Final Technical Report

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1. Executive Summary

The basic concept of the REFABB project was that by genetically engineering the biomass crop switchgrass to produce a natural polymer PHB, which is readily broken down by heating (thermolysis) into the chemical building block crotonic acid, sufficient additional economic value would be added for the grower and processor to make it an attractive business at small scale. Processes for using thermolysis to upgrade biomass to densified pellets (char) or bio-oil are well known and require low capital investment similar to a corn ethanol facility. Several smaller thermolysis plants would then supply the densified biomass, which is easier to handle and transport to a centralized biorefinery where it would be used as the feedstock. Crotonic acid is not by itself a large volume commodity chemical, however, the project demonstrated that it can be used as a feedstock to produce a number of large volume chemicals including butanol which itself is a biofuel target. In effect the project would try to address three key technology barriers, feedstock logistics, feedstock supply and cost effective biomass conversion.

This project adds to our understanding of the potential for future biomass biorefineries in two main areas. The first addressed in Task A was the importance and potential of developing an advanced value added biomass feedstock crop. In this Task several novel genetic engineering technologies were demonstrated for the first time. One important outcome was the identification of three novel genes which when re-introduced into the switchgrass plants had a remarkable impact on increasing the biomass yield based on dramatically increasing photosynthesis. These genes also turned out to be critical to increasing the levels of PHB in switchgrass by enabling the plants to fix carbon fast enough to support both plant growth and higher levels of the polymer. Challenges in the critical objective of Task B, demonstrating conversion of the PHB in biomass to crotonic acid at over 90% yield, demonstrated the need to consider up-front the limitations of trying to adopt existing equipment to a task for which subsequent basic research studies indicated it was not suitable. New information was developed in the most complex of the chemical conversions studied, advanced catalysis to make acrylic acid, a chemical used widely to make paints, and this was published in a scientific journal.

In regard to the technical effectiveness, the crop science aspects were for the most part remarkably effective in addressing the underlying objectives indicating the soundness of the technical approach. With time, it should be possible to fully develop the advanced biomass biorefinery feedstock. Challenges within the thermolysis step to recover crotonic acid meant that by the end of the project we were not able to demonstrate an economic case based on data from scaled up equipment. Solving this will take further research and development work.

As a general statement, the broadest public good is in demonstrating the value of funding a very unique approach to the complex problem of enabling large-scale biomass biorefineries which resulted in significant progress towards the ultimate goal and a clearer understanding of the technical hurdles remaining. Perhaps not surprisingly, some of the broader benefits to the public come from the use of the REFABB project innovations in areas unrelated to the initial objective. It is worth highlighting the breakthrough developments in identifying three single global regulator genes which can be engineered into plants to dramatically increase photosynthesis and carbon capturing ability. These genes have tremendous potential for use in major food crops, in particular corn to enhance grain yield and based on recent findings, increase the root density, a critical key to

increasing carbon sequestration in agriculture and improving the sustainability of global food and biofuel production.

2. Comparison of the actual accomplishments with the goals and objectives of the project.

The objective of this project was to create a disruptive technology to enable economic renewable biomass energy production by co-producing commodity chemicals at low cost with net CO_2 fixation. The strategy was to produce PHB biopolymer within the green tissue of switchgrass and develop technologies for thermolysis of the PHB containing switchgrass biomass to produce crotonic acid, bio-oil, and char. Work was also done to establish chemistry to convert crotonic acid to commodity chemicals. The program was divided into three main tasks to achieve these goals, Task A, Core plant science activities for producing high levels of PHB in switchgrass; Task B, Develop and validate key process technologies for an integrated biorefinery; and Task C, Life cycle analysis of the project.

Task A. Core plant science activities for producing high levels of PHB in switchgrass

Success factors for this task were to achieve 10% PHB in switchgrass, engineer a gene containment method for switchgrass, and develop a novel plastid transformation technology to increase transgene expression to increase PHB production. There were six subtasks to achieve these goals.

A.1. Develop model system Setaria for high throughput transformation.

We intended to use Setaria as a model system to speed up the engineering and evaluation of PHB producers allowing quicker results to be obtained. We successfully established a tissue culture system for transformation of Setaria but found it to be less efficient than our current switchgrass transformation system. Since there was no benefit to incorporation of the model system into our work, this subtask was terminated at a project Go/No Go Decision and all further work was performed directly in switchgrass.

A.2. Increase carbon flow to polymer production in model system Setaria.

Since our switchgrass transformation system is more efficient than the Setaria system, we elected to continue this work directly in switchgrass and terminated this subtask.

A.3. Transformation of switchgrass.

Multiple genetic constructs were transformed into switchgrass to increase PHB production and improve the health of the resulting plants. The best ones co-expressed a global transcription factor with the PHB pathway and reduced the negative effects on plant health and biomass production that high levels of polymer production often have on plants. We were able to isolate decent sized plants producing up to 10% PHB in a portion of their leaves. Without the expression of the GTF, many of these plants would have died or have been very small. We also made significant increases in total tiller PHB content over our previously published results of 2.3% PHB in a whole tiller.[1] We anticipate that further methods to significantly enhance gene expression, such as the plastid transformation strategies in task A.5, and methods to increase PHB production in stems, will allow 10% PHB in the weight averaged biomass to be achieved.

[1] Table 3 in Somleva, M.N., O.P. Peoples, and K.D. Snell, *PHA bioplastics, biochemicals, and energy from crops.* Plant Biotechnol J, 2013. **11**, 233-252.

Importantly, the GTFs isolated as part of this effort have the ability to increase the photosynthesis and thus biomass production of wild-type switchgrass. We have found orthologs of these GTFs in major crops. These genes have tremendous potential for use in major food crops, in particular corn to enhance grain yield. A patent was filed for the three global transcription factors.

A. 4. Development of novel gene containment technology in switchgrass

Our objective was to develop a gene containment system where genes would be present in switchgrass biomass for PHB production, but would be excised from the reproductive tissues preventing the flow of transgenic pollen. We succeeded in engineering the novel recombinase based system into switchgrass and demonstrated the excision of transgenes in pollen. We were however not able to achieve complete excision from all pollen. This could likely be achieved through a combination of higher recombinase expression or the use of a different promoter. A paper was published describing this work.[2]

A.5. Increase transgene expression via alternative plastid transformation strategies

Our objective was to develop a plastid transformation system for switchgrass. To our knowledge, no one has succeeded in plastid transformation of a monocot and subsequent achievement of homoplasmy (all plastids in all cells transgenic) despite multiple efforts. We tried a complex alternative approach. We were able to demonstrate integration of our DNA into the plastome of plants but were not able to achieve homoplasmy. This is something that we will continue to work on after the end of the REFABB program.

A.6. Scale up PHB producing switchgrass lines to supply feedstocks for Task B

Sufficient PHB producing biomass was generated for the thermolysis studies in Task B.d

Task B. Develop and validate key process technologies for an integrated biorefinery.

Success factors for this task were to achieve >90% yield of crotonic acid from thermolysis of PHB producing switchgrass and to develop methods to convert crotonic acid to commodity chemicals through hydrogenation, oxidation, and metathesis reactions.

B.1. Develop and optimize torrefaction process and crotonic acid recovery.

Thermal conversion work for Task B.1. was conducted in collaboration with The Sustainable Biofuels & Co-Products Research Unit at The Eastern Regional Research Center (ERRC) of USDA-ARS. Work with the fluidized bed reactor at ERRC succeeded in producing a 45% yield of crotonic acid from PHB. This work was published in a peer reviewed journal.[3]

We tried multiple approaches to improve the yield (goal >90% yield) but were unable to do so in the larger scale equipment. Small scale flask and kiln studies at Metabolix were used to trouble shoot the process. Metabolix was able to demonstrate a ~83% yield of crotonic acid (mix of *trans* and *cis* isomers, primarily *trans*) from thermolysis of PHB in a small scale flask experiment.

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[2] "Transgene autoexcision in switchgrass pollen mediated by the Bxb1 recombinase." Somleva, M. N., Xu, C. A., Ryan, K. P., Thilmony, R., Peoples, O., Snell, K. D., Thomson, J., **2014**, *BMC Biotechnology*, 14, 79.

[3] "Mild Pyrolysis of P3HB/Switchgrass Blends for the Production of Bio-oil Enriched with Crotonic Acid." Mullen, C. A., Boateng, A. A., Schweitzer, D., Sparks, K., and Snell, K. **2014,** *Journal of Analytcial and Applied Pyrolysis,* 107, 40-45.

B.2. Develop catalyst technology to convert crotonic acid.

B.2.1. Testing of hydrogenation catalysts. This work was performed in collaboration with ERRC at ARS. We successfully developed a system for continuous hydrogenation to produce butanol at 85 to 90% yield. This system used butyl crotonate as substrate and could be part of an integrated process for converting PHB containing biomass to butanol. With use of *n*-butyl crotonate as the hydrogenation substrate, two equivalents of *n*-butanol are formed, one half of which can be reused in the synthesis of *n*-butyl crotonate from P3HB/biomass, making it an integrated process. This work was published in a peer reviewed journal.[4]

B.2.2. Testing of oxidation catalysts. This work was performed in collaboration with ERRC at ARS. The key accomplishment of this work was demonstrating the successful conversion of crotonic acid to maleic anhydride with the highest yield achieved being around 50%. Although not acceptable for an economically viable process, we recognize the program was hampered by a combination of the safety concerns of an exothermic reaction due to limited equipment availability, and the lack of commercially available catalysts suitable for the conversion.

B.2.3. Testing of metathesis catalysts. This work was performed in-house at Metabolix and we successfully demonstrated the production of acrylates from crotonates. Turnover numbers for the catalyst (TON for the catalyst), the most important economic driver for these types of reactions, depended on the crotonate starting material (butyl crotonate, TON ~500; ethyl crotonate, TON ~300) and were twice as high other published work using these catalysts with different substrates. They are however not high enough for a commercial process. The turnover-number-limiting factor was found to be the stability of the metathesis catalyst. Metabolix does not have the in-house expertise to carry out sophisticated metathesis catalyst development but can collaborate with companies that have this expertise as the PHB biorefinery technology matures. Our metathesis work was published in a peer reviewed journal.[5]

Task C. Life Cycle Analysis

The goal of this life cycle assessment (LCA) study was to estimate cradle to gate greenhouse gas (GHG) emissions associated with *n*-butanol derived from P3HB in genetically-engineered switchgrass. The LCA was completed by Bruce Dale's group at Michigan State University. Based on the best available information at the time of the analysis, GHG emissions of *n*-butanol derived from P3HB in genetically-engineered switchgrass are less than those of *n*-butanol derived from corn grain or petroleum. However this analysis is strongly dependent on the final values assumed for some sensitive process parameters in the thermolysis/crotonic acid recovery facility. The most sensitive parameters for GHG emissions of *n*-butanol are: 1) recycling of N_2 carrier gas, 2) the thermal energy source used in the thermolysis/crotonic acid recovery facility, 3) energy consumption for crotonic acid purification in the thermolysis/crotonic acid recovery

[4] "Bio-based *n*-butanol prepared from poly-3-hydroxybutyrate: optimization of the reduction of *n*-butyl crotonate to n-butanol." Schweitzer, D., Mullen, C. A., Boateng, A. A., Snell, K. D., **2015**, *Organic Process Research and Development*, 19, 710. This article is part of the Sustainable Chemistry special issue.

[5] "Acrylates via metathesis of crotonates." Schweitzer, D., Snell, K. D., **2015**, *Organic Process Research and Development*, 19, 715. This article is part of the Sustainable Chemistry special issue.

facility, and 4) the characteristics of the biochar, the nitrogen fertilizer application rate, and the carbon credits associated with soil carbon sequestration. More information on this process and the recovery facility is required to make firm conclusions on the LCA.

3. Project Summary

The major goals of this project are to produce 10% PHB in switchgrass, develop a novel gene containment system, and to develop and validate key process technologies for an integrated biorefinery. Progress that has been achieved towards each of these goals is described below.

Task A. Core Plant Science Activities for Producing High Levels of PHB in Switchgrass

***Task A Objectives**:* The objectives of Task A are to increase PHB levels in switchgrass to 10% of the plant dry weight, develop a novel gene containment system for dedicated bioenergy switchgrass, and to scale up the growth of PHB producing switchgrass lines for the production of polymer. Subtasks in Task A included:

- Increasing flux of carbon to polymer production using a model C4 grass system (*Setaria viridis*) for high throughput transformations (Task A.1 & A.2),
- Transferring the best genetic constructs identified in the *Setaria* experiments to switchgrass (Task A.3)
- Development of a novel method for gene containment in switchgrass (Task A.4).
- Enhancing transgene expression using an alternative plastid engineering method (Task A.5)
- Growth of PHB producing switchgrass lines to scale up under greenhouse conditions to produce PHB biomass for Task B (Task A.6).

A.1. Development of model system *Setaria* for high throughput transformations.

The goal of this task was to develop *Setaria viridis* (green foxtail) as a high throughput model system for metabolic engineering of high yielding bioenergy crops, in particular switchgrass, which use the C4 photosynthesis pathway to fix CO_2. *Setaria* was chosen as a model system for switchgrass since it is an annual diploid grass possessing a C_4 photosynthetic pathway, it has a small size (10–15 cm) reducing space issues for large studies, it has a short life cycle (6–9 weeks depending on photoperiod conditions), it is a prolific seed producer (~13,000 seeds per plant), its genome was in the process of being sequenced when the project was initiated, and basic methods were previously reported to be available for tissue culture and *Agrobacterium*-mediated transformation of the plant.[6]

Transformation procedures were evaluated in this program for the rapid production of transgenic lines of *Setaria* using *Agrobacterium*. We successfully initiated callus cultures from various explants from plants from different accessions. Using our expertise in switchgrass tissue cultures, we also succeeded in the induction of somatic embryogenesis in both mature caryopsis- and immature inflorescence-derived callus. However, the frequency of embryogenic callus formation was very low and the cultures could not be maintained for prolonged periods of time. Compared to switchgrass callus cultures, *Setaria viridis* cultures

[6] Brutnell, T.P., et al., *Setaria viridis: a model for C_4 photosynthesis.* Plant Cell, 2010. **22**, 2537-2544.

also have a very low regeneration potential. A comparison of the S. viridis and switchgrass callus systems in our hands is shown in Table 1. Taken together, these findings indicated that *Setaria* tissue cultures do not provide a good basis for the establishment of a highly efficient transformation procedure required for the development of a plant model system and that the Metabolix in-house switchgrass system is more efficient for such purposes.

Table 1. Comparison of switchgrass and *Setaria* tissue culture systems developed at Metabolix.

