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Final Technical Report

Project Title

Multidisciplinary Graduate Education in Bioprocess Engineering

Project Objective

The project objective was to provide advanced engineering training to produce discipline-based biological engineering graduates who can effectively integrate an understanding of renewable resources and their markets, bioprocess engineering, molecular biology, social and environmental issues and advanced mathematics in the implementation of a comprehensive research program.

Project Abstract

This report describes the accomplishments of the University of Georgia in establishing an academic program geared toward the emerging biobased products industry. By virtue of its strengths and structure, the University of Georgia is particularly well-suited for developing a program focused on plant- and microbial-based bioproducts, and it was in this general area that this program was developed.

The program had several unique characteristics. First, we implemented a distinguished lecture series that brought outstanding scientists and engineers to our University to interact with students and share their vision of the biobased economy. Second, we offered industrially-oriented and multidisciplinary courses that provided students with a broad background on various facets of biobased business and technology. Third, we provided the students with opportunities to expand beyond the classroom by engaging in research lab rotations and industrial internships. Fourth, each student was engaged in a creative research project as led by a multidisciplinary faculty team. Throughout the implementation of these activities, we maintained a student-centered, mentoring approach to education.

The most tangible outcome of this project was the graduation of two students who participated in a variety of scholarly activities, culminating in research toward the completion of a thesis and dissertation. Both research projects involved the use of microorganisms to produce industrial products from agricultural substrates via fermentation processes. The research advanced our understanding of microorganisms as used for industrial processes and products, as described in several articles published in scholarly journals and presentations made at scientific conferences (see information on pp. 14-15). Another outcome is one graduate course, Fermentation Engineering Laboratory, which is a unique experiential and multidisciplinary course. This course will be offered in the future as an elective to graduate students in several engineering and science degree programs.

Other significant developments have arisen as direct or indirect consequences of this project. The University of Georgia has established a B.S. Biochemical Engineering degree and an M.S. Biochemical Engineering degree. A strong component of these degree programs is education toward a biobased economy. We will integrate particularly positive components of this project (such as the distinguished lecture series) into these degree programs. The University of Georgia is establishing a Center for Biorefining and Carbon Cycling. This multidisciplinary Center houses a pilot scale biorefinery, comprising a pyrolysis unit and an ethanol plant. Together with new faculty positions that are currently being advertised, this project has encouraged the University of Georgia to assume a leadership role in the preparation of students in the biobased industries of the future.

Technical Report

Background

We wanted to put together a program that would serve as a model for the education of students interested in contributing to the future biobased economy. We anticipated an evolutionary process. The program was more about preparing us, the faculty of the University of Georgia, than about preparing a couple of specific graduate students. If we were able, at least partly, to change our approach to research and instruction and extend ourselves, then we would be prepared to continue supporting the education of students for years to come. In a sense the two students supported by this Graduate Fellowship Program were test cases, individuals who could help us achieve the long term objectives that underlie this project. It is intended that the students will go out to make contributions to society and propagate this continuum of knowledge, and we at the University of Georgia who served as project "directors" will also continue to make contributions by virtue of our experiences. If the project ends merely with the departure of the two students and the completion of this report, then it really would not have been successful.

How did we set out to develop and implement an academic program for the future biobased economy? From the comments made above, about the long term objectives, as well as informal input from external sources, learning the process of learning would seem to be more important than learning about specific information or techniques. A graduate student fifteen years ago would never have learned about DNA microarrays, BLAST or the myriad of biological/computational tools available on the internet. These 1990 graduates, entering mid-career in 2005, would be obsolete if they relied only on the information that they learned while in school. So, the underlying concept we recognized was that, while learning the current state of science and technology was important, we had to create a strong environment of inquiry and independence. This goal would seem at first to be obvious, and would seem to be no different from what any academic program would set out to accomplish. However, this is not the case. Curricula are largely constrained by historical precedent. Disciplines are isolated by language and University structure. We needed to break free from the formulaic, prescribed curriculum that is common at most graduate engineering programs. The funds enabled us moreover, and importantly, to break free from the prescribed research program that most students are faced with once they have entered a program. U.S. graduate education is currently based (largely as a result of the mechanism through which Universities are funded) on a model through which the research project is the focus, and the presumption is that "quality" education will occur as a natural consequence of the research process. We wanted to begin at least to shift this thinking toward a model in which the education was the focus, with the anticipation that "quality" research would occur as a natural consequence of the educational process. Therefore we strived to make our program student-centered. Throughout our project, it was in this area that we struggled the most, and which we also found quite difficult to document.

To achieve the objective of training graduate students in bioprocess engineering, we established the following principles to guide the creation and implementation of the proposed training program:

1) Graduate students must complete a rigorous program of lecture-based coursework, which provides advanced education in fields of economics, mathematics, applied biological sciences, and engineering sciences, which are integrated together to provide a foundation for bioprocesses and bioproducts. We tried to make courses as student-centered as possible. That is, within the courses, the students are engaged in presentations, etc. This goal is difficult when courses are taken outside of the "domain" of the project directors and numerous other students are enrolled in them.

2) Graduate students must complete laboratory-based coursework which provides hands-on experiences with pertinent instrumentation and equipment for bioprocesses, and develops their skills in design of experiments, interpretation of data, and presentation of results.

3) Graduates must be engaged in discussions with technical and business leaders central to the transition to a biobased economy, as well as complete meaningful industrial internships pertinent to a biobased economy, which together integrates coursework and research with real-world technical, social and economic situations. These discussions can occur informally at scientific conferences, or more formally through visits to the University and student internships.

