of 1) the sum of Gaussians over all ligand atom-receptor atom pairs and ligand atom-alpha sphere pairs, 2) the affinity scoring dG function, whose terms address atomic contacts of specific types, and 3) an estimated self-energy of the ligand. The lowest value of this combination was taken as the best conformation.

The preferred (S)-enantiomer and the unreactive (R)-enantiomer were docked individually using MOE. The best result from each docking was superimposed in the active site of CALB in order to see any difference when binding. The (R)-enantiomer is centered in the active site, while the (S)-enantiomer is positioned more to the right, closer to the catalytic residues of the active site (Figure II.1.A). The (S)-enantiomer binds in an orientation in which the carbonyl carbon is closer to the catalytic residue, serine 105, than the (R)-enantiomer (Figure II.1.B).

![Figure II.1.](image-url)
Uppenberg et al. (15) discusses the importance of the $\text{Ne}_\text{e}$ of histidine 224 in the catalysis of the hydrolysis reaction by CALB. The alcohol oxygen of the substrate must be able to receive a proton from the nitrogen of the enzyme. When looking at the interactions between the two enantiomers it is noticeable that the $(S)$-enantiomer is closer to the histidine, with only 3.91 Å distance between the two atoms (Figure 13), than the $(R)$-enantiomer at a distance of 5.20Å (Data not shown). When considering this important interaction the only probable reactive enantiomer is the $(S)$-enantiomer.

Based on the previously described analysis method and the crystal structure of CALB with n-hexylphosphonate ethyl ester as a bound ligand (Figure 14), a template of the CALB active site was formed. Molecules that are accepted as substrates of the enzyme were compared using a feature of MOE called Flexible Alignment of small molecules. This feature ranks the molecules according to their similarity. Several of the molecules were then docked and analyzed to determine their orientation in the active site.

With this information, a tool was created that will continue to help our lab, as well as others, predict substrate acceptance or preference between enantiomers for molecules based on their functional groups. This is a valuable, time saving, tool when designing enzymatic syntheses or polymerizations.

**Enzyme Catalyzed Michael Addition**

We successfully used CALB as a catalyst to synthesize novel compounds via Michael addition reactions, that can further be used as monomers in polymerization reactions via enzymatic and non-enzymatic catalyzed reactions. Primary and secondary amines and thiols are being used as nucleophiles. The Michael addition reaction is a common reaction in synthetic organic chemistry between a nucleophile and an $\alpha,\beta$-unsaturated carbonyl compound. Recently, the reaction has been employed by polymer chemists for the synthesis of linear, graft, hyperbranched, dendritic, and network polymers. Usually these reactions are catalyzed by
strong bases, causing unwanted side reactions with starting materials and products. Common catalysts for Michael additions in organic chemistry are transition metals and lanthanides.48

In the base catalyzed Michael addition mechanism. Scheme II.1. (above left), the Michael acceptor is first deprotonated by the base resulting in a carbanion. The nucleophile then reacts and proton extraction by the enolate leads to the final Michael adduct.

An alternative to base catalysis is the use of enzymes. The oxyanion hole of the lipase active site stabilizes the negative charge of the transition state and the His-Asp pair acts as a proton shuttle in the enzymatic mechanism. Amines and thiols have been shown to be good nucleophiles for lipase catalyzed Michael additions.49

Scheme II.2. (above right) demonstrates the Michael addition mechanism in the active site of Candida antarctica lipase B. The carbonyl oxygen of the α,β-unsaturated carbonyl compound is bound in the oxyanion hole formed by Gln106, Ser105, and Thr40. His224 acts as a general base to activate the nucleophile.50

Experimental data has shown that CALB is able to catalyze several Michael additions, resulting in products that can be used as monomers for novel polymers. Two examples of the enzymatically catalyzed Michael addition can be seen below. The reaction of 1,2-ethanediethiol and acrylonitrile results in thioether-nitrile mono- and di-Michael adducts. The nitrile group can easily be converted to the free acid through acid hydrolysis and can be further polymerized using a variety of monomers to give a novel polymer containing thioether functional groups within the backbone. The free sulfide can be used as a nucleophile in further polymerizations.