	Switchgrass	***S. viridis***
Callus initiation/embryogenic callus frequency:		
Mature caryopses	Yes/Very high	Yes/Very low
Immature inflorescences	Yes/Very high	Yes/Very low
Seedlings	Yes/Low	Yes/None
Plant regeneration frequency	Very high	Very low
Long-term callus cultures producing plants	Yes (up to 2 years)	No
Cryopreservation of callus cultures producing plants	Yes (up to 4 years)	n.t.

n.t., not tested due to the lack of long-term embryogenic callus cultures.

Based on the experimental results obtained with *Setaria* transformation and the fact that the existing switchgrass transformation system is very robust, we believed that to spend more time and resources on this was detracting from the objective of achieving 10% PHB in switchgrass. The work on this subtask was discontinued (A.GN.1a decision point) as reported in Q2, 2013.

A.2. Increasing carbon flow to polymer production in model system *Setaria*.

This work was scheduled to begin after the *Setaria* transformation system had been developed and validated. This subtask was terminated based on the No Go decision on Task A.1 reported in Q2, 2013 and all planned activities were transferred to Task A.3.

A.3 Transformation of switchgrass.

Due to the No Go decision on the use of Setaria as a model system we initiated the transformation of switchgrass in the third quarter of 2012, a year earlier than originally planned. This work originally focused on introducing genes encoding proteins to stabilize the PHB granules, as well as novel global transcription factor genes to increase overall photosynthetic carbon fixation in switchgrass and increase the substrate availability for the PHB pathway (Transformation module 1). Later transformation modules were added to test more genes and make a polymer with a different monomer unit composition. The management team responsible for this high risk research project planned to test each of the specific approaches independently recognizing that the ultimate success of this task which was to demonstrate 10% by weight of PHB in switchgrass would most likely come from combining the results of the individual Task elements. This in fact proved to be the case.

A.3 Transformation module 1:

This work focused on transforming genes encoding proteins to mitigate any negative impacts of producing high levels of PHB in the plant chloroplasts, due to the diversion of carbon from biomass synthesis to polymer, or potential negative impacts of the presence of the hydrophobic PHB granules on the chloroplast membranes. Genes investigated for this purpose included novel global transcription factors to enhance photosynthesis and proteins which have the potential to mitigate negative impacts of the PHB granules on chloroplast membranes

For co-expression of the PHB biosynthesis pathway with genes encoding proteins to stabilize chloroplast membranes, two plant transformation vectors containing either of these genes and a marker gene were constructed in Q1, 2013. Immature inflorescence-derived callus cultures were co-transformed with these vectors and the vector for PHB production. Following callus selection and plant regeneration and selection, PCR was used to identify co-transformants which contained the PHB genes and genes to help stabilize the membranes. Plants with the correct gene expression profiles were then grown in the greenhouse prior to carrying out PHB analysis.

Measurements of plant biomass and polymer content were performed. Although the average PHB content in leaf biomass of the co-transformed plants was lower than the content in the leaves of the plants expressing the PHB genes only, the polymer yield in the PHB plants engineered to stabilize the chloroplast membrane was higher mainly due to the increased average leaf biomass. Individual co-transformants with improved biomass yield and phenotype compared to PHB producing control plants accumulating polymer at similar levels were identified.

In conclusion, most of the re-transformed and co-transformed plants had improved phenotype and biomass production compared to the corresponding controls (PHB genes only) containing similar amounts of polymer resulting in a significant increase in the average PHB yield. However the addition of genes encoding proteins to stabilize the chloroplast membranes with the PHB gene system was not enough to dramatically change the outcome. The expression of one of the chloroplast membrane enhancing proteins in some of the advanced PHB producing lines developed toward the end of the project, produced in experiments after this task was completed, could however be very useful to further improve PHB production. These results marked the achievement of the milestone A.3.ML.2 PHB levels determined for Transformation Module 1.

Transcription factors. The focus of this activity, initiated in Q1, 2012, was to identify genes which when overexpressed in switchgrass would significantly increase overall carbon fixation, resulting in higher levels of metabolic intermediates and increased biomass production. We believed if we could successfully identify such genes, they could be used in combination with the PHB pathway genes to increase PHB levels without yield drag on biomass production towards our goal of 10% by weight PHB in switchgrass. Basically if more carbon was available then this should support more biomass and PHB accumulation. This Task was very high risk given all of the previous efforts in the crop field to increase photosynthesis and the fact that switchgrass is already a high yield C4 monocot. Transcription factors (TFs) are recognized as promising alternatives to single-

enzyme transgenic approaches.[7] Despite the rapid expansion of the discovery of transcription factors only a few genes, namely *Dof, Golden2* and *MNFs* are associated with regulation of genes involved in C_4 photosynthesis.[8] In these studies, we sought to identify transcription factors whose modified expression would specifically result in improved photosynthetic capacity. Since biomass production is a complex multi-gene quantitative trait, the newly identified TF genes will also be required to up-regulate key metabolic pathways leading to efficient conversion of photosynthates to biomass. Activities on this Task were carried out over multiple years and here we summarize the key findings ignoring the chronological order of their discovery.

Identification, Validation and Analysis of Novel Transcription Factors. In this study, we investigated the possibility to increase biomass production in switchgrass by overexpressing novel transcription factors with a predicted function as regulators of genes related to primary carbon metabolism. Initial efforts to increase carbon flow to polymer production involved overexpression of transcription factors (TFs) regulating photosynthesis-related genes. Several novel transcription factor genes were identified and their tissue specific expression analyzed by RT-PCR. Various levels of transcript accumulation were detected in leaves at different developmental stages, stem tissues (culm, leaf sheath and panicle) and roots.

In order to study the impact of overexpressing each of these three genes in switchgrass, binary vectors containing the genes were created and used for switchgrass transformation. More than 210 T_0 plants representing 58 independent transformation events were identified by PCR. Prior to transfer to soil, the primary transformants were tested for stable transgene expression by qRT-PCR. Based on the results from this screening, 102 transgenic switchgrass plants expressing the introduced TF genes at levels significantly higher than the background levels detected in the wild-type plants were grown in a greenhouse for further analyses. These plants represented 10 independent transgenic lines (2-5 plants per line) from the two Alamo genotypes for each transcription factor gene. Non-transgenic, wild-type plants (regenerated from untransformed callus cultures) grown under the same conditions served as controls in analyses of transgenic lines. To evaluate the effect of the overexpression of the identified TF genes on plant growth and metabolism, biochemical and physiological analyses were performed with transgenic and wild-type switchgrass plants grown in soil for two months. At this stage of rapid growth, the plants contain only vegetative tillers at different stages of leaf development and stem elongation. All the analyses were performed with the leaf attached to the second visible node from the base of the stem of vegetative tillers with emerging 4th leaf (E2 stage according to Moore *et al*., 1991).[9] The increase in biomass production, an increase of over 60% in some of the transgenic lines, was the most readily observable phenotype (Figure 1).

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[7] Grotewold, E., *Transcription factors for predictive plant metabolic engineering: are we there yet?* Current Opinion in Biotechnology, 2008. **19**, 138-144; Century, K., T.L. Reuber, and O.J. Ratcliffe, *Regulating the Regulators: The Future Prospects for Transcription-Factor-Based Agricultural Biotechnology Products.* Plant Physiology, 2008. **147**, 20-29.

[8] Weissmann, S. and T.P. Brutnell, *Engineering C4 photosynthetic regulatory networks.* Current Opinion in Biotechnology, 2012. **23**, 298-304.

[9] Moore, K.J., et al., *Describing and quantifying growth stages of perennial forage grasses.* Agron. J., 1991. **83**, 1073-1077.

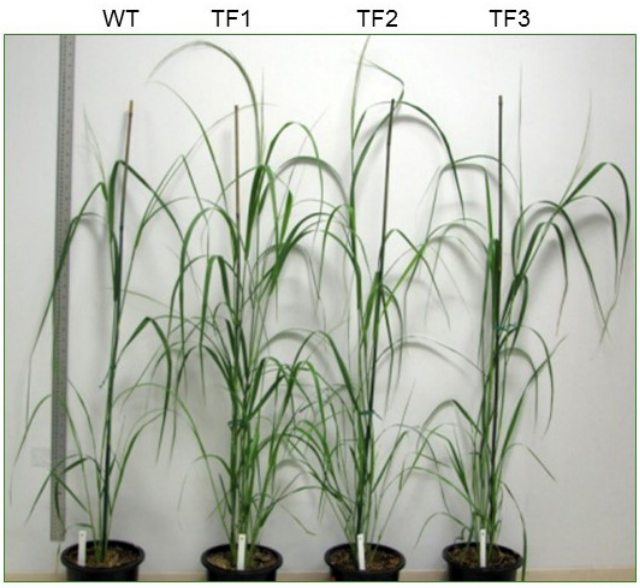

Figure 1. Comparison of biomass yield in representative plants transformed with global transcription factors. WT, wild-type. TF1, TF2, TF3 are lines transformed with different global transcription factors.

Comparative measurements of various photosynthetic parameters were conducted with light adapted leaves. The functioning of photosystem I (PSI) and photosystem II (PSII) was mainly studied in terms of photochemical quantum yield (Y) and electron transport rate (ETR). In some of the transgenic lines analyzed, the effective quantum yield of PSII, which represents the portion of absorbed quanta that is converted into chemically fixed energy by the PSII reaction centers, was considerably enhanced at photosynthetically active radiation (PAR) in the range of 46-849 μmol m^{-2} s^{-1}. An increase in ETR(II), the rate of the non-cyclic electron transfer, was detected in all of the lines analyzed at PAR ≥77 μmol m^{-2} s^{-1}. ETR(I), the electron transport rate of PSI (both cyclic and non-cyclic electron transfer), was significantly increased in lines expressing one of the transcription factors and slightly elevated in lines expressing the other two transcription factors in comparison to the controls. Marked changes (up to 185% to control) in the efficiency of quantum energy absorption by the PSI reaction centers, Y(I), were observed in lines expressing one of the transcription factrors.

Transgenic lines with a significant increase in the content of non-structural carbohydrates as primary products of photosynthesis which act as intermediary metabolic carbon sinks were also identified. Both starch and soluble sugars (sucrose and glucose) were found to be present at increased levels in most of the TF lines analyzed. The total chlorophyll content was significantly increased in some of the lines expressing two of the transcription factors, while no considerable changes were detected in the abundance of this pigment in lines expressing the other transcription factor compared to the wild-type plants. Similarly, the total content of carotenoids was significantly elevated in some of the lines.

Based on these remarkable outcomes, Metabolix elected to investigate these phenotypic outcomes at the transcriptome level to determine the impact on global gene expression profiles in high yield switchgrass lines. It was anticipated that this could provide

additional insight on the metabolic pathways responsible for the observed phenotypes and how they could be utilized to enhance PHB accumulation.

Microarray analysis. These studies were initiated in Q2, 2013. For gene expression profiling of the best TF lines, total RNA was isolated from 27 transgenic plants (3 lines per TF gene; 3 plants per line) and 6 wild-type plants. Affymetrix switchgrass cDNA microarrays were used for these studies. The gene expression microarray processing and data analysis was outsourced.

The gene ontology (GO) analysis for categorization of differentially expressed genes involved in major biological processes was performed using a publicly available database (http://bioinformatics.cau.edu.cn/easygo/). The GO analysis indicated that the transcription factors up and down regulated a significant number of genes suggesting that they are global transcription factors. The results also indicate that the transcription factors regulate the expression of genes involved in key biological processes and metabolic pathways by different mechanisms.

A patent application was filed and reported to DOE. A more detailed account of the identification and characterization of these novel transcription factors is being written up for publication.

Expression of the PHB Pathway Genes in Combination with the Transcription Factors. Three different approaches can be used to generate plants expressing the transcription factors in combination with the genes encoding the PHB biosynthetic pathway: a) re-transform transgenic lines which produce PHB with the transcription factor constructs b) re-transform transgenic lines which express the transcription factor at high levels and have significantly increased biomass and storage carbohydrate levels with the DNA constructs encoding the PHB pathway and c) develop new expression vectors having both the PHB genes and the individual transcription factor gene of interest in a single construct. We believed that approaches b) and c) would have the highest chances of success and although we did carry out approach a) beginning in Q1, 2013 we did not see lines with significantly increased PHB content using this approach.

In Q4, of 2013 we started work on approach b) and generated 184 independent events by re-transformation of callus cultures initiated from 11 TF lines with the PHB biosynthesis genes. In total, 1531 plants were identified by PCR. Another 82 plants from 12 independent callus lines transformed with the PHB genes only were also identified for use as controls in the experiments on the evaluation of the effect of the TF genes on PHB production and plant fitness. These plants represented 184 independent events obtained through re-transformation of immature inflorescence-derived cultures from 11 TF lines with the genes of the PHB biosynthesis pathway. In addition, 80 plants from 17 independent lines transformed with the PHB genes alone were also analyzed.
PHB producing plants were transferred to soil and grown in a greenhouse for further analyses. Measurements of the polymer content in mature leaves of vegetative tillers from the TF + PHB expressing lines as well as the corresponding PHB controls were performed. Although numerous additional measurements of transgenic lines were carried out in subsequent quarters the main conclusion was that the combination of the transcription factors with the genes encoding the basic PHB biosynthetic pathway did not result in a step change in PHB polymer levels in switchgrass. However, it was also clearly observed that

the switchgrass plants with the combination were healthier overall and produced higher levels of biomass as compared to switchgrass lines with the PHB genes alone at similar levels of PHB accumulation.

To evaluate the genetic perturbation caused by the PHB production in lines overexpressing a TF gene, we initiated a series of gene expression analysis studies in Q4, 2014. RNA was isolated from leaves of tillers at a defined developmental stage and the gene expression microarray processing was outsourced. The gene expression levels were analyzed in TF+PHB lines. A PHB producing line and wild type plants were used as controls. For each line, total RNA was isolated from the phytomer 2 leaf of E2 tillers[9,10] from 2-3 plants (independent biological replicates). PHB content was measured in the phytomer 3 leaf of the tiller sampled for RNA isolation from each plant.

The data normalization was performed using Robust Multiarray Averaging. A linear model was used to average data between array replicates and to detect genes with significant changes in their expression and a scatter plot was used to assess gene expression variation between the replicates.

In total, 723 genes with ≥2-fold change in their expression were identified in the PHB producing plants (in a wild-type background) in comparison to the wild type control. To investigate the biological functions of the differently expressed genes, gene ontology (GO) analysis was performed (http://bioinfo.cau.edu.cn/agriGO/index.php) using the biological process category. The results revealed the massive down-regulation of numerous genes involved in general metabolic processes, nitrogen and carbohydrate metabolism as well as photosynthesis. The numbers of the up-regulated genes involved in these processes are significantly lower.