4) Graduate students must be intimately involved in the design, implementation and conclusion of a research project making a contribution to the biobased industry.

Recruitment of Fellows

Although the project start date was November 1, 2000, the nature of the academic calendar at the University of Georgia resulted in graduate students not commencing their program until the following fall (about September 1, 2001). That is, a normal graduate student does not matriculate November 1, and a starting date of January 1 (roughly the start of the spring semester) is similarly uncommon. Students generally fall into our cycle of matriculating in the fall. What we did not do, was provide fellowships merely to students who had already been accepted into our graduate program for the fall of 2000. It was very important for the students to be engaged in the process, and this engagement therefore required that a period of recruitment occur in the ten months preceding the students' matriculation. Moreover, because the "DOE Fellows" were proposed to have additional responsibilities and expectations beyond those of "normal" graduate students, the tasks of recruitment and selection were particularly important.

The recruitment of fellows began about the same time that the project commenced in November 2000. A web-site was quickly established to explain the DOE fellowship and the requirements for acceptance into this program. The entire proposal was available on the web. Letters were sent to faculty at departments which have disciplines appropriate to our biobased graduate program. Letters were sent to student organizations at Universities in the Southeastern United States. Any inquiry made to the University from a prospective student was similarly informed of the opportunity of training in Bioprocess Engineering. All these letters and contacts directed students to the web-site and invited applications into the program. The results of all these efforts were eight applicants, three from the U.S., one from India, and four from China. Of the applicants four were male and four were female. These recruits were specific to the biobased products program, and thus can be thought of as "additional" to our regular graduate student pool, most of whom would have no particular interest in biobased products or the additional program requirements.

These eight students were of outstanding quality, each with GRE scores exceeding 2000, and having an average "quantitative GRE" score of 765.

Selection of Fellows

Having accomplished the recruitment task, the next task was to select the fellows. This activity occurred in March 2001. For this activity the complete materials submitted from the eight applicants were distributed to four faculty. These four faculty ranked the eight candidates (1 through 8) independently. In general, the faculty used the following criteria for the selection:

1) applicant demonstrates academic preparedness in the engineering sciences,
2) applicant demonstrates academic preparedness in the biological sciences,
3) applicant demonstrates commitment to study biobased products, and
4) applicant demonstrates outstanding communication skills.

The faculty then met to discuss their rankings, then an additional "round" of ranking was completed. The result of this selection process was an overall consensus ranking of the eight candidates. Our proposal called

for the support of two fellows. Therefore, the two highest ranking candidates were sent letters of offer, the third and fourth ranked candidates were sent letters indicating that they were “alternates”, and the lowest four ranking candidates were sent rejection letters. One of the top two candidates declined the offer, and therefore the first of the alternates was extended an offer, which ultimately was accepted. The result of this activity was (by May 15, 2001) two “DOE Fellows” also known as “Fellow A” and “Fellow B”. Fellow A entered the Ph.D. program, while Fellow B entered the M. S. program. Both were male. One was a foreign national, while the second was a U.S. citizen.

Distinguished Lecturer Series

During any graduate student’s first academic year (that is in this case, the fall of 2001 and the spring of 2002), the primary responsibility is to complete a set of core courses (and these will be described with other courses in the “Courses” section). We imposed a couple of additional responsibilities on the Fellows. The Fellows with their graduate student colleagues were to select distinguished scientists and engineers for a lecture series.

In March 2001 (prior to the selection of the fellows), the graduate students of the Engineering Department met to brainstorm possible distinguished lecturers. A list of approximately 20 names was generated. Once the 2 fellows were selected and committed in May, they contributed to this list and were engaged in the discussions. By a process analogous to the selection of fellows, a group of 4 graduate students and two faculty ranked the candidate lecturers. The lecturers selected were both highly rated and as a group constituted a range of backgrounds and expertise. One in particular was selected due to his experience with this Biobased-Products Education Initiative from another University.

The selected lecturers were:

Dr. Helena Chum, Director of Renewable Chemical Technologies, NREL;
Dr. Doug Cameron, Director of Biotechnology, Cargill;
Dr. Greg Zeikus, Professor of Biochemistry, Michigan State University;
Dr. Michael Ladisch, Professor of Biological and Agricultural Engineering, Purdue University,
Dr. Greg Luli, BCI Corp.;
Dr. Jarrold Lalman, Oklahoma State University (now University of Windsor);
Dr. Terry Walker, Clemson University; and
Dr. David Mousdale, Managing Director of beocarta Ltd.

Three individuals (Chum, Cameron, Zeikus) were able to visit in the Spring of 2002, while four individuals (Luli, Lalman, Walker, Mousdale) visited the University of Georgia in the Spring of 2003. Dr. Ladisch had several scheduling conflicts arise, and he was never able to visit.

While the selection of lecturers was a very “student-centered” process, the visits were similarly not the usual “meet-faculty, present-seminar-and-leave” type of activity, but instead were also very “student-centered.” Generally, the students assisted in planning the lecturer’s visit and developing an itinerary. Also, the first meeting on the lecturer’s schedule was breakfast with the two Fellows. Such an initial meeting was a crucial beginning to set the tone for the day and establish the purpose for the visit. Meeting with the two Fellows sent a very clear message to the visitors (on which a couple commented) that their fundamental role was one of education, one of imparting their experiences to students. This approach worked surprisingly well. Having the students meet with the distinguished lecturer first allowed the students a sense of responsibility, engagement and elevated their self-esteem. During the day, each visitor was escorted by students around campus to visit other labs and faculty. At about the midpoint of the day, the lecturer provided a seminar before numerous students, staff and faculty. During the day, there were additional opportunities for lecturers to visit with individual students and small groups of students in their laboratories. On some occasions, the

lecturers joined a group of students to “go out for a beer”. In most cases, the day closed with dinner with the visitor, two faculty and the two Fellows.