Scheme III.3. CALB catalyzed Michael addition of 1,2-ethanediethiol and acrylonitrile.
The Michael addition of dithiols and acrylonitrile results in the thioether-nitrile mono- and di-Michael adducts. For example, see the CALB catalyzed Michael addition of 1,2-ethanedithiol and acrylonitrile (Scheme II.3.). After the enzyme catalyzed Michael addition, the nitrile group can easily be converted to the free acid through acid hydrolysis and can be further polymerized, chemically or enzymatically, using a variety of monomers resulting in a novel polymer containing thioether functional groups within the backbone. The free sulfide of the mono-Michael adduct can be used as a nucleophile in further polymerizations.

Amines were also used as nucleophiles for CALB catalyzed Michael additions. As seen in Scheme II.4., the addition of methylmethacrylate and N-phenylethane-1,2-diamine only occurred in the presence of CALB. Again the product contains a functional group for further polymerization.

**Scheme II.4.** CALB catalyzed Michael addition of methylmethacrylate and N-phenyl-1,2-diamine.

**Amine Functionalized Polyesters**

CALB was used to catalyze monomers that could easily be used in the polymerization of polyesters with pendent amine groups. Different functional groups can be incorporated into the same polymer giving the polymer completely different properties. These functional groups may be incorporated into the polymer as end groups, as a part of the polymer backbone or as pendent groups. Pendant functionalization allows the same polymer backbone to be used in many different applications depending on the properties of the pendent functional groups. The amount of pendent groups can be controlled during polymerization, allowing for tuning of the polymer properties or for control of the number of molecules that will be covalently attached to the polymer.

Common pendent functional groups used in biodegradable polymers include carboxyl, hydroxyl, and vinyl groups. These functional groups all increase biodegradability and hydrophilicity. Amine functional groups increase hydrophilicity and biodegradability, allow for crosslinking, and for covalent attachment of bioactive molecules and prodrugs.51,52,53,54

During polymerization of amine functionalized polymers, it is often necessary to protect and deprotect the amine group. An example is seen in Scheme 5.55 This step can be eliminated by the use of enzymes, more specifically lipase. Using the CALB active site and knowledge of its reaction capabilities, monomers can be chosen so that the pendent amine groups do not react with other functionalities on the monomers. The percentage of amine functional groups can be controlled during polymerization, by controlling the concentration of the amine and diacid monomers. The polyester with amine pendent groups could be used as a water soluble carrier for compounds that will be covalently attached to the amine groups.
**Scheme II.5.** Example of protection and deprotection used in polymerization of amine functionalized polymers.

\[
\text{Scheme II.6. Proposed polymerization of tunable polyester with amine pendent groups catalyzed by CALB.}
\]

**Enzymatic Synthesis of Polyurethane Polyesters**

Polyurethanes are one of the most important classes of polymers. They convey a wide range of physical properties that have made it possible for them to be used in every aspect of life, from synthetic heart valves to cushioning foams. The conventional synthesis of polyurethanes consists of reacting a polyol with a diisocyanate. Diisocyanates are derived from highly toxic phosgene, which is known to cause respiratory problems, and is not highly biodegradable. A purely enzymatic diisocyanate-free polymerization method would not only eliminate the hazards of using diisocyanates but would also allow for lower reaction temperatures and offer the possibility of regiospecificity, enantiospecificity or chirality of functionalized reactants. With this in mind, an attempt to create a poly(ester-urethane) alternating copolymer in a two-step, one-pot enzyme-catalyzed reaction was carried out (see Scheme II.6).

**Scheme II.7.** Enzymatic synthesis of bis-carbamate monomer.

The monomer, di(hydroxyethyl)-hexamethylene bis-carbamate, was successfully made following a procedure from the literature (33) (First step, Scheme 7). This monomer synthesis was irreproducible when a modified procedure containing CALB was tried. The enzyme may be performing a ring-opening polymerization on the ethylene carbonate and the multiple products of this polymerization could react with the diamine in the system.