In contrast, GO analysis for the biological process of the commonly up-regulated genes in PHB producing plants co-expressed with TFs indicates that the up-regulation of key metabolic pathways delivers a sufficient increase in carbon fixation to enable both PHB production and plant growth. Several genes involved in primary metabolic processes like photosynthesis, carbohydrate metabolic processes and polysaccharide metabolic processes are notably among the down regulated processes in all PHB producing lines, however the extent of downregulation of genes in these pathways is significantly less in PHB producing lines co-expressed with transcription factor genes. These results suggest that several key genes and pathways involved in primary metabolic processes are significantly affected by PHB polymer production in switchgrass plants and that co-expression of TFs can help, in part, to alleviate the negative effects of PHB production.

A.3 Transformation module 2:

Additional metabolic Engineering Strategies to Increase PHB production. In the 2014 PMP, the transformation work to produce PHB was expanded to include the evaluation of additional genes with the potential to increase the efficiency of the PHB biosynthetic pathway in switchgrass (Transformation module 2). The first of these was a novel PHA synthase gene expected to increase PHB polymerase activity in switchgrass thereby increasing the rate of PHB production (referred to as gene 1 below). The second gene enabled a novel method to produce one of the PHB pathway intermediates (referred to as gene 2 below). Vectors for the expression of these genes were constructed and

[10]Moore, K.J. and L.E. Moser, *Quantifying developmental morphology of perennial grasses.* Crop Sci., 1995. **35**, 37-43.

transformed into switchgrass. Independent callus lines producing plants were obtained from transformation experiments and transgenic plants were isolated from each construct transformed. Although the average levels in the plants expressing gene 1 were higher than the levels in the control PHB producing plants, the increase is not significant enough to indicate that this synthase performs better than the previously used synthase. This task was therefore considered to be completed.

PHB levels using gene 2 (enables novel method to produce one of the PHB pathway intermediates) were however much higher than control PHB producing plants. Up to 10.27% DW PHB was produced in tissue culture. However, only two of the plants containing gene 2 survived in peat pellets and were able to be established in soil. One of the two plants did not grow normally and died two months after transfer to soil probably due to the high polymer content. From these initial studies we concluded that this novel method employing gene 2 to generate the PHB pathway intermediate was diverting most of the available substrate to polymer to the detriment of plant growth and survival.

Based on these results and the improved PHB yield and biomass production demonstrated by combining the transcription factors with the conventional PHB pathway in Transformation Module 1, we decided to focus the remainder of the project on combining the PHB pathway containing gene 2 with the global transcription factors in switchgrass, and to do so by re-transforming high yielding transcription factor switchgrass lines with vectors for expression of the gene 2 containing PHB pathway.

Transformation of the different transcription factor lines with the gene 2 containing PHB pathway, with the conventional PHB pathway as a control, were continued through Q4 of 2015 and large numbers of transgenic lines were selected and screened for PHB. 334 re-transformed plants representing 49 lines engineered for co-expression of the gene 2 PHB pathway and a TF gene were identified by PCR. In addition, 79 plants from 14 events expressing the gene 2 PHB pathway without a TF were also identified. PHB content was measured in 1466 plants in tissue culture. The highest PHB values, up to 10.82% DW in a section of leaf tissue, were measured in a plant co-expressing the gene 2 PHB pathway and a TF. Most of the high PHB producing gene 2 PHB pathway + TF plants had a better phenotype than the gene 2 PHB pathway controls with similar polymer content. Based on the results from this screening, 341 plants were transferred to the greenhouse for further analysis.

PHB content was measured in these 341 soil-grown (greenhouse) plants during the following quarter. About 47% of the TF + gene 2 PHB pathway and 33% of the gene 2 PHB pathway control plants died within 3-10 weeks after transfer to soil. The whole above ground part of some of these plants was harvested and used for measurements of the polymer content. The highest PHB values detected in these whole plants were 13.42% DW (gene 2 PHB pathway + TF) and 10.45% DW (gene 2 PHB pathway control), respectively.

Amongst the highest PHB producers, mainly TF + gene 2 PHB pathway plants showed normal growth and development. Most of them had an improved phenotype compared to gene 2 PHB pathway controls with similar levels of PHB (Fig. 2). The distribution of polymer in leaf and stem tissues of vegetative and reproductive tillers from high producers was also analyzed. The highest PHB content was measured in a TF + gene 2 PHB pathway plant. For this plant, the weight averaged PHB content in the whole tiller was 4.05% DW. The weight averaged content in the leaf tissue only was 7.31%. The weight averaged PHB content in the stem tissues (nodes, internodes, leaf sheaths, and the

panicle) was 1.01% DW. These are the highest weight averaged levels that we have observed in switchgrass to date.

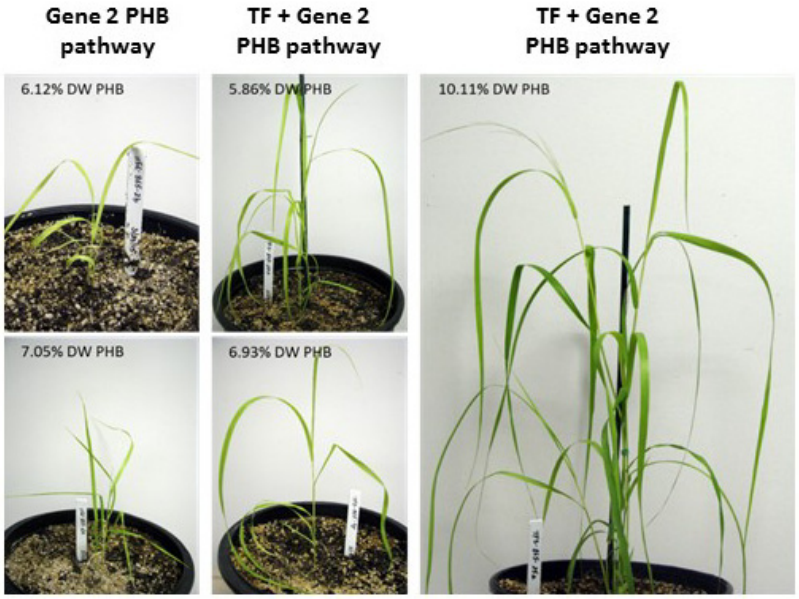

Figure 2. **Phenotype of high PHB producing plants engineered with the gene 2 PHB pathway or the TF + gene 2 PHB pathway after 10 weeks growth in soil.**

Most switchgrass plants with PHB content higher than 10% are generally not healthy looking and do not follow a typical growth pattern. They are often stunted, do not develop normal tillers and leaves appear very chlorotic. However, when comparing plants with similar PHB levels in the 7-10% PHB range, plant co-expressing the TFs with the gene 2 containing PHB pathway provide a better phenotype than control plants expressing only the gene 2 containing PHB pathway (Figure 3).

Metabolic profiling of engineered lines. In winding down the program in Q4, 2015/Q1, 2016, we initiated a series of experiments for global biochemical analysis (metabolic profiling) of PHB producing lines engineered with the novel gene 2 containing PHB pathway in a wild-type or transcription factor expressing background. This experiment included (a) Monitoring the growth pattern of PHB producing plants with their appropriate controls; (b) Harvesting of leaf tissue samples from two independent tillers of the same plant from phytomer 2nd leaf; (c) analyses of PHB content from the portion of the leaf tissue; (d) selection of tissue samples with PHB content ≥5 (% dry weight); and (e) processing of samples (ground in liquid nitrogen and freeze drying) and shipping to a contract metabolic profiling company for analysis.

Metabolomics data was received and while this data is in the process of being analyzed by Metabolix researchers at the time of this report writing, several key observations have been found in the initial analysis.

- A total of 504 compounds were detected in the datasets. Of these 347 (69%) were identified as known biochemicals whereas 157 were unnamed compounds.
- Key metabolic signatures of transcription factor expression, PHB production, and gene 2 containing PHB pathway +TF co-expression were identified by a statistical comparison of the biochemical changes between various groups

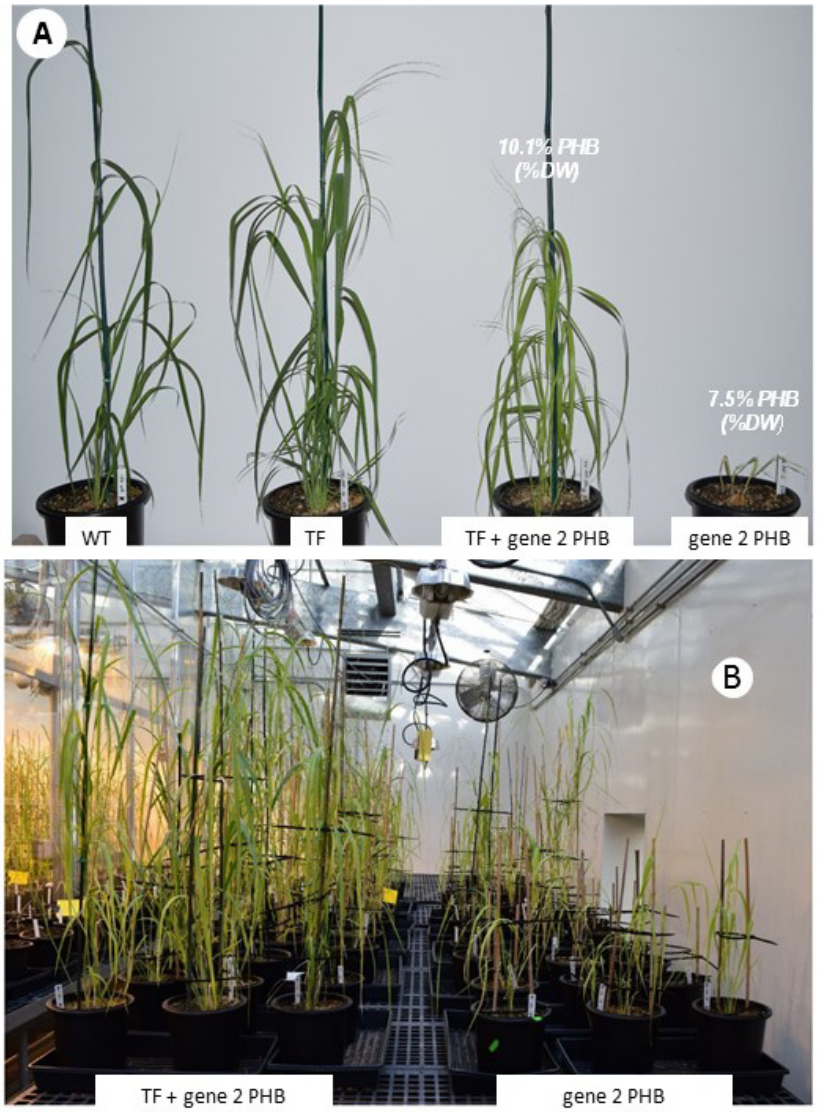

Figure 3. Effect of overexpression of transcription factors on PHB producing switchgrass lines. (A) Phenotype of switchgrass plants overexpressing transcription factor (TF) and co-expression of TF in high PHB producing plants engineered with the gene 2 containing PHB pathway. (B) Phenotype comparison of TF + gene 2 PHB pathway plants (left) and control gene 2 PHB pathway plants (right) growing in the greenhouse.

- Expression of each of the transcription factors in plants showed an alteration in compounds associated with carbon and nitrogen metabolism consistent with higher biomass observed in these plants.
- PHB production by the engineered gene 2 containing PHB pathway caused a perturbation in carbon flow, carbon partitioning and C: N balance. Further, markers generally induced for mitigation of plant abiotic stress were observed in these engineered plants, suggesting that alteration in intermediary metabolism by diverting carbon into PHB production was detrimental to plant health.
- While the co-expression of transcription factors did not appreciably alter PHB production, one of the transcription factors effectively reduced the perturbations in carbon flow and restored the homeostatic balance in C: N metabolism. These results show that this transcription factor mediated an increase in carbon assimilation that most likely allows both the production of PHB and normal growth and development of TF + gene 2 containing PHB pathway plants.

These results will continue to be analyzed over the next several months and are expected to provide guidance for engineering additional increases in PHB production after the REFABB program.

A.3 Transformation module 3:

Direct production of a novel co-polymer in plants. Transformation module 3 was added in Q1, 2014 to try and demonstrate that we could produce an alternative PHA polymer with a different monomer composition. This was performed in switchgrass as a possibly commercially more attractive route to produce one of our targeted commodity chemicals based on direct themolysis of the biomass.

By the following quarter (Q3, 2014), 38 independent transformation events had been generated and over 20 primary transformants representing 6 independent events having the complete pathway for production of the alternative PHA were confirmed by PCR resulting in the achievement of the milestone A.3.ML.3 ahead of the schedule. Over the following two quarters, over 400 transgenic lines were analyzed for polymer production However no alternative polymer was detected in these plants suggesting that the pathway needs to be optimized. This activity was therefore terminated in Q3, 2015.

A.4 Development of novel gene containment technology in switchgrass.

Since most forage and turf grasses including lignocellulosic bioenergy crops are outcrossing species, a gene containment system to prevent transfer of transgenes to other crops would be highly advantageous for large scale planting of such crops. In this task, two potential gene containment systems were evaluated for use in switchgrass. In the first approach, *Transgene Excision by Site-Specific Recombination,* the objective was to develop a genetic technology using recombinase genes and tissue specific promoters to enable selective excision of transgenes from reproductive organs but not vegetative tissues resulting in transgene-free pollen and/or seeds. While the use of site specific recombination systems to create marker-free transgenic plants has previously been reported, select excision by using promoters functional in the floral meristem has not to our

knowledge been developed. The second approach was designed to produce reactive oxygen species that are cytotoxic to the pollen cells. In addition to promoters isolated in-house, Metabolix obtained several candidate promoters as well as some novel recombinase genes from USDA Labs for testing.

The first step in implementing this technology was to identify promoters with the appropriate tissue specificity for the strategy and to test the activity of site-specific recombination systems in switchgrass.

Promoter Selection. Inducible and organ- or germline-specific promoters provide potent and flexible systems for the temporal and spatial control of transgene expression necessary for effective gene containment. In addition to promoters isolated in-house, Metabolix obtained several promoters from the USDA-ARS for testing in switchgrass. Although additional promoters and their variants were evaluated over the course of the project, the ones chosen to develop the system included a floral meristem-specific promoter, a heat shock promoter, and two rice pollen-specific promoters.