A central premise motivating the design of the visits in this way was that through informal interactions, students could understand the approaches, values and motivations of these leaders/lecturers. The students (and indeed the faculty participating) learned not only the historical facts and activities of each individual, but understood their view of the world, with emphasis on the “biobased-world”. At the onset of their graduate research, when the students were full of ideas and open to possibilities (hopefully they remain so), these visits had a substantial impact.

The leader/lecturers each gained from their experiences and the University community gained as well from their visits. Three of the seven leader/lecturers have returned to the University on their own time to follow-up on ideas that were initiated during their seminal visit. Not only the two Fellows, but also other students interacted with the visitors and benefited. Finally, the program itself, including curricular development, was impacted by the suggestions made from the leader/lecturers.

In summary, the process of the distinguished lecture series was ideal for accomplishing the student-centered goal, and will be a model for future series that are established related to on-going biobased-products activities at this University.

Lab Rotation

A second component of this project was that each fellow selected one or more temporary academic advisors who acted as mentors. The idea was to expose the student to faculty, activities and graduate students in a variety of research areas. This aspect of the project was moderately successful. The students were indeed exposed to several other faculty, including ones in Microbiology, Pharmacy and Engineering. The Fellows gained a variety of experiences. For example, one Fellow worked briefly on a fermentation project to produce alanine by recombinant microorganisms. One Fellow was mentored by a crystallography professor, and worked on cloning diaminopimelate decarboxylase, an enzyme involved in the lysine pathway.

Although these activities provided the fellows with a broad range of experiences, they also took time and as such detracted from the ultimate objectives of completing courses, a specific research project, and a thesis/dissertation. The goal of attaining a “broad experience” probably does not fit well in the context of a research project. The students would do better (i.e., spend their time more efficiently) to focus on one specific research project. The problems in trying to break-away from the traditional model of graduate education are two-fold. First, Universities ultimately require the completion of an independent dissertation. Any time spent on a lab rotation must be balanced with the time that is required to complete the independent work. Developing a new skill is perceived as beneficial only so far as it may assist a specific project. Another way to consider this point is to calculate the “value” of completing a 9 month lab rotation compared with graduating 9 months sooner and thereafter presumably earning a salary. Second, faculty (including those whose labs a rotating student would occupy) are commonly funded because of a specific area of expertise or for a specific research objective. Thus, the student entering a lab for a rotation is generally perceived as “free-hands” for that lab to assist them in completing a different but nevertheless specific research objective. Rotation for all students in diverse areas and for a period of one semester is an excellent way to facilitate cross-fertilization and expose the students to new ideas. Most of the “breadth” though should and can be obtained through formal coursework, in addition to the lectures and internships.

Coursework

The two fellows were required to complete a set of courses. Many University and Departmental requirements to some extent constrain the selection of coursework. Fortunately (or unfortunately), our

engineering curriculum does not have a core set of graduate engineering coursework. Thus, at the time the students matriculated, there was only one required engineering course. This fact allowed us to compose a comparatively tailored curriculum for each student. We continue to be convinced that having only a limited number of required courses provides the student with the flexibility to engage in interdisciplinary programs, and also would allow the program itself to adapt quickly to changing demands for education. There is really no reason not to permit this tailored approach (aside from ever-evolving University regulations where applicable).

Both students selected a "molecular track", which integrated molecular techniques in biotechnology with engineering coursework. Ultimately, their interest was in fermentation processes. This molecular biological aspect of their interests of course was also associated with their selection of research projects.

Fellow #1 (Ph.D. student) completed the following courses:

Sensors and Transducers, Research Methods, System Simulation, Statistical Analysis, Biotechnology, Biochemical Engineering, Monitoring and Control of Biological Processes, Numerical Analysis, Microbial Genetics Laboratory, Bioinformatics, Industrial Energy Management, Advanced Fermentation Laboratory, Genomics, Nucleic Acids

These courses represent a combination of engineering, mathematics, biochemistry and mathematical biology courses. The student gained a deep appreciation of gene regulation and expression, in the context of a quantitative engineering degree. Having teaching experiences is an additional important means of receiving instruction. Therefore, Fellow #1 has also had the experience of teaching the "Biochemical Engineering" class to undergraduate and graduate students two years after it was completed. Fellow #1 received extensive mentoring from the course instructor for the duration of the semester course, but still retained independence over homework assignments, testing, and evaluation of student performance. This instructional experience was very positive in solidifying the student's understanding of the subject matter, and relating it to other courses and his research project.

Fellow #2 (M.S. student) completed the following courses:

Biochemistry and Molecular Biology, Physical Biochemistry, Gene Technology, Research Methods, Statistical Methods, Economics of Conservation and Sustainable Development, Industrial Energy Management, Advanced Fermentation Laboratory

Two important courses in these lists of courses completed include "Industrial Energy Management" and "Advanced Fermentation Laboratory".