The bis-carbamate monomer synthesized by the unmodified procedure was then used in an enzyme-catalyzed polymerization (Step 2 in Scheme II.7). Analysis of the reaction products by gel permeation chromatography failed to show the presence of a high molecular weight polymer. This may have been due to small amounts of water in the reaction system that stopped the polymerization before the polymer reached a high molecular weight, or caused competing side reactions in the system.
**Enzymatic Synthesis of Poly-\(\beta\)-Hydroxybutyrate**

Polyhydroxyalkanoates are a relatively large class of thermoplastic biopolymers of which poly-\(\beta\)-(\(\beta\)-hydroxybutyric acid (PHB) is the simplest. PHB is produced when certain bacterial strains are stressed, typically by nutrient limitation, and can be produced in yields up to 95% of the bacterial dry cellular weight. Since they can be produced from renewable resources and are biocompatible and biodegradable, PHB copolymers are attractive as environmentally-friendly alternatives to traditional plastics for a number of applications.

We chose to focus on *in vitro* enzymatic synthesis of PHB, which differs from the conventional approach of producing this polymer within bacterial cells via fermentation. *In vitro* polymer production obviates the necessity for polymer extraction from bacterial cells. A second consideration is that polymer synthesis within cells is subject to limitations related to the cellular uptake and possible toxicity of substrates, which limits the range of polymers that can be produced *in vivo*. In contrast, the enzymes in the PHB pathway accept a variety of alternative substrates, thus making possible *in vitro* production of novel homopolymers or copolymer.

We have successfully developed quantitative HPLC methodology to monitor metabolite concentrations and reaction time courses during the *in vitro* enzymatic synthesis of PHB. These experimental results were then used to develop the first metabolic model of *in vitro* PHB biosynthesis. All previous modeling studies of PHB production have been designed to analyze *in vivo* biosyntheses, typically in bacteria but also in yeast and plants. Furthermore, previous simulations were not based on quantitative analyses of the concentrations of the PHB pathway metabolites: acetyl-CoA, CoA, acetoacetoyl-CoA, and \(\beta\)-hydroxybutyryl-CoA. Our results can therefore serve as a basis for further development of the potential of *in vitro* enzymatic syntheses of polyesters as a compliment to the more widely used approach of *in vivo* production of these polymers.

**CIBER Website (www.ciber.chemistry.gatech.edu)**

A website has been created to provide general and scientific information on biomass sources, biomaterials, polymers, and enzymes. The site includes a continuously expanding searchable database of literature abstracts covering the areas mentioned above. A glossary of definitions is included to help those readers who are not familiar with the area of biomaterials. This website is designed to be useful to high school readers as well as scientists in the field.
The CIBER website design has a navigation pane on the left side with major topics that expand as the links are followed. A search application appears in the upper right corner that allows the viewer to quickly search the entire website for a specific term or phrase. There is an option to print or email the viewed page and breadcrumbs appear in the upper bar to allow the viewer to trace their path through the website. These features are seen on every page of the website containing content as shown in Figure II.4.

Selecting the topic **Enzymes** goes to a page, shown in Figure II.5, where links to several subtopics are available along with a general discussion of enzymes. Figures II.6 shows that following the subtopic link, **Lipases**, (from the page in Figure II.5) goes to a general discussion of lipases with links to a specific lipase such as, **Candida antarctica** lipase.

Pages for each of the exchange programs to Portugal have been added and linked to the About CIBER page, Figures II.7 and II.8. These pages contain pictures of the exchange program participants, titles and PDFs of research presentations, and itineraries. Exchange groups consisted of Georgia Tech professors and graduate students and local Atlanta high school teachers.
Summaries of biomaterials related scientific articles can be searched topically by going to the Literature Cross-Reference page, shown in Figure II.9, and scrolling through the list of topics of interest in the body of this page. Following a link to a literature summary from this page will bring up that specific literature summary page with a list of other related literature organized by topics on the lower left side of the page. For example, following the link to Kaga 1998 brings up the page seen in Figure II.10.

An alphabetical list of all the literature summaries can be seen by following the Literature Summaries link. A typical literature summary page is shown in the three panels of Figure II.11.

A Teacher’s Resource page (Figure II.12) has been added in order to provide teachers of all levels (K-12) with an easily accessible compilation of websites containing numerous activities, lesson plans, and laboratory experiments for use in teaching biomaterials and biomass sources and conversion. The websites included in this list are reputable sources of facts and information on the topics and include the U.S. Dept. of Energy and the Energy Information Administration. Linked to this page is a list of downloadable PDFs of classroom activities and laboratory experiments aimed towards high school teachers.
The CIBER website is constantly being updated and is serving as a tool to introduce the area of biomass conversion to the public and to focus attention on the major problems that still remain to be solved by researchers in this area. As seen from the most recent 6-month statistics, generated by Awstats Website (Figure II.13, left), the site is being used by an average of 243 different users each month and is generating an average of 26,865 hits per month. Navigations to the website have been referred by search engines such as Google, Yahoo and Bing.