Characterization of the spatiotemporal activity of the floral meristem-specific promoter in switchgrass plants indicated that this particular sequence is not suitable for controlling the expression of site specific recombinases or genes encoding cytotoxic products specifically in the floral meristem and/or developing inflorescences. Additional variants of this promoter were investigated further throughout the project. In Q3 2013, the expression analyses of transgenic plants engineered for testing the activity of the deleted versions of the putative floral meristem-specific promoter were completed. The results for the spatiotemporal activity of the promoter suggest that it can be used for engineering of total transgene biocontainment in switchgrass and other monocots through cell ablation.

Site-specific recombination system testing. Two of the recently characterized serine resolvase site-specific recombinases[11] were chosen for testing in switchgrass. Metabolix obtained plasmids containing the coding regions of the recombinase genes from USDA-ARS. In Q1, 2012, three plant transformation vectors were constructed for testing our novel technology for gene biocontainment. The vectors were introduced into switchgrass callus cultures by *Agrobacterium*-mediated transformation. The results of these studies have been published in a peer reviewed journal.[12] These studies demonstrated the possibility to achieve transgene biocontainment in switchgrass through site specific recombinase-mediated autoexcision of transgenes from germinal tissue and its transmission to the next generation resulting in the production of stable lines free of undesirable exogenous DNA sequences. The complex genetics of switchgrass, an exclusively outcrossing tetraploid crop, however presents a serious challenge for achieving complete gene containment. Despite this obstacle, we have successfully demonstrated the possibility to obtain transgene-free progeny by conventional plant breeding. Further studies are

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[11]Thomson, J.G. and D.W. Ow, *Site-specific recombination systems for the genetic manipulation of eukaryotic genomes.* Genesis, 2006. **44**, 465-476; Ow, D., W. and J. Thomson, G, *Site-Specific Recombination Systems for Use in Eukaryotic Cells.* WO/2006/026537, 2006.

[12]Somleva, M.N., et al., *Transgene autoexcision in switchgrass pollen mediated by the Bxb1 recombinase.* BMC Biotechnol, 2014. **14**, 79.

necessary to optimize all the genetic elements required for 100% gene containment in switchgrass.

Use of a novel gene to produce reactive oxygen species in pollen to generate sterile plants. A novel approach based on the manipulation of the production of reactive oxygen species in pollen to generate sterile transgenic switchgrass plants through cell ablation was initiated in Q4, 2011. Genetic constructs harboring the novel gene encoding a hydrogen peroxide-generating enzyme were constructed. A rice promoter was used to control the expression of these genes in developing and mature pollen grains.

Highly embryogenic immature inflorescence-derived switchgrass cultures were transformed with these vectors to generate plants for analysis around Q3/4 of 2012.

In Q3, 2012, A series of experiments were also performed to evaluate pollen viability and functionality in transgenic switchgrass plants as follows:

a. Determination of pollen developmental stages. Since switchgrass is an exclusively outcrossing species, the development of spikelets from the same panicle as well as of the florets in the same spikelet is asynchronous. The precise time of anthesis (when the mature pollen is released) was determined by microscopic examination of the floret morphology and pollen developmental stages.

b. Optimization of techniques for evaluation of pollen viability and germination rate. The optimal conditions for *in vitro* pollen germination in terms of medium composition, incubation temperature, etc. and for staining with a fluorescent dye have been established and used for analyses of switchgrass lines engineered for male sterility through pollen ablation. Abnormal pollen grains were observed in some of the lines transformed with the vector for glucose oxidase activity in the cytosol.

c. Identification of transgenic lines with reduced pollen germination rate. Mature pollen from transgenic and wild-type plants was harvested and germinated following the optimized techniques described above.

Screening of lines was completed and a significantly reduced pollen germination rate in some of the lines, combined with phenotypical changes, indicate abnormal pollen development likely due to the accumulation of hydrogen peroxide at cytotoxic levels in the transgenic pollen grains. 5 lines engineered with the novel gene showed significantly reduced (up to 50%) pollen germination rate

In Q4 2012, the genetic analysis of switchgrass lines engineered with the novel gene for reactive oxygen species production was completed. In total, 1,361 T_1 seeds were obtained from reciprocal crosses between: (1) three transgenic lines with significantly reduced (up to 50%) pollen germination rate and wild-type plants and (2) a transgenic line with functional pollen and a non-transgenic plant. After stratification for 2 weeks, the seeds were germinated following our procedure for germination of freshly harvested seeds resulting in the generation of 1,159 T_1 plants (57.4-98.6% seed germination rate). More than 94% of the seedlings were analyzed for their response to the selectable marker co-expressed with the novel gene for reactive oxygen species production, followed by PCR of randomly selected tolerant and sensitive plants. The segregation analysis of the T_1 progeny revealed that the expected ratios of transgenic:non-transgenic (herbicide tolerant:herbicide sensitive) plants were obtained from all crosses when the transgenic plants were used as the female parent. However, when the transgenic plants were the male parent the calculated

ratios were different than the expected ones except for a line without reduction in the pollen germination rate, as confirmed by the χ^2 test analysis.

The observed differences in the segregation ratios most probably reflect the reactive oxygen species-mediated formation of abnormal pollen. Although most of the plants analyzed by Southern blot hybridizations have rather complex integration patterns, a line with a single locus was identified. This analysis also revealed the presence of two copies of the T-DNA in reverse orientation in the locus. The segregation ratio of 1:1 (62 tolerant plants:61 sensitive ones) in the T_1 progeny of this plant when used as the female parent confirmed the integration of the transgenes in a single locus. However, when the transgenic plant was used as the male parent, the number of the transgenic T_1 plants was reduced with about 30% (63 plants) compared to the number of the non-transgenic ones (93), which correlates with the previous finding that 20-30% of the pollen grains had abnormal phenotype. Some of these T_1 plants are grown under greenhouse conditions for further analyses.

The data presented here combined with the previously reported results on the recombinase mediated transgene excision demonstrate the successful development of two different strategies for transgene biocontainment in switchgrass –autoexcision by site-specific recombination and reactive oxygen species-mediated pollen ablation. The milestone A.4.ML.7 and the deliverable A.4.DL.1 were achieved ahead of schedule.

A.5 Increasing transgene expression via alternative plastid transformation strategies.

A.5.1. Stable integration of nucleic acids w/ marker genes into plastid genome. A parallel approach to improve polymer production was employed that involved increasing the expression level of transgenes encoding the PHB biosynthetic pathway. Expression of genes from the plastid genome can increase levels of gene expression beyond what can be typically obtained via nuclear-encoded expression strategies. In proof of concept work in tobacco, Metabolix has engineered genes encoding enzymes of the PHB biosynthetic pathway into the plastome of tobacco generating stable, fertile lines that produce up to 20% dwt PHB in areas of leaf tissue and 8% dwt PHB in whole tobacco plants.[13] These levels of polymer are significantly higher than previous yields obtained in tobacco using nuclear transformation of transgenes with a plastid targeting sequence to direct the enzymes to the plastids[14] or plastid transformation.[15] Plastid-encoded expression of the PHB pathway in switchgrass may similarly increase polymer production, however, a method for switchgrass plastid transformation is not currently available. Efforts to develop plastid transformation systems for switchgrass or other monocots have proved unsuccessful to date and stable transplastomic monocot plants in which the majority of plastids are transgenic have not been reported. Tissue culture procedures for regenerating plants from chloroplast rich

[13] Bohmert-Tatarev, K., et al., *High levels of bioplastic are produced in fertile transplastomic tobacco plants engineered with a synthetic operon for the production of polyhydroxybutyrate.* Plant Physiol, 2011. **155**, 1690-1708.

[14] 0.3% dwt PHB produced in leaf tissue; Bohmert, K., et al., *Constitutive expression of the β-ketothiolase gene in transgenic plants. A major obstacle for obtaining polyhydroxybutyrate-producing plants.* Plant Physiol., 2002. **128**,1282-1290.

[15] 1.7% PHB produced in leaf tissue in unstable, infertile lines; Lössl, A., et al., *Polyester synthesis in transplastomic tobacco (Nicotiana tabacum L.): significant contents of polyhydroxybutyrate are associated with growth reduction.* Plant Cell Rep, 2003. **21**, 891-899.

explants, such as leaf segments, that would be most suitable for particle bombardment procedures for plastid transformation are not available for switchgrass and many other monocots of commercial interest. The ability to use callus cultures for plastid transformation procedures, such as the caryopses (seed) derived callus cultures, would remove a major hurdle for plastid transformation of this crop as well as other monocots. In this task, a method for using such callus cultures was pursued.

This project activity started in Q2, 2012 based on the hiring of a research scientist to carry out the project completing milestone A.5.ML.1. The construction of two vectors was completed in Q3/4 of 2012 (milestone A.5.ML2).

Transformations of highly embryogenic immature inflorescence-derived switchgrass cultures with these vectors were initiated and a number of potential plastid transformants were identified. After 3 weeks using various selection protocols, all the cultures were analyzed by PCR using different sets of primers. PCR products indicating operon integration in the plastome were detected in ~27 lines. Sequencing analysis of the amplicons revealed the precise junction between the transgenes and the plastid genome on both flanks thus confirming the transgene integration through homologous recombination.

The cultures were subjected to repeated cycles (2 weeks each) of selection with the chosen selection agent for enrichment of transgenic plastomes. Every 4 weeks, portions of the resistant callus were transferred on to medium for plant regeneration and selection. Transplastomic plants regenerated from these callus cultures were identified by PCR based on the presence of the amplicons indicating the operon integration into the plastome. These results marked the achievement of the milestone A.5.ML.5 Isolation of five regenerated lines.

In Q1, 2015 a modified strategy for selection of transplastomic cultures and plants was initiated. After each selection cycle, selective agent tolerant plants were transferred to a fresh medium containing a higher concentration of the selective agent.

In Q2-Q3, 2015, southern blot hybridizations were performed to verify the integration of the synthetic operon into the plastome of switchgrass cultures and plants. A band of the expected size was detected in both callus and plant cells. The presence of the band in soil-grown plants demonstrates the stability of the integration events. These results confirm the data from PCR analysis for identification of transplastomic callus cultures and plants regenerated from them. These results marked the achievement of the milestone A.5.ML.6 Alternative plastid transformation system demonstrated in switchgrass. Based on these results, we decided to continue work with the plastid transformation strategy (A.GN.5 Go/No Go decision) to address the remaining technical hurdle, developing an improved selection strategy to generate homoplasmic switchgrass lines.

In an attempt to get homoplasmic plants, immature inflorescence derived (ii) cultures were initiated from heteroplasmic plants. After a few cycles of subculture, it was envisioned that more copies of the wild type plastid genome might be replaced by the transplastomic genome. We were not however able to achieve hompolasmy.

By Q1, 2016, seventy-eight transplastomic plants from different treatments were generated and analyzed to be positive by PCR. Six of them were transferred to the green house for the further evaluation. The demonstration for the first time of the successful integration of a transgene construct into the plastid genome of the monocot switchgrass is a major technical accomplishment and the essential first step toward successful plastid

engineering of monocots, a technology with high economic value. Metabolix plans to continue this work after the program ends, including methods to obtain homplasmic plants.

A.6. Scale up PHB producing switchgrass lines to supply feedstocks for Task B.

PHB producing plants that were already in hand at Metabolix at the initiation of the REFABB program were scaled up to produce PHB producing biomass for Task B. A portion of the biomass generated in this subtask was transferred to ARS for thermolysis testing.

Task B. Develop and Validate Key Process Technologies for an Integrated Biorefinery Producing Biofuels and Bio-Based Commodity Chemicals

Task B Objectives*:* The objective of Task B is to demonstrate and optimize the use of torrefaction, a low temperature thermal conversion process, with PHB containing biomass to produce a densified biomass and recover high purity crotonic acid. This crotonic acid is then chemically converted to a series of commodity chemical intermediates.
Subtasks in Task B included:

- Develop and optimize torrefaction process and crotonic acid recovery (Task B.1),
- Development of catalyst technology to convert crotonic acid using
    - Hydrogenation catalysts (Task B.2.1)
    - Oxidation catalysts (Task B.2.2)
    - Metathesis catalysts (Task B.2.3)

B.1. Develop and Optimize Torrefaction Process and Crotonic Acid Recovery

Thermal conversion work for task B.1. was conducted in collaboration with USDA-ARS.

B.1.1. Torrefaction with PHB biomass produced through fermentation.

This project got underway in Q1/2 of 2012. Pyrolysis experiments on dried whole broth based PHB biomass obtained from microbial fermentations were performed in a fluidized bed reactor system. The pyrolysis experiments on the PHB/ fermentation biomass material were all characterized by a high level of fouling of the system. In experiments with a sand bed, the material acted as an adhesive, creating chunks in the bed which caused it to lose fluidization and resulting in a large pressure drop. This could not be undone by combustion of the material. Therefore, the pyrolysis experiments were attempted without any sand in the bed. While this approach was more successful, material still collected in the reactor tube and deposited on surfaces throughout the system. Mass balance was very difficult to obtain due to solids fouling the system throughout. Mass balance was best obtained for a run performed at ~230 ^{o}C. The liquid product obtained for the pyrolysis of the material was analyzed and contained a large amount of the desired product crotonic acid (i.e. 37%). Fermentation salts are present in the dried, fermentation-derived PHB containing biomass. These salts are thought to be the primary reason for the bed agglomeration observed with the bench scale pyrolysis unit.

B.1.2. Torrefaction with wild-type switchgrass blended with PHB

Fluidized bed reactor runs

Scoping pyrolysis runs using a 10% blend of powdered PHB polymer with dried wild-type switchgrass biomass were performed in Q2 2012. This blend of polymer with plant material was made in order to simulate processing of genetically engineered switchgrass plants producing 10% PHB that are scheduled to be available at the end of this program. Two scoping experiments were performed using a fluidized reactor. The results from initial experiments involving the thermolyis of a blend of switchgrass and polyhydroxybutyrate polymer does indeed show that fluid bed technology is a viable technology for the recovery of crotonic acid from various PHB containing plant biomass sources. The observed conversions in these initial studies (34 and 45%, Table 2, Experiments 1 and 2) indicate additional work is necessary in order to obtain higher yields of crotonic acid.