"Industrial Energy Management" was taught in the Spring 2002 semester, through a subcontract with an engineer employed with the State's Economic Development Institute associated with Georgia Tech. The course itself was composed of about 8 students, which in addition to the two Fellows included other full-time students as well as professionals working for state agencies. The backgrounds and varied perspectives of the course participants were outstanding and very enriching across the board. The course covered the practical application of scientific, physical, engineering and accounting concepts as applied to energy use and purchasing in industry. While the techniques apply to all industries, there was some focus on industries relying on biologically-based resources (such as the Paper and Pulp industries, which are major industries in the state of Georgia). The course was taught based on case studies, and covered: energy use in industry,

electricity cost and demand charges, electricity purchasing, natural gas purchasing, standby fuels, boilers and fired systems, steam systems, compressed air, electric motors and drives, dry processing systems, wet processing systems, instruments and measurement, process analysis and integration, HVAC systems, energy management controls, industrial lighting, energy auditing practices, productivity considerations, pollution prevention. The students participated in a detailed energy audit at an industrial facility, and used software tools to determine energy use, costs, major energy-using processes, air emission quantities, and options for reducing energy use and costs. The major means of evaluating performance in the course was through a final written report (which was provided to the industry). Response from the course was very positive. Fellows benefited from being exposed to participants who themselves worked in industry or in state agencies and thus had a different perspective on the subject matter of the course. A common theme in feedback was that the students "hadn't thought about energy use in this way before."

"Advanced Fermentation Laboratory" was taught by a team of two instructors in the spring 2002 semester. Students were required to have substantial background in biochemical engineering as prerequisite to the course. Each instructor covered two separate laboratory experiences. The four general topics covered were: mass transfer of oxygenation, secondary metabolite production by fungi, ethanol production (by Z. mobilis) and continuous fermentation of mixed substrates. Each lab was presented to the students initially, through a two-hour lecture providing background information. In this background lecture numerous references were provided to the students as background reading. At the end of the lecture, a problem was posed to the students with little additional information on how to go about addressing and studying the problem. The students were then required to prepare a written proposal outlining the (experimental) steps they intended to take in addressing the problem posed. Furthermore, the students were required to make an oral presentation of the work before a "board of directors" (i.e., faculty), in which they had to defend their proposal. After completion of this oral report, the students worked in teams to complete the experimentation and deliver a final report. Of course, the intent of this course was to encourage creative thinking while the students also developed skills in design of experiments, team problem solving, project planning and time management. All these "soft" factors were placed in the backdrop of specific technical information on bioprocess engineering. In general, response from the course was fair. Students felt extremely uncomfortable without the usual guidance and without being told "what to do". This type of "problem" permeated into all aspects of the course, such as trying to figure out what variables needed to be measured, when, and how they needed to be interpreted.

After this course was taught for the first time, faculty agreed to a variety of modifications. First, students need to be provided with greater guidance while still permitting them to develop project planning and time management skills. Some faculty in other departments (outside of engineering) indicated a strong interest in this course and agreed to help with its evolution and second offering in the spring of 2004.

The multidisciplinary "Fermentation Engineering Laboratory" course was offered a second time during the spring semester 2004. Eight students registered for the course. Now with the assistance of a faculty member in Microbiology, the course was again offered to Microbiology students and to Engineering students. The course covered five experiments including ethanol production using a recombinant organism, a chemostat using yeast cells, fed-batch culture for secondary metabolite production and oxygen transfer experiments. Students were grouped in teams of four for each experiment, and had to complete a written and/or oral report after each experiments' completion. The membership in the groups rotated, so that everyone had the chance to serve as a "group leader" and to be grouped with everyone else. There was also the opportunity for the groups to make comparison studies. For example, one group could study the use of glucose as the substrate while the other group could study the use of fructose as the substrate. Details of the course are available on the course website: http://www.engr.uga.edu/~eiteman/miboengr/details.html

The response to this course was very positive. One significant benefit of this course is the unique setting of having students from multiple backgrounds interact and learn from each other. The students are also having

the opportunity to “problem solve” in real time. Several students mentioned that they had never before had the opportunity to work on technical projects in diverse teams. Additional support of about $1,000 was provided from the widow of a departmental alumni to purchase supplies and consumables for this course.

The two Fellows completed this course when it was first offered two years before and thus did not complete the course this second time. However, Fellow #2 served as a teaching assistant for the course. This activity further benefited his knowledge and experience in this area.

At this project draws to a close in the fall of 2005, the “Fermentation Engineering Laboratory” course is formally being considered as a graduate level course, BCMB 8810, for a M.S. Biochemical Engineering degree program.

To further expand their molecular biology experiences, both Fellows also enrolled in an independent study course in which a molecular biologist provided them with instruction on the preparation of strains, specifically performing one gene knockout and one gene transformation. Fellow #1 was involved in knocking out the pps gene encoding for PEP synthase in E. coli while Fellow #2 was involved in knocking out the sfcA gene encoding for malic enzyme in E. coli. Both of these activities were successfully completed. Additionally, Fellow #2 isolate genomic DNA from E. coli for the acs gene encoding for acetyl CoA synthase, constructing a plasmid with that DNA, and transforming E. coli with that plasmid. This activity was also successful, and an enzyme assay was developed to measure the acetyl CoA synthase activity. Although Fellow #1 received the fdh gene from S. cerevisiae encoding for formate dehydrogenase, this Fellow did not complete the construction of a plasmid with the DNA.