**Figure II.13**

An Undergraduate Student Laboratory Experiment: Bacterial Production of Poly(3-hydroxybutyrate)

As part of the Educational Mission of CIBER, we developed and published an undergraduate student laboratory experiment for culturing, isolating, and purifying the biopolymer poly-(β)-hydroxybutyric acid (PHB). PHB accumulates in the cytoplasm of bacterial cells under specific growth conditions, and it has attracted much recent interest since its biodegradability makes it "environmentally-friendly" and its biocompatibility makes it an attractive choice for medical and therapeutic applications. This laboratory experiment is a valuable addition to any traditional chemistry or polymer science curriculum, since it exposes the student to the field of biotechnology and teaches biological methodologies that are very different from traditional methods of polymer synthesis. The experiment is designed so that the students perform the PHB cultivation and extraction processes in three stages that are easily accommodated within the scheduling confines of a typical undergraduate laboratory course. Moreover, the bacterially-produced PHB can then be used by students in subsequent exercises for comparison with polymers produced using chemical synthesis. In addition to chemistry and polymer science curricula, this laboratory experiment is also suitable for a variety of biology, biochemistry, or biotechnology curricula.

This laboratory experiment was incorporated into an undergraduate multidisciplinary course that is cross-listed between five departments at the Georgia Institute of Technology, and student reactions in course evaluations have been very favorable (e.g., "The material was very interesting and I learned a lot."). Students were required to write detailed laboratory reports and also to give end-of-semester oral presentations. The bacterially-produced PHB was then used by the students in subsequent laboratory exercises later in the course. For example, in a laboratory session focusing on NMR spectroscopy, students compared the bioPHB with synthetically prepared PHB (via ring-opening polymerization of β-butyrolactone). They also conducted a laboratory experiment that focused on thermal analysis in which they compared the DSC (differential scanning calorimetry) and TGA (thermogravimetric analysis) scans of the bioPHB with synthetic PHB. They were then able to relate the thermal properties to tacticity determined from NMR,
which they could then connect to the preparation method (bio versus synthetic). Students were able to confirm from their NMR and IR analyses that bioPHB has enantiomerically pure form and isotactic configuration which results in a crystalline solid state, whereas synthetic PHB consists of a mixture of several configurations and amorphous form.

**Website that Acknowledge NSF Support**

CIBER Website: www.ciber.chemistry.gatech.edu. As seen from the most recent 6-month statistics, generated by Awstats Website (Figure II.13, above), the site is being used by an average of 243 different users each month and is generating an average of 26,865 hits per month. Navigations to the website have been referred by search engines such as Google, Yahoo and Bing.

**Professor A. Ragauskas/Georgia Institute of Technology:** Prior studies by Ragauskas had demonstrated the ability to functionalize cellulosics by periodate oxidation and sulfonation could provide a unique super-absorbent materials when nanocellulose structures (i.e., whiskers and balls) were derivatized in this manner (see Scheme III.1).

![Scheme III.1. Oxidation and sulfonation of cellulosics.](image)

The results of this investigation were reported last year in Cellulose {(2008), 15(3), 489-496} and highlights of these studies are presented in Figure III.1. The difference in water absorption properties were attributed differences in the dimensionality of the cellulosics materials employed.
Figure III.1. Water retention values for softwood ECF bleached kraft fiber (C0), cellulose nanospheres (C1: 360 nm diameter & C2: 80 nm diameter) and cellulose whiskers (C3 8 nm diameter, 300 nm length) native and after low (L) and high (H) chemical charge of sodium periodate oxidation (O)/sulfonation (S).

During these investigations, it was observed that employing excessively high charges of periodate followed by sulfonation lead to complete dissolution of cellulose in water. Although this was initially attributed to oxidative fragmentation of cellulose, it became apparent that this was not the case and indeed a new water soluble cellulose derivative had been discovered. Given the general interest in water soluble biopolymers we studied this chemistry further.