Scoping pyrolysis runs were continued in Q3, 2012 using a 10% blend of powdered PHB polymer with dried wild-type switchgrass. Lime was added as a potential catalyst for the process since it has been used effectively at Metabolix in other thermolysis processes. During the pyrolysis experiment with the switchgrass/PHB/lime blend the pressure in the bed was observed to rise relatively quickly over the course of the experiment. This was due to agglomeration of the sand bed, which was not observed during the experiments with the switchgrass/PHB blends that did not contain lime. This was similar to the initial experiments with the fermentation/PHB biomass feedstock, although the agglomeration was not as severe in this experiment. However, material from this experiment did collect on the feed injection auger into the fluidized bed which caused it to fail. This material was not easily removed and resulted in the need to replace the injector auger screw on the system. Analysis of liquids from the reaction revealed that the addition of lime to the PHB/switchgrass blend greatly depressed the yield of crotonic acid (Table 2, Experiment 3).

The results of seven additional thermolysis runs that were performed in the absence of lime under various conditions (different nitrogen flow rate, different PHB particle size, etc.) are shown in Table 2. In summary, the highest yields of crotonic acid were obtained in Experiment 3 producing 45.2% overall crotonic acid yield. A peer reviewed manuscript describing eight of these experiments was published in The Journal of Analytical and Applied Pyrolysis.[16]

Analyses of biomass residue from fluidized bed reactor pyrolysis

GC butanolysis was used to determine the amount of P3HB present and HPLC was used to determine the amount of crotonic acid present in char samples. Only traces quantities of P3HB and crotonic acid were detected in the thermolysis/pyrolysis chars. Additionally, the char samples were extracted with $CDCl_3$ (24 h stirring at RT, + 3 h ultrasound at 50 °C, + 3 h stirring at 60 °C) and analyzed by ^1H NMR. The relative amounts of crotonic acid observed mirror those determined via HPLC. For the crotonic acid present, about 80 % was *trans*-crotonic acid and about 20 % was *cis*-crotonic acid.

[16]Mullen, C.A., et al., *Mild pyrolysis of P3HB/switchgrass blends for the production of bio-oil enriched with crotonic acid.* J Anal Appl Pyrol, 2014. **107**: p. 40-45.

Table 2. Summary of 10 different fluidized bed reactor experiments using purified PHB mixed with wild-type switchgrass.

Fluid Bed Pyrolysis/Thermolysis - Results
as per analysis at the USDA

		Experiment									
Parameter	**Unit**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Date of Experiment		07/11/12	07/17/12	09/18/12	10/25/12	01/23/13	01/31/13	06/04/13	06/18/13	11/19/13	12/09/13
$Temperature_{Reactor}$	°C	350	375	375	375	350	375	375	375	290	250
Lime added	%w/w	0.0	0.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total Feed (P3HB+switchgrass)	kg	1.390	1.280	1.130	1.920	1.660	1.330	2.280	2.050	1.750	2.350
P3HB	%w/w	10.0	10.0	9.5	10.0	10.0	10.0	10.0	25.0	10.0	10.0
P3HB Particle Size		fine	fine	fine	coarse	coarse	coarse	fine	fine	?	?
$m_{P3HB\ fed}$	kg	0.139	0.128	0.107	0.192	0.166	0.133	0.228	0.513	0.175	0.235
N_2 Flow Rate	l/min	75	75	75	70	60	60	60	60	75	75
$time_{experiment\ overall}$	h	2.0	1.7	1.7	2.4	2.0	2.2	2.4	2.0	2.1	3.0
Char, Mass collected	kg	0.245	0.195	0.225	0.185	0.325	0.325	0.315	0.300	0.360	1.575
Char, % formed	%w/w	17.6	15.2	19.9	9.6	19.6	24.4	13.8	14.6	20.6	67.0
Liquid, Mass collected	kg	0.558	0.620	0.190	0.790	0.760	0.630	1.030	0.980	0.710	0.400
Liquid, % formed	%w/w	40.1	48.4	16.8	41.1	45.8	47.4	45.2	47.8	40.6	17.0
Total Mass recovered	kg	0.803	0.815	0.415	0.975	1.085	0.955	1.345	1.280	1.070	1.975
% Mass recovered	%w/w	57.8	63.7	36.7	50.8	65.4	71.8	59.0	62.4	61.1	84.0
% Crotonic Acid in Liquid	%w/w	8.54	9.33	0.49	6.29	8.86	8.38	9.64	22.08	9.38	22.09
Overall Crotonic Acid Yield	**%w/w**	**34.3**	**45.2**	**0.9**	**25.9**	**40.6**	**39.7**	**43.5**	**42.2**	**38.1**	**37.6**

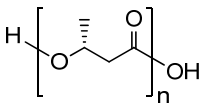
Laboratory scale fundamental studies of key unit operations during thermolysis

A technical challenge of the thermolysis process is the heat transfer from the solid heating apparatus to the solid biomass. We thus decided that achieving the targeted recovery of crotonic acid from PHB containing switchgrass biomass requires that we develop a complete understanding of all of the variables which could potentially result in loss of product and develop thermolysis conditions to minimize losses. For this reason we initiated a series of fundamental studies at laboratory scale of the key unit operations in the overall thermolysis process. The key unit operations are the thermolysis conditions to convert the PHB to crotonic acid and the distillation recovery of the crotonic acid produced. These studies were initiated by optimizing the process for the distillation of crotonic acid as this is the key operation for determining the yield from thermolysis work. We then determined the purity and particle size of PHB polymer we intended to use for fundamental PHB thermolysis work and finally carried out PHB thermolysis studies using the purified PHB.

Distillation of crotonic acid. Crotonic acid has a melting point of 70 °C and a boiling point of 180 °C**.** Although employing a vacuum would allow the distillation of crotonic acid to be conducted at a temperature below its normal pressure boiling point, it was decided to conduct all experiments at atmospheric pressure. Atmospheric pressure allows for a higher pot temperature, which is necessary since even at the temperature of the atmospheric pressure boiling point of crotonic acid (180 °C) the rate of PHB thermolysis is very slow. A number of alternative experimental setups were evaluated to develop the best conditions for conversion of PHB to crotonic acid. The critical issue addressed was optimizing the heat transfer to the PHB polymer in the glass reaction vessel so as to deliver a temperature greater than 180ºC across all elements of the distillation apparatus. Several scoping experiments were carried out and the best apparatus configuration was identified. This apparatus has a cup-shaped heating mantle, such that the maximum possible surface of the distillation apparatus can be heated. To minimize the amount of glass required to be heated, a miniature distillation apparatus was designed with a 90° tube connecting two flasks. Using this set-up efficient distillation of crotonic acid was accomplished resulting in a 99.1 % recovery of crotonic acid. Summary results of this experiment are presented in Table 3.

Purity and particle size of PHB polymer used for fundamental studies. We fully expect that the kinetics and yield of the thermolysis of PHB to crotonic acid could be impacted by the presence of contaminants and the PHB particle size which may impact heat transfer. In order to get definitive data on the optimum reaction conditions for the conversion of PHB to crotonic acid, a series of experiments using PHB which had been purified from microbial cells was carried out.

The purity of the PHB to be used in thermolysis studies was determined by carrying out elemental analyses (performed by Galbraith Laboratories, Knoxville, TN). The white, purified PHB powder was shown to have a purity of 99.6 %. The particle size distribution of the purified PHB was determined using a series of sieves.



P3HB

Chemical Formula of Monomer Unit: $C_4H_6O_2$
Elemental Analysis of Indefinitely Long, Pure P3HB:
C, 55.81 %; H, 7.02 %; O, 37.17 %

PHB thermolysis studies. Pure PHB was heated in the equipment (50 ml starting flask, 90° connecting adapter, 250 ml 2-necked receiving flask) previously optimized for the distillation of crotonic acid (Figure 4). Within 15 minutes of raising the mantle temperature, all PHB melted to form a rapidly boiling liquid, while at the same time a white oily solid

Table 3. Results of Distillation of Crotonic Acid using the Optimized Experimental Set-Up

Distillation of Crotonic Acid - Mass Balance experiment DS05-52					
item	**m_{tare} [g]**	**$m_{before/+CA}$ [g]**	**m_{after} [g]**	**$m_{material\ on\ it\ after\ rxn}$ [g]**	**description of material**
50 ml flask + stirrer	67.00	88.50 **=> 21.50 g crotonic acid used**	67.12	0.12	brown film, burnt sugar smell
90° adapter + 2 teflon sleeves	15.13		15.19	0.06	white & brown solids
2-necked receiving flask	102.56		123.86	21.30	white crystalline solid
				21.30 / 21.50 => 99.07 % crotonic acid collected after distillation	

Figure 4. Reaction set up for thermolysis of pure PHB. Left panel, heating of flask containing PHB. Middle panel, top of receiving flask. Right panel, flasks after conclusion of experiment.

condensed in the 2-necked receiving flask.

Upon completion of the thermolysis/distillation experiment, 4 samples were taken for ^{1}H-NMR analysis.

(A) dark oil from left side of 90° adapter
(B) white crystals from right side of 90° adapter
(C) oily white solid of receiving flask
(D) 2nd sample of produced oily white solid

Based on ^{1}H-NMR, our thermolysis product mixture is composed of 82.4 % *trans*-crotonic acid, 2.0 % *cis*-crotonic acid, and 15.6 % oligomers containing crotonyl end-groups. Compared to results in a previous study wherein P3HB was thermolyzed at 260 °C under high vacuum,[17] our product mixture contains more *trans*-crotonic acid (82.4 vs. 67.7 %) and less oligomers (15.6 vs. 29.2 %). It is expected that a subsequent re-distillation of this crude thermolysis + distillation product will result in a more complete conversion to

[17]Ariffin, H., et al., *Determination of multiple thermal degradation mechanisms of poly(3-hydroxybutyrate).* Polymer Degradation and Stability, 2008. **93**, 1433-1439.

trans-crotonic acid. The oligomer size can also be estimated based on the ^{1}H-NMR integrations. About 73% of the oligomer molecules are dimers (crotonic acid-3hydroxbutryate) while the remaining 27% are trimers (CA-3HB-3HB). Since the molecular formula, and thus molecular weight, of *trans*-crotonic acid ($C_4H_6O_2$ 86.089 g/mol) is identical to that of the monomeric subunit of P3HB, its molecular conversion/yield to *trans*-crotonic acid can easily be calculated. In this experiment, an 81.6% molecular conversion/yield of P3HB to *trans*-crotonic acid was achieved (Table 4).

This series of fundamental laboratory scale studies was crucial in identifying an experimental setup and improved conditions for the conversion of PHB to crotonic acid and its recovery by distillation. They also served to identify areas of concern regarding heat transfer in the apparatus.

B.1.3. Torrefaction conversion of engineered switchgrass containing PHB

Work with PHB containing transgenic switchgrass in Metabolix kiln.

In Q2, 2013 we evaluated the thermolysis of genetically engineered P3HB producing switchgrass in Metabolix's laboratory Kiln thermolysis/pyrolysis apparatus. The inside of the Kiln reactor was filled with finely ground P3HB switchgrass biomass powder (100 g). The P3HB switchgrass biomass used was from the leaves collected from plants that produce 2 to 6 % of P3HB in mature leaves of vegetative tillers. Its moisture content was determined to be 7.1 %. Two temperature-probes, a reflux condenser, and receiving flask were attached, and the Kiln reactor was heated for a total time of 90 min to a maximum temperature of 325 °C. This experiment was featured in an article in the *MIT Technology Review* about our plant-science program.[18]

Table 4. PHB Thermolysis and distillation mass balance.

item	m_{tare} [g]	$m_{before/+P3HB}$ [g]	m_{after} [g]	$m_{material\ on\ it\ after\ rxn}$ [g]	description of material
50 ml flask + stirrer	69.45	90.81 **=> 21.36 g P3HB used**	69.47	0.02	black powder & dark oil
90° adapter + 2 teflon sleeves	15.13		15.17	0.04	dark oil on left side & white crystals on right side
2-necked receiving flask	102.56		123.71	21.15	oily white solid
				21.15 / 21.36 => 99.02 % of material collected after distillation	
				based on NMR	**Conversion/Yield**
				82.4 % *trans*- crotonic acid	17.43 g => 81.6 %
				2.0 % *cis*- crotonic acid	0.42 g => 2.0 %
				15.6 % oligomers with crotonyl end-groups	3.30 g => 15.4 %

[18] https://www.technologyreview.com/s/515486/plastic-from-grass/
"Plastic from Grass" *MIT Technology Review*, **2013**, *116,* 84-87.

While the temperature within the Kiln reached above 300 °C, the temperature outside the reactor did not reach more than 91 °C, indicating that all thermolysis/pyrolysis occurs within the Kiln and the pyrolysis products, which include crotonic acid, distill out of the Kiln. In future experiments the metal tubing outside the Kiln could be heated with heating tape to ensure that *trans*-crotonic acid remains in the gas phase, and only condenses once it has reached the reflux condenser.

Following cooling back to room temperature, the reactor was disassembled and the following fractions were collected:

- biomass residue recovered from the Kiln (48 g)
- material recovered from the metal tubing (washed out with *i*-PrOH, 7 d evaporation under hood, 0.5 g).
- material present in the reflux condenser (washed out with *i*-PrOH, 7 d evaporation under hood, 0.7 g).
    - brown solid at the top of the reflux condenser
    - brown film at the bottom of the reflux condenser
- black, viscous oil collected in the 3-necked flask (washed out with *i*-PrOH, 7 d evaporation under hood, 5.3 g).

Once recovered the weight of the material in each of these samples was determined and reported in Table 5.

From the 100 g of P3HB switchgrass loaded into the kiln, around 38 g was lost as non-condensable vapor. During the experiment the loss of a white smog was noted from the open port of the 3-necked flask and in future experiments this will be controlled. The biomass residue recovered from the kiln was analyzed for the presence of P3HB and for crotonic acid. No P3HB was detected in the residue and HPLC analysis indicated only a trace amount (~7 ppm) of crotonic acid. As the intention is to use residual biomass as a solid fuel, the heat of combustion was determined and found to be about 12,460 BTU/lb (28,980 kJ/kg) which is very similar to charcoal. It was noted that in this laboratory process the biomass did not lose its grass like appearance. Each of the other samples taken was analyzed by ^{1}H-NMR for the presence of crotonic acid and the presence of *trans*-crotonic acid was identified as the major component in all of the samples. Despite the low yield of crotonic acid, these results confirm the feasibility of thermally degrading P3HB in engineered switchgrass to produce crotonic acid and a biomass residue suitable as a solid fuel.