Industrial Internship

One component of this project was that the Fellows should complete an industry internship. We tried to have this internship accomplished early in the project period, when the research project had not yet been formulated. Both Fellows commenced a six month industry internship at small pharmaceutical companies. The selection of companies was limited to those companies who sought interns, and the small pharmaceutical company sector was one of the few employing interns. Though this industry sector was not ideal for the overarching objectives of this proposal (i.e., a future "biobased economy") the sector did represent a mature biobased industry. Furthermore, the internship period clearly permitted each student to understand “real” problems and to formulate relevant research projects. We found the six month period ideal in that it was long enough for the intern to be treated more like an “employee” rather than merely a temporary intern. Thus, the Fellows were able to engage in realistic company activities, and could devote time to a specific and more meaningful project. It was important that the students’ find their own internships, with support and assistance of faculty as necessary.

Both Fellows had good, educational experiences through the industrial internship. Fellow #1 completed several projects for the company, and helped them implement several process modifications which benefited their production processes. Fellow #2 worked with yeast fermentations until the company elected to “save money” and abruptly terminated all internships after the student had been employed for about 4 months. While this result was first considered to be unfortunate, the experience that was gained by the student (which was shared to other students) could not have been replicated in such a relatively harmless way. This result, and the way the termination was conducted, turned out to provide great insights into companies which were shared among many graduate students.

The benefits of these internships were derived not so much by the technical details of their work (although this benefit did occur), but more by the overall experience of working in an industrial company. The students learned how each company got things done. In their accounts of experiences, both students expressed surprise at the significance of profit-motivation in the companies, and their exposure to the politics and

bureaucracy. They gained an exposure into the value-system in corporate America. Having students more knowledgeable about industrial settings made them better participants and critics to academia. It also made these students better prepared to conceive and plan a meaningful research project. The students who participated in internships have returned to the academic setting to become mavens for other students. That is, they have shared their insights into the values of companies more effectively than professors can in a classroom setting.

One primary disadvantage of the internships was the time they took. Professors as well as the University, must get used to the idea of their “workers” being gone for a block of six months. During that time “nothing” is getting accomplished that is relevant to the faculty members’ usual benchmarks for success (such as publications, presentations, more funding, etc.). Similarly, performance during this interval is difficult to document for a federal or state funding agency. Thus, the culture of both the University and granting agencies do not generally encourage this type of activity. Finally, it is challenging to find internships directed at a "future" biobased economy, in that the number of industries is still small, and the particular companies may not have established relationships with Universities and internship programs, etc. Our experience makes clear that a good partnership between a consortium of Universities and industries would be very beneficial in bringing "realistic" problems to University research and instruction, as well as in communicating to industries about the activities (and students) at Universities working in the biobased area.

Research

After the engagement of the students through industry internships, distinguished lecture series, and other University scientists through lab rotations, we intended that the two Fellows would be able to contribute to mankind’s knowledge through a creative research project. The research is not the end, however. As noted in the Background section, the research is a consequence of the educational experience, not the other way around. Moreover, success in research as defined by the usual indicators (i.e., number of publications, intellectual property, etc.) does not necessarily indicate success in education. Conversely, failure to publish results entirely (by virtue of their ambiguity, for example) does not mean that the student involved in that project did not “become educated.”

One Fellow (#1) became interested in overflow metabolism. Overflow metabolism is the condition where cells generate an undesirable byproduct when they quickly consume a substrate like glucose beyond a threshold rate. In the case of bacteria like E. coli, the cells accumulate acetate when the glucose consumption rate exceeds this threshold. In the case of yeast like S. cerevisiae, the cells accumulate ethanol when the glucose consumption rate exceeds this threshold. These products are generated regardless of the availability of other nutrients including oxygen, but are merely a consequence of their fast consumption of the substrate. We speculated that overflow metabolism centered on a couple of issues. First, pyruvate is the biochemical precursor of acetate or ethanol, and the accumulation of this compound could trigger overflow metabolism. Second, it is well-known that oxygen consumption reaches a plateau at high growth rates. That is, cells have a limited capacity to consume oxygen even when this gas is readily available. If cells cannot consume oxygen beyond a particular rate despite glucose consumption increasing, then the reduced cofactor NADH generated during glucose consumption could itself accumulate, an event which could trigger overflow metabolism.

This research involved three lines of inquiry. One approach was to redirect carbon away from acetate or pyruvate the bacteria E. coli through the use of pyruvate carboxylase and via the knockout of pyruvate oxidase. This work led to one publication. The results clearly demonstrate that pyruvate carboxylase is able to redirect carbon toward the tricarboxylic acid cycle and away from acetate. Moreover, the results demonstrate that pyruvate oxidase is important in acetate accumulation.

The results of this first line of inquiry led to another question. Why does pyruvate accumulate and what is the relationship between pyruvate accumulation and the cofactor NADH? In order to address this question, a very complex set of experiments were undertaken. First, we had to construct a strain of bacteria that overexpressed the enzyme NADH oxidase. NADH oxidase is a way for the cell to consume any "extra" NADH without any other physiological consequence. Normally, NADH reconversion to NAD occurs via the electron transport chain and is accompanied by ATP generation. NADH oxidase permits the decoupling of NAD regeneration from ATP generation. Thus, NADH oxidase would act as a relief valve, permitting the cell to get rid of excess NADH freely. So, we now had two strains to compare, one was the "wild-type" E. coli, while the second was the same strain expressing NADH oxidase. We grew these two strains separately in chemostats where we could carefully control the rate of glucose consumption. For each strain, some of the rates selected were below that threshold rate where acetate appears, while others were above the threshold glucose consumption rate. We measured intracellular concentrations of NADH and carbon metabolites. But we did one more thing. We took samples at 7 of these controlled glucose consumption rates and determined their genome-wide gene expression (i.e., the simultaneous expression of all 4400 genes of E. coli). We then compared the expression of all these genes as a function of glucose consumption rate for the two strains, and also compared the expression between the two strains for any given glucose consumption rate. Statistical analysis was used to determine which genes were expressed at significantly different levels among the comparison groups.