To investigate this phenomena, we conducted a series of sodium periodate oxidations of a bleached hardwood kraft pulp (see Table III.1).

Table III.1. Copper number and carbonyl content of oxidized cellulose samples.

<table>
<thead>
<tr>
<th>Samples</th>
<th>NaIO₄/Cellulose (w/w)</th>
<th>Copper number (mmol g⁻¹)</th>
<th>Carbonyl content (mmol g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting Cellulosic</td>
<td>0.0</td>
<td>0.037</td>
<td>0.002</td>
</tr>
<tr>
<td>DAC0.2</td>
<td>0.2</td>
<td>1.20</td>
<td>0.12</td>
</tr>
<tr>
<td>DAC0.3</td>
<td>0.3</td>
<td>1.41</td>
<td>0.14</td>
</tr>
<tr>
<td>DAC0.4</td>
<td>0.4</td>
<td>1.67</td>
<td>0.17</td>
</tr>
<tr>
<td>DAC0.5</td>
<td>0.5</td>
<td>1.74</td>
<td>0.18</td>
</tr>
<tr>
<td>DAC0.7</td>
<td>0.7</td>
<td>2.10</td>
<td>0.22</td>
</tr>
<tr>
<td>DAC1.0</td>
<td>1.0</td>
<td>2.66</td>
<td>0.28</td>
</tr>
</tbody>
</table>

DAC: Dialdehyde cellulose
The cellulose copper number was determined experimentally from the reductive reaction of dialdehyde cellulose with CuSO₄ and then it was converted to carbonyl content using equation III.1.

\[
\text{Carbonyl Group Content (mmol/100 g o.d. pulp)} = \frac{(\text{Cu}^\# - 0.07)}{0.6} \quad (\text{III.1})
\]

It can be observed from Table 1 that as the ratio of sodium periodate to cellulosic fiber increased the carbonyl group content of oxidized fiber is increased.

The presence of the aldehyde group in oxidized fiber was further verified by using FT-IR spectroscopy. The characteristic band for the carbonyl group at 1736 cm⁻¹ and for the hemiacetal structure at 880 cm⁻¹ in the spectra of oxidized fibers clearly indicates the formation of dialdehyde cellulose after periodation. In the case of cellulose a band also appears at 900±20 cm⁻¹ due to the vibration of C⁵C⁶H and O⁶C⁶H groups which overlaps with the hemiacetal band of dialdehyde cellulose. Thus, both the copper number calculation and FT-IR spectroscopy confirms the conversion of cellulose to dialdehyde cellulose. It is also evident from the FT-IR spectra that with increasing the concentration of sodium periodate the oxidized product exhibits a gradual enhancement in carbonyl band intensity.

The dialdehyde cellulose samples were treated with sodium bisulfite to yield the corresponding sulfonated cellulose. Sulfonic acid group content in each sample was determined by conductometric titration (Table III.2) and the results indicate an increase in sulfonic acid content from 0 to 0.16 (mmol g⁻¹ o. d. fibers) with the charge of sodium periodate employed. It can also be noticed that the samples having higher carbonyl content afforded sulfonated cellulose with higher amounts of sulfonic acid groups.

**Table III.2.** Sulfonic acid group content of oxidized and sulfonated cellulose samples.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Sulfonic acid content (mmol g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting</td>
<td>0.00</td>
</tr>
<tr>
<td>Cellulosic</td>
<td></td>
</tr>
<tr>
<td>SC0.2</td>
<td>0.09</td>
</tr>
<tr>
<td>SC0.3</td>
<td>0.10</td>
</tr>
<tr>
<td>SC0.4</td>
<td>0.14</td>
</tr>
<tr>
<td>SC0.5</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Figure III.2 (a-g) shows the SEM images of the initial fibers and its oxidized product. Cellulosic fibers appear long, well separated from each other and are in the range of 15-18 µm in width. In the case of oxidized samples, the fibrous form is retained but the thickness of the fibers is greater than that of the cellulosic fibers, a distribution of fiber width from 15 to 40 µm (b-g). However, conversion of dialdehyde cellulose to sulfonated cellulose exhibits a significant change in surface morphology (Figure III.3), which could be due to the mass losses during the sulfonation process. These samples have a smooth surface compared to both initial fiber and dialdehyde cellulose. In conclusion, the surface morphology of cellulose fibers has been affected by the oxidation/sulfonation sequence.
Figure III.2. SEM images of (a) cellulose (b) DAC0.2 (c) DAC0.3 (d) DAC0.4 (e) DAC0.5 (f) DAC0.7 (g) DAC1.0.
The structure of the oxidized/sulfonated product was also confirmed by $^1$H and HSQC NMR. Determination of the solubility of sulfonated cellulose samples in water was accomplished using $^1$H NMR with DMSO as an internal standard (see Figure III.4). Solubility was calculated based on the integration ratio of the sample signal to an internal standard signal. It is interesting to note that the solubility of the sulfonated cellulose increases dramatically from 2.85 to 28.57 g/L with the increased level of oxidation and sulfonation.
**Figure III.4.** Solubility of oxidized and sulfonated cellulose samples determined from $^1$H NMR spectra.