Table 5. Material mass balance for kiln experiment

PHB switchgrass	100 g
moisture	7.1 g
pyrolysis residue	48 g
metal tubing material	0.5 g
reflux condenser material	0.7 g
3-necked flask material	5.3 g
	38.4 g

Work with transgenic PHB containing switchgrass in fluidized bed reactor

In Q4 2014, we pursued the thermolysis/pyrolysis of switchgrass that was genetically modified to contain P3HB in a fluidized bed reactor. Although PHB content in

the switchgrass material was low, thermolysis was performed using our best conditions [375 °C, 75 l_{N2}/min; Table 2] that previously provided a crotonic acid yield of 45% with non-transgenic switchgrass/P3HB blends. The recovered masses of char and liquid closely match those of a pure switchgrass control experiment performed under similar conditions. From the P3HB-producing transgenic switchgrass a conversion to crotonic acid of 28.5% was achieved. Considering the more diluted presence of P3HB in the substrate feed stream and the fact that the P3HB is confined within the cells and plastids of the plant, these results of the pyrolysis/thermolysis of P3HB-producing transgenic switchgrass are promising.

Decarboxylation of crotonic acid

Yields in task B.1. pyrolysis studies may be impacted by a competing decarboxylation of crotonic acid. The kinetics and mechanism (Figure 5) of the gas-phase thermal decarboxylation of *trans*-crotonic acid has been investigated in detail.[19] At 360 °C [634 K], 15% of the crotonic acid produced from PHB decarboxylates within 15 min. It is thus of interest to know the rate of P3HB thermolysis/pyrolysis at lower temperatures. We investigated the thermal properties of pure P3HB using thermogravimetric analysis (TGA) [~ 15 mg sample, 20 K/min to the desired temperature, then isothermal, N_2 gas, balance purge flow = 40 ml/min, sample purge flow = 60 ml/min]. While the P3HB sample was heated (20 K/min) to the desired temperature of 350 °C, loss of mass due to its thermolysis/ pyrolysis had already commenced during the heating-up period and, indeed, went to completion prior to reaching the desired temperature. When another P3HB sample was heated to the desired temperature of 300 °C, its thermolysis/pyrolysis also had already commenced during the heating-up period and reached completion near the time point at which the desired temperature was reached.

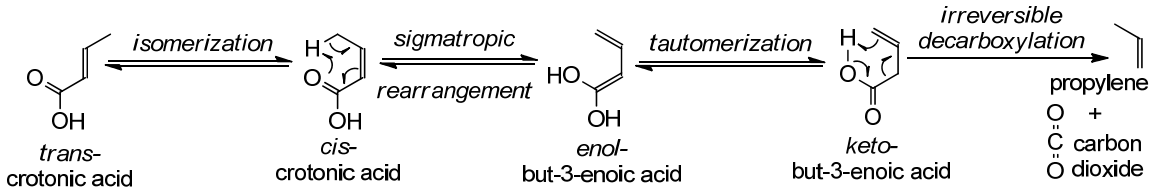


Figure 5. Mechanism of decarboxylation of crotonic acid

Heating another P3HB sample to the desired temperature of 250 °C, loss of mass due to its thermolysis/pyrolysis commenced near the time point at which the desired temperature was reached, and went to completion within minutes. In comparison, when another P3HB sample was heated to the desired temperature of just 200 °C, loss of mass due to its thermolysis/pyrolysis commenced about 10 min after the desired temperature was reached, and went to completion within about 40 minutes. Even at 190 °C, P3HB undergoes thermal decomposition/pyrolysis within minutes. The lower temperature limit at which a thermal decomposition/pyrolysis of P3HB can be detected via the loss of mass is given by the boiling point of its eventual thermolysis product *trans*-crotonic acid (bp = 180 °C). This suggests that the PHB pyrolysis reactions should be performed at a lower temperature than the low of 250°C that we tried with the available fluidized bed reactor. Generally speaking,

[19] *Journal of the Chemical Society, Perkin Transactions II* **1982**, p. 1 - 6

it is difficult to operate a fluidized bed at a low temperature such that we were unable to further optimize temperature to achieve increased crotonic acid in the fluidized bed reactor. The sand in the bed often is no longer fluidized and sticks together. Thus the lower temperature that would likely increase the yield of crotonic acid is outside the operability window of the fluidized bed reactor equipment that was available to us.

Key Conclusions and Future work.

Successful accomplishment of the goal of 90% conversion of PHB in biomass to crotonic acid was a very challenging objective further complicated by equipment limitations with the highest conversion yield reached being just over 45% in experiments of PHB mixed with switchgrass biomass. Laboratory studies did however demonstrate that PHB can be converted at over 80% efficiency (Table 4) but developing a successful commercial process still has significant technical challenges to overcome.

B.2. Develop Catalyst Technology to Convert Crotonic Acid

B.2.1. Testing of hydrogenation catalysts.

We originally intended to work with a 3rd party to evaluate hydrogenation catalysts to convert crotonic acid to *n*-butanol. However, at the beginning of the REFABB program, the 3rd party informed Metabolix that they could no longer perform work on the REFABB program due to assignment of personnel to other projects. During discussions with USDA-ARS, we learned that they possess the capability to test hydrogenation catalysts for crotonic acid and their scientists expressed interest in performing this work. Metabolix informed DOE of the third party's inability to complete the work on the REFABB project and requested a modification of the work agreements to allow USDA-ARS to perform the work. Metabolix submitted a formal request to DOE for a modified work agreement so that hydrogenation and oxidation work (subtask B.2.2) could be performed at USDA-ARS. This modification to the work agreement was formally approved in Q3, 2012 and the project initiated in Q4, 2012.

n-Butanol can either be produced by the hydrogenation of crotonic acid or its esters, such as *n*-butyl crotonate. Literature references show that the ease of hydrogenation of an ester is greater than a carboxylic acid, and that a -CO_2H group can leach metal ions affecting the stability of the catalyst.[20] The preferred hydrogenation substrate is therefore an ester of crotonic acid. With use of *n*-butyl crotonate as the hydrogenation substrate, two equivalents of *n*-butanol are formed, one half of which can be reused in the synthesis of *n*-butyl crotonate from P3HB/biomass, making it an integrated process (Figure 6). This is the process that was developed by the end of this study.

The initial experiment employed a commercial CuO and ZnO containing catalyst (Johnson-Matthey PRICAT CZ 40-18 T) at a reaction temperature of 200 °C and an H_2 pressure of 200 psi. These conditions resulted in complete consumption of the starting *n*-butyl crotonate. While about 45 % of the material remained at the intermediate *n*-butyl butyrate stage, a 55% conversion/yield of *n*-butanol was achieved. Increasing the reaction temperature to 250 °C resulted in an increased conversion/yield of 77 % however a small amount of by-products were detected. Doubling the H_2 pressure to 400 psi while maintaining a reaction temperature of 200 °C enabled a similarly high conversion/yield of

[20] *Journal of the American Chemical Society* **1932**, *54*, 1145 – 1154; *Helvetica Chimica Acta* **1947**, *30*, 39 - 43

76 % without the formation of by-products. Combining a H_2 pressure of 400 psi and a reaction temperature of 250 °C further increased the conversion/yield to 86 %, however, detectable quantities of by-products were again formed.

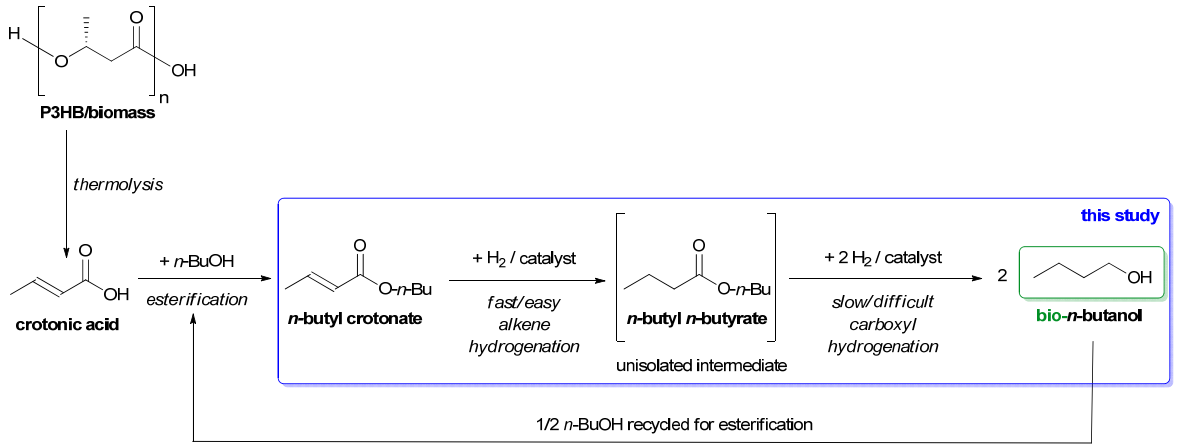


Figure 6. Integrated process for converting PHB containing biomass to *n*-butanol developed in this project.

The proof of concept conversion of *n*-butyl crotonate to *n*-butanol achieved milestone B.2.M.1. For optimization of the hydrogenation of *n*-butyl crotonate to *n*-butanol, the conversion of butyl-crotonate to *n*-butanol needs to be maximized, while any decomposition products need to be minimized. From a process economic perspective, separating the *n*-butanol produced from intermediate *n*-butyl butyrate is preferred to having a higher conversion to *n*-butanol but more loss of material due to decomposition. During Q4, 2013 we evaluated a series of heterogeneous catalysts, substrate flow rate in the reactor (residence time), the effect of temperature and the effect of hydrogen pressure and flow rate on the conversion of butyl-crotonate, as well as the formation of decomposition or by-products, and the results are summarized in Table 6. The best reaction conditions obtained for converting butyl crotonate to butanol to were those using the Cu 1986 catalyst, a pressure of 400 psi, temperature of 200°C, and a substrate flow rate through the reactor of 0.5 ml/min (Reaction 7, Table 6). Using these conditions, a 90% conversion was achieved with only 5% loss of material.

To test the reproducibility of the reaction conditions, they were repeated (experiment 15, Table 6). In the repeat experiment less intermediate *n*-butyl butyrate (0.9%) remains under the steady state conditions as compared to the original experiment (4.9%). Additionally, somewhat less of the desired hydrogenation product *n*-butanol was formed in the repeat experiment as compared to the original one (87.8 vs. 91.0%). These two comparisons suggest that in the repeat experiment noticeably more material underwent decomposition as compared to the original one (13 vs. 5 mol%). In conclusion, under optimized conditions, high 80s conversion of *n*-butyl crotonate to *n*-butanol appears to be reproducible (86 vs. 90%).

We also tested a new commercial catalyst, BASF-X 540 T 1/8'' [60/10/30 $CuO/Al_2CuO_4/MnO_2,Al_2O_3$] for the hydrogenation of *n*-butyl crotonate to *n*-butanol. In order to be able to compare these results to the earlier studies, we carried out these

Table 6. Results of hydrogenation of butyl-crotonate

									in 100 g						
#	$p(H_2)$ [psi]	T [°C]	Catalyst	BuCr Flow Rate [ml/min]	BuCr [wt %]	BuBu [wt %]	BuOH [wt %]	Others [wt %]	n(BuCr) [mol]	n(BuBu) [mol]	n(BuOH) [mol]	n(Others, as 2-EHal) [mol]	n(BuCr, starting) [mol]	Conversion to BuOH [mol %]	mol % decomposed
								=100-BuCr-BuBu-BuOH	MW 142.20	MW 144.21	MW 74.12	MW 128.12	$=n_{BuCr}+n_{BuBu}+0.5*n_{BuOH}+n_{2EHal}$	$=100*n_{BuOH}/(2*n_{BuCr,starting})$	$=100*n_{others}/n_{BuCr,starting}$
1	200	200	PRICAT CZ 40	0.5	0.0	23.1	56.1	20.8	0.000	0.160	0.757	0.162	0.701	54	23
2	200	250	PRICAT CZ 40	0.5	0.0	19.1	78.0	2.9	0.000	0.132	1.052	0.023	0.681	77	3
3	400	200	PRICAT CZ 40	0.5	0.0	14.7	76.1	9.2	0.000	0.102	1.027	0.072	0.687	75	10
4	400	250	PRICAT CZ 40	0.5	0.0	8.4	86.7	4.9	0.000	0.058	1.170	0.038	0.681	86	6
5	200	200	Cu 1986	0.5	0.0	18.0	65.2	16.8	0.000	0.125	0.880	0.131	0.696	63	19
6	200	250	Cu 1986	0.5	0.0	8.3	85.7	6.0	0.000	0.058	1.156	0.047	0.683	85	7
7	400	200	Cu 1986	0.5	0.0	4.9	91.0	4.1	0.000	0.034	1.228	0.032	0.680	90	5
8	400	250	KATALCO 83-3	0.5	0.0	2.6	74.1	23.3	0.000	0.018	1.000	0.182	0.700	71	26
9	400	250	PRICAT CZ 30	0.5	0.0	3.5	74.6	21.9	0.000	0.024	1.006	0.171	0.698	72	24
10	400	250	Cu 1986	0.25	0.0	0.6	68.3	31.1	0.000	0.004	0.921	0.243	0.708	65	34[b]
11	400	250	Cu 1986	0.5	0.0	1.7	70.1	28.2	0.000	0.012	0.946	0.220	0.705	67	31[b]
12	400	200	Cu 1986	0.25	0.0	0.6	90.7	8.7	0.000	0.004	1.224	0.068	0.684	89	10
13	600	200	Cu 1986	0.5	0.0	0.7	74.2	25.1	0.000	0.005	1.001	0.196	0.701	71	28
14	400[a]	200	Cu 1986	0.5	0.0	0.6	82.2	17.2	0.000	0.004	1.109	0.134	0.693	80	19
15 [r7]	400	200	Cu 1986	0.5	0.0	0.9	87.8	11.3	0.000	0.006	1.185	0.088	0.687	86	13
16	400	200	X 540 T	0.5	0.0	1.1	86.0	12.9	0.000	0.008	1.160	0.101	0.688	84	15
17	400	250	X 540 T	0.5	0.0	2.4	75.5	22.1	0.000	0.017	1.019	0.172	0.698	73	25[c]
18	400	200	Cu 0860	0.5	0.0	14.7	69.2	16.1	0.000	0.102	0.934	0.126	0.694	67	18[d]
19	400	250	Cu 0860	0.5	0.0	3.9	60.9	35.2	0.000	0.027	0.822	0.275	0.713	58	39[e]
20 [r3]	400	200	PRICAT CZ 40	0.5	0.0	5.0	71.5	23.5	0.000	0.035	0.965	0.183	0.700	69	26
21 [r4]	400	250	PRICAT CZ 40	0.5	0.0	6.6	77.5	15.9	0.000	0.046	1.046	0.124	0.693	75	18
22	400	300	PRICAT CZ 40	0.5	0.0	3.0	30.5	66.5	0.000	0.021	0.411	0.519	0.746	28	70[f]

BuCr = *n*-butyl crotonate; BuBu = *n*-butyl butyrate; BuOH = *n*-butanol; 2-EHal = 2-ethyl-hexanal.