What we found from this investigation is that NADH and pyruvate accumulation is correlated with acetate accumulation. Moreover, the arcA regulatory system was highly correlated with acetate production and NADH accumulation. The arcA gene regulates numerous other genes in the tricarboxylic acid cycle, and is known to be involved in respiration. So, the next step was for us to knockout the arcA gene and create two additional strains, one only with the arcA knockout and a second with the arcA knockout which also overexpressed NADH oxidase. When we conducted fermentations with the arcA(–) NADH oxidase(+) strain, no acetate was formed even at the highest glucose consumption rates. We took a temporary detour and examined whether this result could enhance the production of any product known to be inhibited by acetate formation, and it did, so we filed for a patent on this concept.

The third and final line of inquiry was to examine overflow metabolism in yeast instead of bacteria. Do yeast behave essentially the same as bacteria, despite the product being ethanol instead of acetate? Both E. coli and S. cerevisiae exhibit overflow metabolism by forming acetate or ethanol, respectively, at high glucose consumption rates. The proposed model of this regulatory mechanism for E. coli was that when the redox ratio (NADH/NAD ratio) increases above a critical value of 0.06, the ArcAB regulon is induced, which in turn represses aerobic respiration as a mechanism to avoid the accumulation of NADH. Is ethanol formation in S. cerevisiae also related to redox? The question was complicated because glucose metabolism in S. cerevisiae is compartmentalized, and the mitochondrial membrane is impermeable to NADH. The first step in testing this hypothesis was to overexpress water-forming NADH oxidase in S. cerevisiae.

In batch cultivations using glucose minimal medium, the ethanol concentration in the NADH over expressed strain was identical to that in the control strain. However, the glycerol concentration reduced five-fold.

S. cerevisiae generates glycerol as a mechanism to oxidize the excess NADH that is formed from the EMP pathway at high glucose consumption rates. Reduced glycerol in a strain over expressing NADH oxidase could be explained by increased NADH oxidation in NOX, which precluded the need to generate glycerol. Glycerol generation in response to excess NADH occurs in the cytosol, and since this process was affected by introducing heterologous NADH oxidase it appears that this enzyme is present in the cytosol. NADH is generated in the cytosol (from glycolysis) as well as in the mitochondria (from the TCA cycle), but it cannot be transported between the two compartments. Therefore, NADH generated in each compartment has to be oxidized in the compartment where it is generated. The three main enzymes responsible for cytosolic NADH oxidation are two NADH dehydrogenases (encoded by NDE1 and NDE2) and the glycerol-3-phosphate

shuttle (GUT2). A triple deletion mutant (.NDE1 .NDE2 .GUT2) produced twice as much glycerol compared with the control strain. However, the presence of NADH oxidase in this strain restored the glycerol to the concentration found in the control. This result confirms the localization of heterologous NADH oxidase in the cytosol and also that this enzyme can functionally replace the native NADH oxidation system.

Since a significant amount of NADH is also generated in the tricarboxylic acid cycle, we expected that a means to enhance the oxidation of mitochondrial NADH would cause noticeable physiological changes. This was achieved by over expressing the aox gene from Histoplasma capsulatum, which expresses the "alternate oxidase". This enzyme is responsible for the cyanide-insensitive alternative respiration mechanism, and naturally localizes in the mitochondria. Over expressing this gene in S. cerevisiae (resulting in the strain AOX) did not affect glycerol maximum concentration, but decreased ethanol concentration four-fold compared with the control.

Although additional studies will be required to confirm the localization of this enzyme, it seems very likely that it is functional in the mitochondria. An increase in the biomass yield and growth rate of AOX along with reduced ethanol suggests increased functioning of the TCA cycle activity, which generates the precursors for biomass faster. The utilization of more carbon for biomass and CO2 generation possibly eliminated the need to generate ethanol. Therefore, from these preliminary studies it appears that ethanol formation in S. cerevisiae is the consequence of saturated TCA cycle functioning, probably due to inhibition by NADH.

The three strains (control, with NADH oxidase, with alternative oxidase) were grown in chemostats to study the differences in their respiratory capacities and to study the network utilization. A detailed analysis of transcription as well as metabolite profiling will also be performed for these strains simultaneously to provide a complete picture of the redox-mediated regulation in S. cerevisiae.

The research of Fellow #1 required the involvement of several outside researchers. In order to learn and perform DNA microarray techniques, Fellow #1 required an expert in this area. So, he traveled to the University of Minnesota to collaborate with Arkady Khodursky who works with genome-wide E. coli microarrays. Support was obtained (non-DOE funds) for an initial, organizational trip. A second trip of a couple months was used to prepare the arrays and analyze experimental results. When we wanted to turn our attention to yeast, we needed to find someone with expertise in that area. The Fellow developed a relationship with Jens Nielsen in Denmark who in addition to studying yeast, uses NMR analysis to quantify carbon flux through metabolic pathways. Fellow #1 traveled to Denmark for one year (with non-DOE funds) to receive specialized training and to participate in additional research activities. So, the research project was extended far beyond the walls of the University of Georgia. The research results have been published in 4 articles for peer-reviewed journals. Two additional manuscripts are currently in preparation.