The molecular weight and polydispersity index of cellulosic fiber and sulfonated samples was obtained by size exclusion chromatography (Table III.3). Due to the limited solubility of lower degree sulfonated samples in water, the data was only obtained for samples having high sulfonic acid content. An increase in molecular weight of sulfonated samples compared to starting material indicates the addition of sulfonic acid group to the cellulose chain through oxidation/sulfonation reaction.

**Table III.3.** Molecular weight and polydispersity index of oxidized and sulfonated cellulose samples by size exclusion chromatography.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Number average molecular weight Mn (g/mol)</th>
<th>Weight average molecular weight Mw (g/mol)</th>
<th>Polydispersity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting Cellulosic</td>
<td>1.87×10^5</td>
<td>7.66×10^5</td>
<td>4.09</td>
</tr>
<tr>
<td>SC0.5</td>
<td>2.44×10^5</td>
<td>1.68×10^6</td>
<td>6.87</td>
</tr>
<tr>
<td>SC0.7</td>
<td>2.34×10^5</td>
<td>1.72×10^6</td>
<td>7.38</td>
</tr>
<tr>
<td>SC1.0</td>
<td>2.77×10^5</td>
<td>1.76×10^6</td>
<td>6.36</td>
</tr>
</tbody>
</table>

In summary, a series of sulfonated cellulose samples were prepared by following standardized sodium periodate oxidation and sodium bisulfite sulfonation of hardwood bleached kraft fibers. The products were obtained in good yields and the increase in periodate concentration has led to a corresponding increase in sulfonic acid group in the sulfonation step. The resulting product was shown to have water solubility properties provided that the sulfonic acid content is above 0.16 mmol g$^{-1}$ of the sample. Thus, a novel water soluble derivative of cellulose was successfully prepared at room temperature through periodation/sulfonation sequence which has a promising
future as a biocompatible and biodegradable material in biomedical and other industry applications.

As a compliment to oxidation sulfonation of cellulosic fibers, we have previously reported novel properties acquired by crosslinking cellulose whiskers with the poly(methyl vinyl ether co-maleic acid) (PMVEMA) – polyethylene glycol (PEG) matrix. The resulting crosslinked nanocomposite was shown to have unique properties that were distinctly different from the starting components and of special interest were the hydrogel properties that facilitated up to ∼900% mass absorption of water.

PMVEMA is a polycarboxylic acid containing polymer that is currently used in health care applications. However, PMVEMA has recently been investigated for its potential as a bioadhesive polymer for drug delivery options. There has been interest in expanding its use with cellulosic materials. Barcus and Bjorkquist developed a procedure of reacting PMVEMA and PEG with wood fibers using a thermal dehydration reaction as an alternative method for grafting cellulose without metal catalysts to create a crosslinked material with increased water sorption properties, while Khutoryanskaya et al. have investigated blends of hydroxyethylcellulose and PMVEMA.