[a] H_2 flow rate = 4000 ml_n/min, all other experiments used 3000 ml_n/min.

[b] Hydrocarbons as side-products identified.

[c] Heptanes, heptanol, and heptanone as side-products identified.

[d] A trace amount of di-*n*-butyl ether as a side-product identified.

[e] A greater amount of di-*n*-butyl ether as a side-product, as well as 3-heptanone and 3-heptanol.

[f] Hydrocarbons are the major side-products: mainly C7 and C8 ones, with 2-methylheptane being the most abundant.

experiments as a direct comparison with the previous best catalysts under the same conditions and the results are presented below:

Reaction conditions 400 psi H_2, 200 °C, 0.5 ml/min

catalyst	experiment	% conversion	% decomposed
PRICAT CZ 40	3	75	10
Cu 1986	7, 15	90, 86	5, 13
X 540 T	16	84	15

Reaction conditions 400 psi H_2, 250 °C, 0.5 ml/min

catalyst	experiment	% conversion	% decomposed
PRICAT CZ 40	4	86	6
KATALCO 83-3	8	71	26
PRICAT CZ 30	9	72	24
Cu 1986	11	67	31
X 540 T	17	73	25

We demonstrated that the catalyst PRICAT CZ 40 appears to provide the best and most reproducible results.

Key Accomplishment

The key accomplishment of this work is the proof-of-concept that (bio-)*n*-butanol can be synthesized from (bio-)*n*-butyl crotonate with high conversion yield. The optimized conditions are:

- neat *n*-butyl crotonate as the substrate,
- a hydrogen pressure of 400 psi,
- a reaction temperature of 250 °C,
- a reactant-residence time of 60 min,
- a CuO-containing catalyst, such as commercial JM-PRICAT CZ 40-18 T [51/32/5/12 CuO/ZnO/$CuCO_3$/Al_2O_3,C].

The experimental hydrogenation findings from this Task were published in the ACS journal *Organic Process Research & Development.*[21]

B.2.2. Testing of oxidation catalysts.

This task was initiated in Q4, 2013 to demonstrate the catalytic conversion of crotonic acid to maleic anhydride, a large volume chemical intermediate. Oxidation catalysts were used at USDA-ARS to demonstrate the production of fumaric acid and maleic anhydride from crotonic acid. The overall reaction scheme for the conversion of PHB to maleic anhydride via crotonic acid is shown in Figure 7.

[21] Schweitzer, D., et al., *Bio-based n-butanol prepared from poly-3-hydroxybutyrate: optimization of the reduction of n-butyl crotonate to n-butanol.* Org Process Res Dev, 2015. **19**, 710-714.

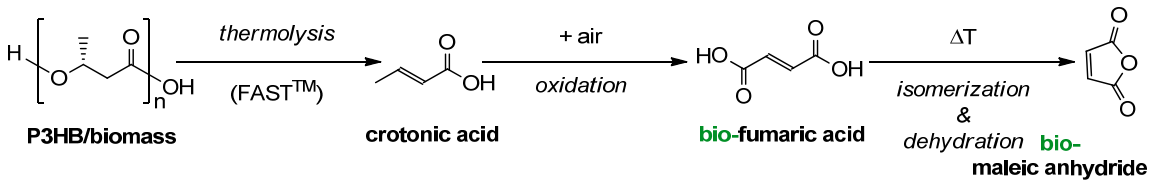


Figure 7. Overall reaction scheme from PHB via crotonic acid to maleic anhydride

Currently maleic anhydride is commercially produced by the oxidation of butane using vanadium pentoxide and molybdenum trioxide catalysts. Since crotonic acid is not an intermediate in the oxidation of *n*-butane (and thus likely also 2-butene) to maleic anhydride, our crotonic acid oxidation to maleic anhydride must proceed via a different mechanistic pathway as shown in Figure 8.

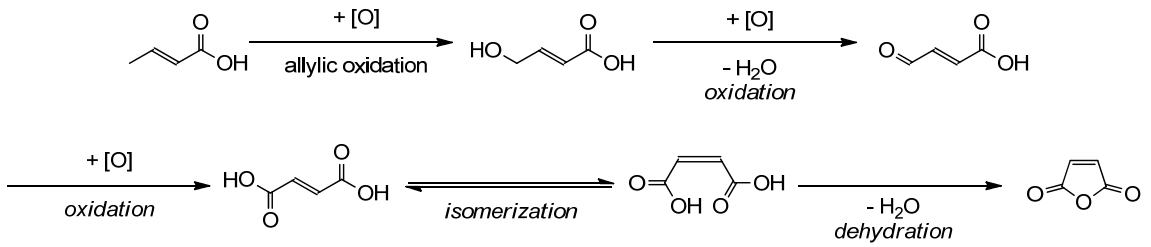


Figure 8. Overall mechanistic pathway for the conversion of crotonic acid to maleic anhydride

The equilibrium state and kinetics of the fumaric acid = maleic acid isomerization have been previously studied in a cyclohexanone solution between 142 (55% fumaric acid) and 190 °C (30% fumaric).[22] Intramolecular H-bonding within maleic acid can explain its high abundance. The kinetics of the subsequent thermal dehydration of maleic acid to maleic anhydride has been studied in detail.[23] A likely by-product of our process will be malic acid,[24] formed by the alkene hydration of fumaric and/or maleic acid.[25]

In this project, two kinds of reactors were used, a tube furnace reactor and a Parr reactor. Work was initiated at USDA-ARS and was performed in a Parr reactor with crotonic acid and air, which supplies the oxygen needed for the oxidation reaction. The reaction was heated at various temperatures and times in the presence of a commercial butane to maleic anhydride oxidation catalyst. In the initial experiments we found that once the reaction system was heated past a reaction activation temperature, rapid near-complete oxidation of crotonic acid occurred. The complete oxidation (combustion/burning) of crotonic acid is exothermic. The initial crotonic acid oxidation reactions were conducted in a 100 ml Parr reactor, using 20 weight % of catalyst and air as the oxidant. Subsequent experiments were performed in glassware with a low air pressure.

In Q4, 2014, we investigated the oxidation of molten crotonic acid in a batch reaction, using a Parr reactor at 100 or 125 °C, under 50 psi of air. A yield of only 1% of maleic acid was achieved. A tube furnace reactor,[26] allowing the oxidation of refluxing

[22] T*ransactions Faraday Soc.* **1955**, *51*, 1506-1517.
[23] *J. Appl. Chem. USSR* **1982**, *55*, 1246-1248.
[24] http://en.wikipedia.org/wiki/Malic_acid
[25] *J. Phys. Chem.* **1924**, *28*, 758-768.
[26] http://mlay.chem.uga.edu/tube%20furnace.jpg.

crotonic acid to occur by a stream of air at normal pressure[27] was also investigated. Outside the reactor, at room temperature, a water bubbler collected all condensable material. Depending on the reaction conditions, the yield of maleic acid in these experiments ranged from < 0.1% to 50 % (exp. 37). The material recovered in the water trap in these experiments was near pure maleic acid.

In general, we had the best results at about 350 °C with the chosen catalyst, getting around 30% maleic acid yield. We believe our process is limited by the efficiency of the delivery of the crotonic acid vapor. Most of the material balance is likely to be unreacted crotonic acid that gets stuck on the catalyst particles and in the inlet of the reactor.

In order to identify potential alternative catalysts, an extensive literature search was conducted using Reaxys and SciFinder to identify known conditions for the oxidation of either one of *n*-butane, *n*-butene, *n*-butanol, *n*-butyraldehyde, or *n*-butyric acid to either one of fumaric acid, maleic acid, or maleic anhydride. Noteworthy, not even a single example for the oxidation of *n*-butyric acid to these products was found in the literature.

We also investigated the batch scale oxidation of crotonic acid catalyzed by another catalyst in a Parr reactor. With this other catalyst we obtained a yield of maleic acid of 49%.

Key Accomplishment

The key accomplishment of this work was demonstrating the successful conversion of crotonic acid to maleic anhydride with the highest yield achieved of approximately 50%. Although not acceptable for an economically viable process, we recognize the program was hampered by limited equipment availability and the lack commercially available catalysts.

B.2.3. Testing of metathesis catalysts.

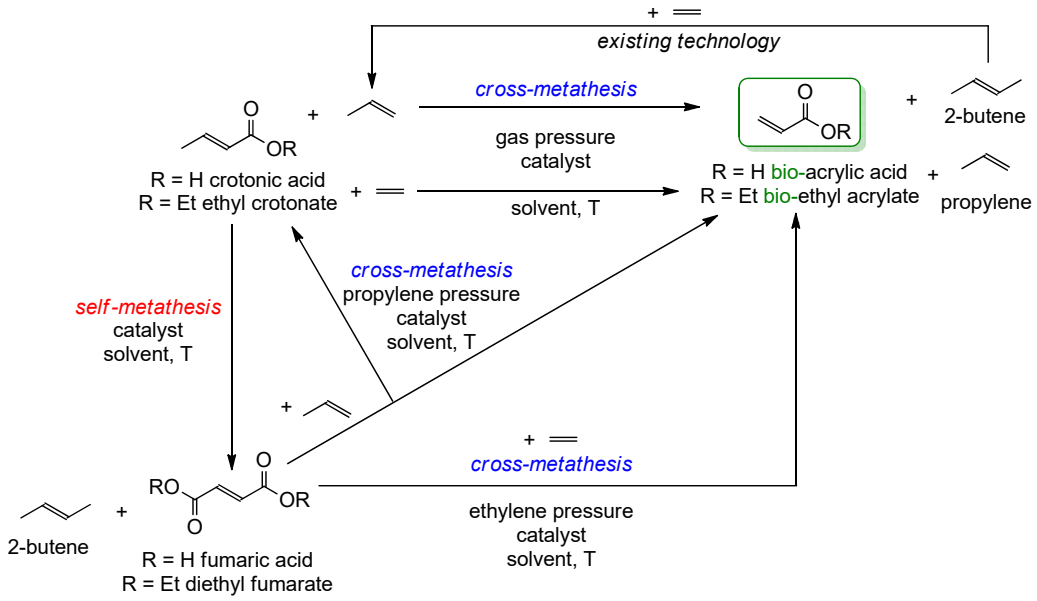
Metabolix was originally planning to work with a third party to proceed with metathesis work on the REFABB project. We however were not able to finalize a Research Agreement. Metabolix informed DOE of this event and requested a modification of the work agreements to allow Metabolix to perform the work in-house with the objective of achieving proof of concept for the conceptual process. A diagram illustrating the reaction network of the proposed metathesis reaction to convert crotonic acid to acrylic acid is illustrated in Figure 9. Results of initial method development and scoping studies to test 12 commercially available metathesis catalysts carried out in Q2/Q3 of 2012 are summarized in Table 7.

Based on the catalyst screening studies, the Hoveyda-Blechert (HB, TON = 168) and SB-H-indene (SB-H, TON = 133) and the Schiff base catalyst SB-NO_2 TON = 114) were selected for studies to optimize the metathesis reaction.

In Q3, 2012/Q4, 2013, research focused on further evaluation of solvent systems and optimizing the pressure and temperature of the metathesis reaction, so as to achieve a maximum turnover number (TON) for the production of ethyl acrylate from ethyl crotonate. TON, defined as % yield/mol % catalyst, is the key economic driver in metathesis catalysis. Figure 10 illustrates the basic metathesis reaction scheme. Using ethylene (R=H) as a metathesis partner has an advantage over propylene (R=Me) from a commercial biorefinery perspective in that it can be readily produced from first or second generation ethanol in a biorefinery.

[27] The heating elements of the tube furnace reactor had a temperature of 350 °C. Inside of the heating zone we expect boiling crotonic acid to be present, thus, the actual reaction temperature is ~ 180 °C.

Figure 9. Diagram illustrating reactions in the metathesis reaction network.



Evaluation of different solvent systems for the metathesis reaction.

Reports in the scientific literature have shown that the use of different solvents can have a significant impact on the metathesis reaction. Relevant factors include the solubility of the metathesis partner ethylene or propylene in the solvent, the state of the Ru catalyst (catalytically active Ru-methylidene vs. dormant Ru-metallacyclobutane), and the rate of Ru catalyst decomposition. Thus, it was decided to expand the investigation of the effect of the solvent onto our cross-metathesis between ethyl crotonate and ethylene or propylene. It was planned to investigate three metathesis catalysts (HB, SB-H, or SB-NO_2) at two temperatures (40 and 60 °C) in four solvents (dioxane, propylene carbonate, CH_2Cl_2, neat) with either ethylene or propylene at a constant pressure of 60 psi, for a total of 48

Table 7. Results of initial metathesis studies

Development Step / Milestone	**Result**
establish analytical methods	Q2, 2012 ● HPLC methods now available to quantify the 4 acids and esters of the reaction system
screen promising commercially available metathesis catalysts with both ethyl crotonate and crotonic acid	Q2-3, 2012 ● screened 12 promising commercially available catalysts, two of the most active ones were chosen for further investigations: Hoveyda-Blechert & SB-H-Indene ● for the neat metathesis reaction of ethyl crotonate with ethylene, turn over numbers (TON) of 168 (Hoveyda-Blechert catalyst) and 133 (SB-H-Indene) were achieved. These TON are higher than previous reports in the scientific or patent literature.
based on these screening	Q2-3, 2012

reactions, decide on a substrate: ethyl crotonate or crotonic acid	● ethyl crotonate is a one order of magnitude more reactive substrate than is crotonic acid
screen solvents to identify the one in which the metathesis performs best	Q3, 2012 propylene carbonate is the preferred solvent

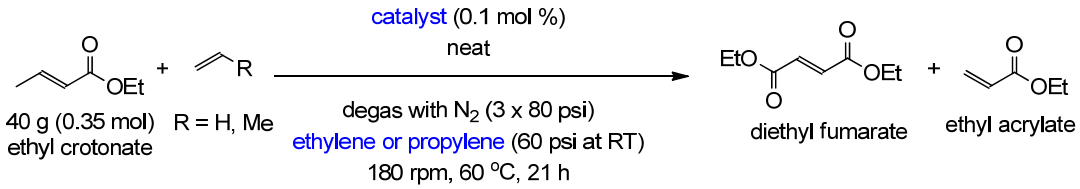


Figure 10. General Metathesis Reaction Scheme

experiments. The results of the experiments performed are presented in Figure 11.