The second Fellow (#2) was interested in the consumption of acetate by E. coli. This research is related to the project completed by Fellow #1. There are certain cases in which an organism must consume acetate in order to generate a desired product. Examples of this are processes to generate pyruvic acid, alanine and lactic acid. Some evidence in the literature exists to suggest that the accumulation of these products can be limited by the consumption of acetate. Therefore, we were motivated to find out if we could speed up the rate of acetate consumption. Theoretically, two ways exist to speed up acetate consumption. One method is to over express the gene encoding a protein which converts acetate into acetyl CoA, the acs gene. This method presupposes that the rate of acetate conversion itself is limiting acetate utilization. A second approach is to over express the gene encoding a protein which converts acetyl CoA (and oxaloacetate) into citric acid. We specifically sought a protein that was not inhibited by NADH, and therefore selected the citZ gene from Bacillus. This approach assumes that acetyl CoA build-up is preventing acetate conversion. The

balance between CoA and acetyl CoA in a cell is tightly regulated, so either one of these approaches, we felt, could be appropriate.

Toward the end of understanding acetate utilization, we over expressed, individually, both the acs gene and the citZ gene. We were not successful with the citZ gene. We learned that this Bacillus gene is not particularly well-expressed in E. coli, and the resulting protein appears to be quite fragile. The protein is a rather complicated hexamer, and it is possible that it did not form properly in the heterologous environment. Also, the Bacillus gene uses a relatively high number of rare codons (for E. coli). Therefore, an optimization of the nucleic acid sequence of this gene might enhance the activity.

We were successful in over expressing the native acs gene in E. coli. However, we learned that the growth of E. coli is extremely sensitive to the level of acetyl CoA synthase activity in the cell. Presumably this is because too much activity would consume free CoA necessary for a wide range of activities. Future work will need to address the balance between CoA and acetyl CoA, perhaps by focusing on citrate synthase.

The two projects provide significant contributions to our understanding of microbial metabolism as they apply to the development of biobased industrial products derived by fermentation processes. Moreover, they were both excellent projects to permit innovative inquiry into broad problems faced in the industrial implementation of such microbial processes.

Publications

M. Lee, G. M. Smith, M. A. Eiteman, E. Altman (2004) “Aerobic production of alanine by Escherichia coli aceF ldhA mutants expressing the Bacillus sphaericus alaD gene,” Applied Microbiology and Biotechnology 65:56-60.

G. N. Vemuri, T. A. Minning, E. Altman, M. A. Eiteman (2005) “Physiological response of central metabolism in Escherichia coli to deletion of pyruvate oxidase and introduction of heterologous pyruvate carboxylase,” Biotechnology and Bioengineering 90(1):64-76.

G. N. Vemuri, M. A. Eiteman, E. Altman (2006) “Increased recombinant protein production in Escherichia coli strains with overexpressed water-forming NADH oxidase and a deleted arcA regulatory protein,” Biotechnology and Bioengineering in press.

G. N. Vemuri, E. Altman, D. P. Sangurdekar, A. B. Khodursky, M. A. Eiteman (2006) “Regulation of overflow metabolism in Escherichia coli: transcriptional basis for physiology,” Applied and Environmental Microbiology in press.

G. M. Smith, S. A. Lee, K. C. Reilly, M. A. Eiteman, E. Altman, “Two-phase production of alanine in Escherichia coli fermentation,” submitted to Biotechnology Letters.

G. N. Vemuri, M. A. Eiteman “Flux Analysis of Escherichia coli expressing NADH oxidase,” in preparation for Metabolic Engineering.

G. N. Vemuri, M. A. Eiteman, J. Nielsen, “Overexpression of NADH oxidase in Saccharomyces cerevisiae,” in preparation for Journal of Biological Chemistry.

Attendance at Professional Conferences

The Fellows attended the following professional conferences:

Fellow A:

Society for Industrial Microbiology, Minneapolis, August 2003
Society for Industrial Microbiology, Anaheim, August 2004
Metabolic Engineering V, Lake Tahoe, September 2004
Society for Industrial Microbiology, Chicago, August 2005

Fellow B:

Society for Industrial Microbiology, Minneapolis, August 2003
American Chemical Society, San Diego, March 2005
Society for Industrial Microbiology, Chicago, August 2005

Presentations

The following presentations were made concerning this biobased program and/or the research activities of the Fellows. Several presentations (marked with *) covered broadly the research activities of the Center of Molecular BioEngineering, including portions of the research results from this project.

G. M. Smith, M. Lee, M. A. Eiteman, E. Altman, "Aerobic production of alanine by Escherichia coli aceF ldhA," Society for Industrial Microbiology, Minneapolis, August 2003

G. N. Vemuri, T. A. Minning, M. A. Eiteman, E. Altman, "Physiological consequences of genetic manipulation at the pyruvate branch point in Escherichia coli," Society for Industrial Microbiology, Minneapolis, August 2003.

*M. A. Eiteman, "Metabolic engineering of Escherichia coli at the pyruvate node," 55th Southeast Regional Meeting of the American Chemical Society, Atlanta, November 2003.

*F. Yang, M. A. Eiteman, E. Altman, "Production of pyruvate by Escherichia coli," Fifth Conference on Recent Advances in Fermentation Technology, St. Petersburg, November 2003.