It is expected that the in-situ co-crosslinking of nanowhiskers in a water soluble biopolymer matrix leads to the development of a well dispersed nanocomposite with enhanced mechanical properties and stability in aqueous medium. Additionally, the crosslinking may enable the dispersed nanowhiskers to be locked within the polymer matrix and develop nanocomposite materials with tailor-made properties depending on the degree of crosslinking, nanowhiskers concentration, and relative humidity conditions. Research results over the last year determined the mechanical properties and transverse relaxation of crosslinked PMVEMA-PEG-CNW nanocomposites and the effect of CNW concentration on these physical properties. We have reported and published the procedure for preparing the PMVEMA-PEG/CNW nanocomposite hydrogels the past fiscal year. The nanocomposite hydrogels are described according to the percent nanowhiskers in each composition (see Table III.4). The mass of PMVEMA to PEG is maintained throughout at 6.70 g PMVEMA: 1.00 g PEG. An unreacted control film of 50 wt-% nanowhiskers and 50 wt-% matrix was also prepared using the same method as the crosslinked nanocomposite hydrogels with the exception of not being cured. The nanocomposite hydrogels were stored in desiccators at varying relative humidity (i.e., 2%, 54%, and 92%) for 1 week prior to physical testing.

Table III.4. Composition of PMVEMA-PEG/CNW nanocomposite hydrogels.

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>CNW, wt %</th>
<th>PMVEMA-PEG, wt %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0CNW</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>25CNW</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>50CNW</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>75CNW</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>100CNW</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>
PMVEMA and cellulose nanowhiskers were shown to undergo an esterification reaction primarily between the primary hydroxyl group of the cellulose and the acid group of the PMVEMA (Figure III.5). In addition, the PEG crosslinks with the PMVEMA in a second esterification reaction. The prior report demonstrated that all three components, PEG, PMVEMA, and cellulose nanowhiskers, were needed to form the reported nanocomposites.

![Figure III.5. Crosslinked PMVEMA, PEG, and cellulose nanowhiskers.](image)

**AFM Imaging.** The size and size distribution of the nanowhiskers used for this study is shown in Figure III.6. The nanowhiskers are well separated needle shaped crystals with diameters between 15-17 nm. The diameter measurements were made using the Nanoscope V software and were determined from the height of the nanowhiskers. This methodology has been shown to be more accurate for the characterization of CNW since the broadening effect due to the AFM tip scanning is considered to be minimized in comparison to width measurements.73
Figure III.6. The height image of the nanowhiskers showing the diameter measurements of the cellulose nanowhiskers.

An overview and detailed view of crosslinked nanocomposites with 25 and 50 CNW are shown in Figure 7. These results demonstrate a relatively homogenous distribution of nanowhiskers throughout the matrix.
In the AFM micrographs, the oval shaped structures are attributed to cellulose nanowhiskers embedded in the PMVEMA-PEG phase. A broad range of size distribution is observed for the nanowhiskers in the nanocomposites images which may be due to the fact that the nanowhiskers are cut at different angles during sample preparation. This is expected as the nanowhiskers are randomly oriented in solution casted films and the AFM image obtained is from the ultramicrotomed cross-section.

**Mechanical properties.** The stress-strain properties of the prepared hydrogels were studied as a function of CNW content and relative humidity (RH) conditions. The nanocomposite hydrogels with 25, 50, 75 and 100 wt.-% CNW content conditioned at 92% RH were evaluated for physical properties and these results are shown in Table III.5. It can be seen that the tensile strength was increased when the nanowhisker content increased from 25 to 50 wt-%. The modulus (stiffness)
of the hydrogels was very low for 25 and 50CNW but increased for the hydrogels with 75 and
100 wt.-% CNW content. All hydrogels exhibited a very high ability to strain, between 120-
306%, with the highest values for lower nanowhisker content. Compared to cellulose nanofiber
networks (nanopaper) toughness values reported by Henriksson et al.74 the in-situ crosslinked
materials have superior toughness but lower stiffness and strength. The effect of the relative
humidity on the mechanical properties is demonstrated in Table III.5 based on the mechanical
performance of the 75 wt.-% CNW content in the composites conditioned at three different
relative humidity conditions (2, 54 and 92%). The percent elongation at break increased as the
relative humidity increased, while the maximum stress at break was achieved at the lowest
relative humidity. From Table III.5, it is possible to see the correlation between the composition
and relative humidity. For example, as the relative humidity increased, elongation at break (%) also increased, while the tensile strength (MPa) decreased as relative humidity increased. This
shows that these materials are capable of a wide range of properties depending on the moisture
conditions.

Table III.5. Mechanical properties of matrix and crosslinked nanocomposite hydrogels at
different relative humidities.

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Relative Humidity [%]</th>
<th>Tensile Strength [MPa]</th>
<th>Strain [%]</th>
<th>E-modulus [GPa]</th>
</tr>
</thead>
<tbody>
<tr>
<td>25CNW</td>
<td>92</td>
<td>2.3 ± 0.8</td>
<td>309 ± 16</td>
<td>0.09 ± 0.06</td>
</tr>
<tr>
<td>50CNW</td>
<td>92</td>
<td>4.2 ± 0.2</td>
<td>227 ± 24</td>
<td>0.19 ± 0.06</td>
</tr>
<tr>
<td>75CNW</td>
<td>92</td>
<td>4.0 ± 0.1</td>
<td>120 ± 2</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>100CNW</td>
<td>92</td>
<td>4.0 ± 1.6</td>
<td>105 ± 0</td>
<td>3.9 ± 0.8</td>
</tr>
<tr>
<td>75CNW</td>
<td>2</td>
<td>36.8 ± 5.3</td>
<td>106 ± 2</td>
<td>14.3 ± 3.3</td>
</tr>
<tr>
<td>75CNW</td>
<td>54</td>
<td>8.5 ± 2.7</td>
<td>116 ± 4</td>
<td>8.7 ± 0.9</td>
</tr>
<tr>
<td>75CNW</td>
<td>92</td>
<td>4.0 ± 0.05</td>
<td>120 ± 2</td>
<td>2.3 ± 0.1</td>
</tr>
</tbody>
</table>

The representative stress-strain curves of the crosslinked composites varied with different CNW
content, with the 92% RH conditioned samples shown in Figure 8. As the nanowhisker content
increased, the stress and modulus increased, while the strain decreased. As can be seen in Table
III.5 and Figure III.8, the strain and the stress varied based on the composition of the materials.
However the relationship between the CNW content and the mechanical properties is non-
linear. It appears as though the 50CNW at 92% RH (7c) is able to balance stress verse strain demands
better than the 25CNW or the 75CNW composite nanocomposite hydrogels. In summary, each
of the nanocomposite hydrogels behaved differently from the others. This furthers our belief that
these materials have developed differing degrees of crosslinking between the matrix and the
cellulose nanowhiskers. This provides the possibility to potentially tailor these materials to a
specific set of conditions depending upon the desired application.
In conclusion, in-situ co-crosslinked nanocomposite hydrogels were developed by dispersing and crosslinking cellulose nanowhiskers with poly(methyl vinyl ether-co-maleic acid) - polyethylene glycol matrix, using solution casting and subsequent crosslinking technique. Atomic force microscopy images show that the nanowhiskers were dispersed homogenously in the matrix even at high nanowhisker concentration indicating that in-situ co-crosslinking of cellulose nanowhiskers with a matrix polymer is a promising new route to prevent nanowhisker aggregation and obtain good dispersion at the nanolevel.

The crosslinked nanocomposite hydrogels exhibited significantly different behaviour both relaxationally, mechanically and morphologically as the material percentages were varied. The mechanical property increased with increase in nanowhisker content and was found to decrease as relative humidity increased. The nanocomposite hydrogels with 50 wt.-% CNW content appear to be a balance between the mechanical properties of the nanocomposites with 25 and 75 wt.-% CNW content. This new methodology of nanocomposite processing is expected to provide an efficient route to develop tailor-made nanocomposite controlled by degree of co-crosslinking, nanowhisker concentration, and relative humidity conditions.

In conclusion, we have demonstrated the unique features of functionalizing cellulosics at the micro and nano level. In the future the results could use waste, cellulosic streams from the forest biorefinery thereby capturing greater value from woody biomass. These results have been reviewed annually by the forest products industrial consortium located at the Institute of Paper Science and Technology at the Georgia Institute of Technology. In addition, our expertise has been sought by several small businesses that are interested in the forest biorefinery concept. We are also actively seeking additional industrial partnerships to advance the concepts developed over the last year of these studies.

**Figure III.8.** The stress-strain curves of prepared hydrogels a) 100CNW, b) 75CNW, c) 50CNW, d) 25CNW nanocomposite hydrogels at 92% relative humidity.
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