*M. A. Eiteman, (invited) "Recent developments in metabolic engineering of Escherichia coli at the pyruvate node: production of succinate, pyruvate and alanine," Fifth Conference on Recent Advances in Fermentation Technology, St. Petersburg, November 2003.

M. A. Eiteman, "Graduate training for the future bioprocess engineer," Creating value for Biobased Products Conference, Kansas City, November, 2002.

G. N. Vemuri, E. Altman, M. A. Eiteman, "Physiological changes in Escherichia coli associated with altered intracellular NADH concentration as a function of growth rate," Society for Industrial Microbiology, Anaheim, August 2004.

*M. A. Eiteman, "Metabolic Engineering of Escherichia coli at the pyruvate node," Institute of Biological Engineering, Fayetteville, Arkansas, January 2004.

G. N. Vemuri, E. Altman, M. A. Eiteman, “Effect of redox engineering on overflow metabolism in Escherichia coli,” Metabolic Engineering V, Lake Tahoe, September 2004.

G. M. Smith, M. A. Eiteman, “Effect of acetyl CoA synthetase overexpression on metabolism and pyruvate formation in mutated strains of Escherichia coli,” American Chemical Society, San Diego, March 2005.

G. N. Vemuri, M. A. Eiteman, E. Altman, “Pyruvate redirection and redox engineering affects overflow metabolism in Escherichia coli,” American Chemical Society, San Diego, March 2005.

G. N. Vemuri, M. A. Eiteman, E. Altman, “Pyruvate redirection and redox engineering affects overflow metabolism in Escherichia coli, Institute of Biological Engineering, Athens, March 2005.

Patents

G. N. Vemuri, M. A. Eiteman, E. Altman, “Recombinant production of polypeptides with reduced acetate formation,” provisional patent application March 3, 2005. 235.0071 0160

Conclusions and Recommendations

Recruiting of graduate students is a very important activity, particularly when the students are asked to commit to a new program. Although we believe that a multidisciplinary graduate program is the best place to develop future technical and business leaders in the biobased economy, such programs are incomplete without students developing an understanding of such programs at the undergraduate level and even before. This conclusion has two consequences. First, it is critical that technically-oriented students be prepared to engage in graduate programs in this area. Therefore, we encourage the integration of biobased and energy themes in a wide range of curricula, including those in the liberal arts and in education. There is no reason that biobased/energy themes could not appear in other core engineering courses such as design, heat transfer, thermodynamics, mass transfer, measurements/sensors, and controls. Second, the development of a technical workforce for the biobased products economy will necessitate a heightened understanding of just what a “biobased economy” is. Such an understanding should be provided not only to potential direct participants in this economy, but also in everyone.

Although outside the scope of this project, we sensed during the course of our interactions as part of this project that the public at-large needs to be better engaged in the development of a biobased economy. It is going to be the non-scientists and the non-engineers who participate most vigorously in this debate and the formulation of associated public policy. What steps are needed to prepare non-technical students to have a sufficient understanding of the technical realities of a biobased economy so that they can participate in informed debates is a question left open but of significant relevance.

A distinguished lecture series which maintains a student-centered approach is an excellent means to heighten the expectation within a graduate program. The relatively small time commitment makes this way of promoting diverse experiences very efficient. Having leaders in the biobased economic developments, as well as persons of differing perspective, truly enhances the education of faculty and students involved in such programs. Designing such a series with a student-centered approach further elevates everyone’s commitment, and the ultimate impact. An educational program, at any level, is incomplete without the engagement such a lecture series provides.

Coursework is still a central component of the educational process. From their early years, students are geared toward learning through a classroom structure, and that mode of learning is not likely to be replaced soon. Some students are quite uncomfortable with the open-ended learning which occurs in a purely research- or investigative- activity. An ideal middle-ground is attained by courses which are focused on group projects and do not have formal “tests”. Such a structure readily permits interactions between students of multiple disciplines. In such a setting many of the students may not have what would be considered appropriate “prerequisites”. Our experience is that at the graduate student level, students should have the motivation and discipline to get what they can out of a class. The benefits from facilitating interactions between microbiology, engineering, biochemistry, pharmacy, etc. students far outweighs the worry regarding whether each one of the students is adequately prepared for a particular course topic. In an industrial setting, people of such diverse backgrounds need to communicate, and the University setting should be the place where these skills are developed. Ideally a course would include law, business and other students. But this greater challenge has not been addressed in the courses implemented as part of this project.

Other means of engagement such as industrial internships and lab rotations are beneficial, but demand a great deal of time. Lab rotations are quite difficult in the current model of funding based on a particular research project. We recommend a lab rotation for one semester only. To obtain the greatest benefit it should be an activity that is completed by all students, so that all faculty view the exercise as a potential “gain” of a worker. An industrial internship is a quite valuable experience to a student (one that contributes significantly to a student’s resume, particularly if she intends to enter the industrial sector for employment). However, graduate programs do not inherently have the ability to incorporate internships in their structure (whereas “coop” experiences are common at the undergraduate level). One problem is that graduate students are largely funded through research assistantships which require participation in a research project, preferably without interruption. Some change in the model of funding graduate work might facilitate greater participation in internships. We recommend greater funding for education-driven projects relative to research-driven projects. Indeed, the funding of graduate students directly (along with a budget for research expenses to the student) as a more significant fraction of overall graduate funding, would not only alter the method by which research was carried out, but it would also motivate faculty to be more innovative in developing meaningful academic programs to attract those “free” students and ensure these students ultimate success in the marketplace.