The main conclusions drawn from these experiments are 1) ethylene is a better metathesis partner than propylene; 2) HB is a much better catalyst than SB-H or SB-NO_2 under these conditions; 3) although CH_2Cl_2 is a better solvent than propylene carbonate or dioxane, in the case of HB the metathesis reaction proceeds best in the neat substrate; 4) the reaction temperature, 40 °C or 60 °C, does not have a large influence on the reaction.

Temperature and Pressure Optimization

Since ethylene is a better metathesis partner than propylene and HB is one of the most active catalysts, it was decided to do further screening on the impact of a range of temperature and pressure conditions on cross-metathesis with ethyl crotonate according to the reaction scheme shown in Figure 12.

Results of these experiments are presented in Figure 13. Two main conclusions can be reached based on this data: 1) the ethylene pressure determines the amount of conversion, as quantified by the TON, that can be reached prior to catalyst decomposition; 2) most of the catalyst decomposition occurs within the first 2 h, even at room temperature, since the TONs of the room temperature reactions after 2 h are not much lower than the TONs of the room temperature reactions after 21 h. A similar conclusion about the HB catalyst stability was previously reached based on metathesis reactions conducted in propylene carbonate as the solvent as described in previous reports. Based on these studies, the current optimum reaction conditions for converting ethyl-crotonate to ethyl acrylate using metathesis chemistry are:

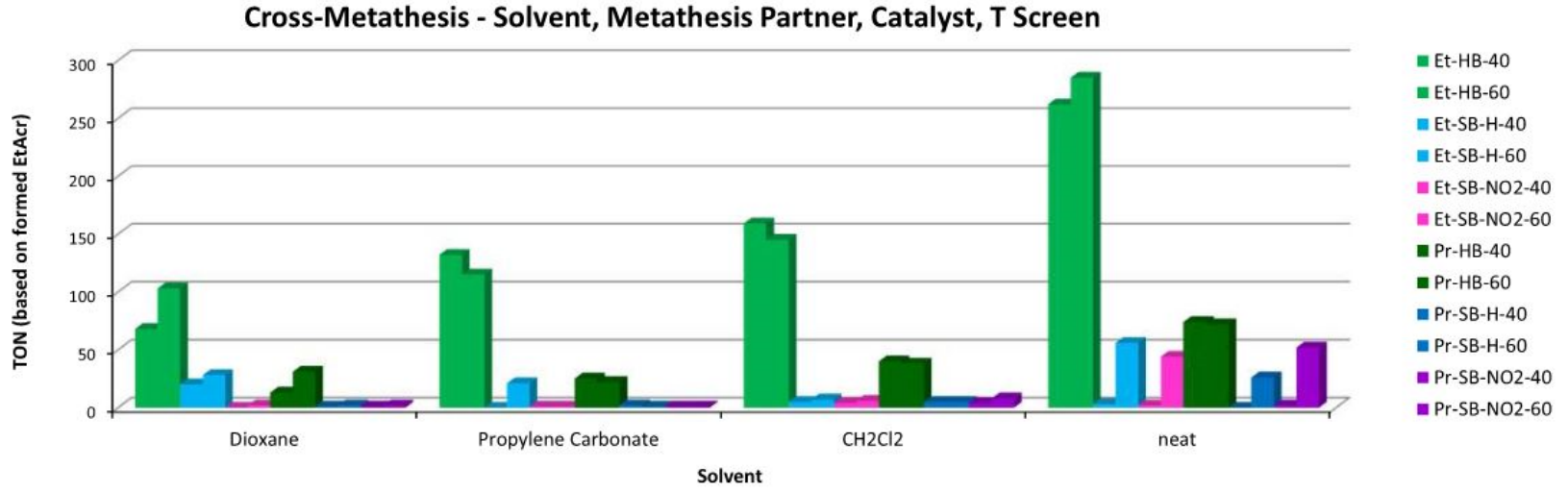
- Neat ethyl crotonate with no solvent as the substrate
- Ethylene as the reaction partner
- Hoveyda-Blechert catalyst (0.05 mol %)
- Temperature: RT to 40 °C
- Pressure: 160 to 255 psi

Under these conditions a TON ~ 350 was achieved.

Figure 11. Effect of different solvents on TON[a]

TON of Ethyl Crotonate Metathesis = f(Solvent, Metathesis Partner, Catalyst, Temperature)

	Metathesis Partner, Catalyst, Temperature											
	Ethylene						Propylene					
Solvent	**Et-HB-40**	**Et-HB-60**	**Et-SB-H-40**	**Et-SB-H-60**	**Et-SB-NO_2-40**	**Et-SB-NO_2-60**	**Pr-HB-40**	**Pr-HB-60**	**Pr-SB-H-40**	**Pr-SB-H-60**	**Pr-SB-NO_2-40**	**Pr-SB-NO_2-60**
Dioxane	68	103	20	28	0	2	13	31	1	2	1	2
Propylene Carbonate	132	115	0	21	1	1	25	22	2	1	1	1
CH_2Cl_2	159	145	5	7	4	6	40	38	5	5	4	8
neat	262	285	3	56	2	44	74	72	0	26	2	52



[a]Catalysts are as follows. HB ■, Hoveyda-Blechert; SB-H ■, SB-H-indene; SB-NO_2 ■, a Schiff base catalyst. Temperature (40 or 60 °C) is indicated next to the catalyst name. Reactions used either ethylene (lighter colored bar) or propylene (darker colored bar) as a metathesis partner.

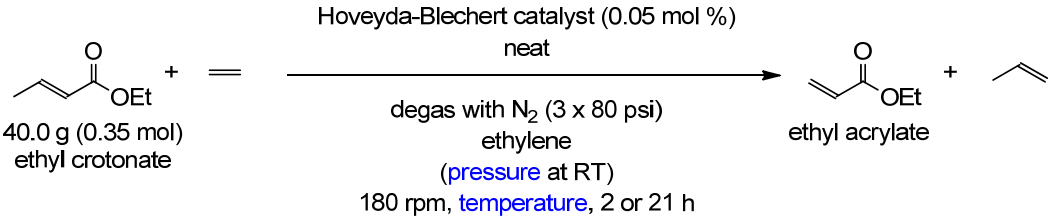


Figure 12. Reaction Scheme for Temperature and Pressure Studies with the HB Catalyst

TON of Ethyl Crotonate Metathesis = f(T, p, t)

Temperature [°C] (Reaction Time [h])	Pressure [psi, prior to heating]					
	10	**20**	**40**	**80**	**160**	**255**
60 (21)	111	187	258	315	297	350
50 (21)	125	200	258	278	321	339
40 (21)	122	195	265	288	331	359
25 (21)	124	203	266	275	336	326
25 (2)	115	175	234	273	298	349

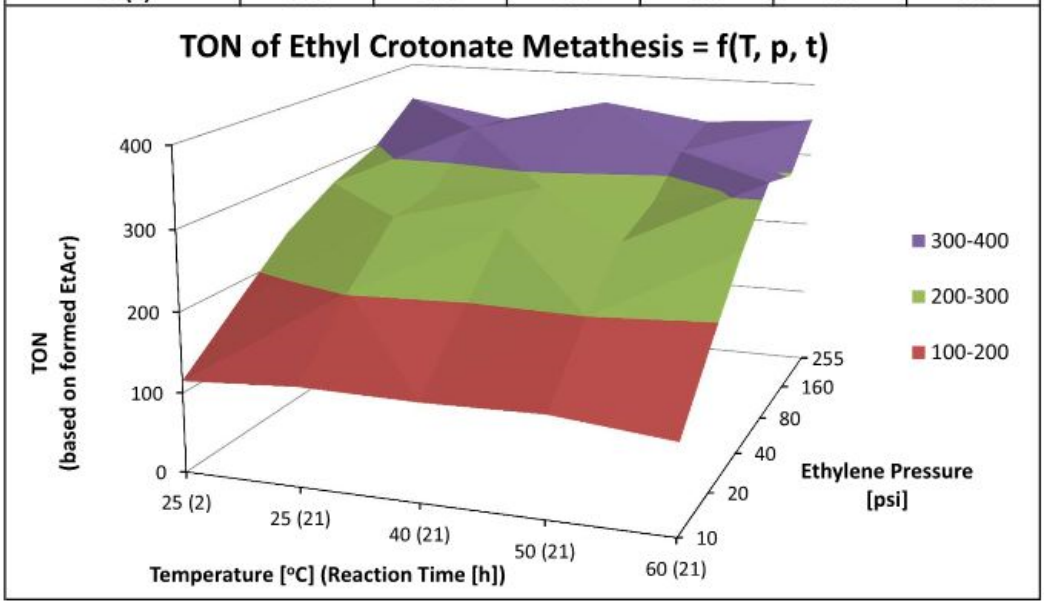


Figure 13. Results of Temperature and Pressure conditions on TON

An optimized HPLC method for separating and quantifying *n*-butyl crotonate and *n*-butyl acrylate was developed in Q2, 2013 to enable us to perform a series of equilibrium and self-metathesis control experiments as well as study the rate of catalyst decomposition versus product formation under optimum conditions in an effort to further improve the metathesis reaction.

Mechanism: Determination of the Metathesis Equilibrium Concentrations

Metathesis reactions are in general equilibrium reactions. Thus, in Q2, 2013 it was decided to investigate if an equilibrium state is reached. To do so, neat ethyl crotonate or ethyl acrylate was exposed to the maximum possible pressure, using our available gas tanks, of ethylene or propylene at room temperature in the presence of increasing amounts of metathesis catalyst. The results of these studies are summarized in Figure 14.

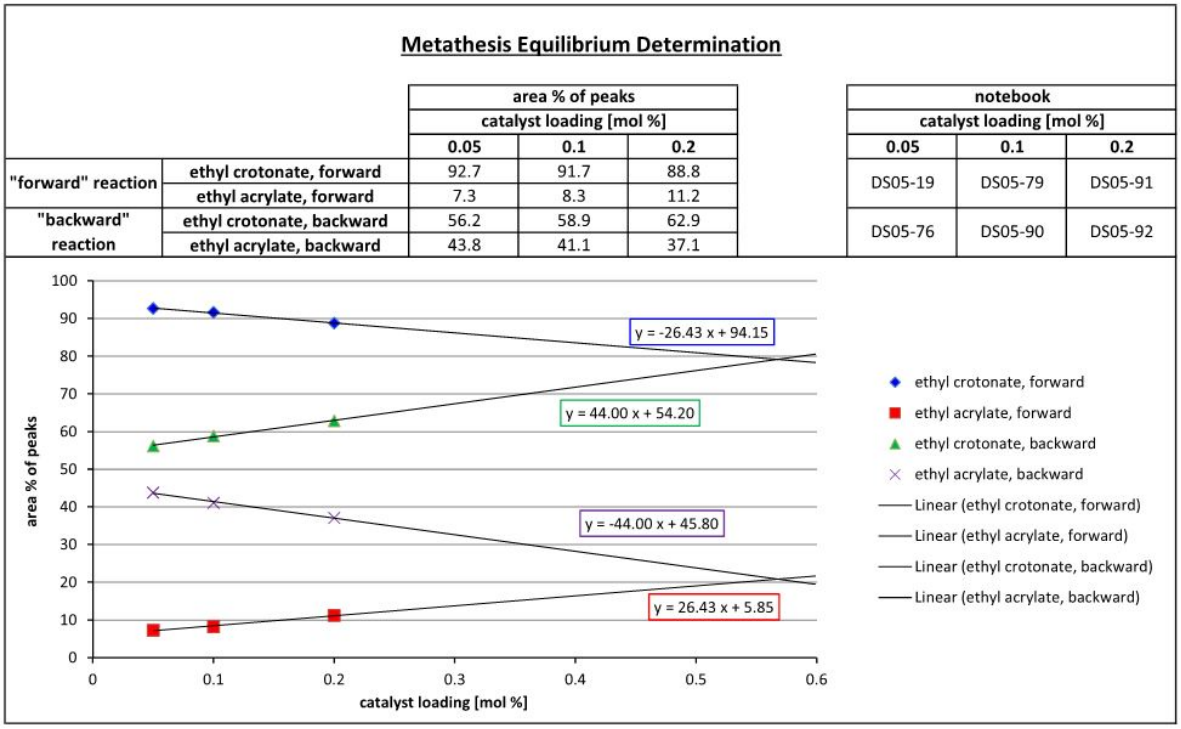


Figure 14. Results of metathesis equilibrium studies with ethyl-crotonate

As a result of these experiments it was determined that the forward reaction, ethyl crotonate + ethylene, and the reverse reaction, ethyl acrylate + propylene, converge on an identical product composition, proving that our metathesis is an equilibrium reaction. Calculations based on these data enabled us to determine that the equilibrium state contains 60.49 mol % of ethyl crotonate and 39.51 mol % of ethyl acrylate. This 3/2 ethyl crotonate to ethyl acrylate ratio represents the maximum achievable amount of ethyl acrylate via the reaction of ethyl crotonate with ethylene. In other words, in all cases separation of the ethyl acrylate produced from the reaction mixture containing an excess of ethyl crotonate will be necessary.

Metabolix previously proposed to conduct the *n*-butyl crotonate + propylene metathesis.[28] The advantage of this process is that the initial metathesis products are *n*-butyl acrylate and 2-butene, meaning that, the metathesis catalyst is never exposed to ethylene and is therefore able to maintain the high reaction rates and high yields needed for industrial biochemical processes. In Q3, 2013, we repeated the equilibrium studies previously

[28] van Walsem, J., et al., *Process for producing a monomer component from a genetically modified polyhydroxyalkanoate biomass.* US2012031681.

performed using ethyl crotonate with *n*-butyl crotonate ester and the results are shown in Figure 15. With the *n*-butyl ester it was again found that both the forward reaction, *n*-butyl crotonate + ethylene, and the reverse reaction, *n*-butyl acrylate + propylene, converge on an identical product composition, proving that the metathesis in the *n*-butyl ester system is also an equilibrium reaction.

Metathesis Equilibrium Determination - *n* -Butylesters

		area % of peaks catalyst loading [mol %]			notebook catalyst loading [mol %]		
		0.05	0.1	0.2	0.05	0.1	0.2
"forward" reaction	*n* -butyl crotonate, forward	89.8	86.7	84.6	DS06-24	DS06-25	DS06-26
	n -butyl acrylate, forward	10.2	13.3	15.4			
"backward" reaction	*n* -butyl crotonate, backward	54.0	60.1	66.2	DS06-28	DS06-29	DS06-30
	n -butyl acrylate, backward	46.0	39.9	33.8			

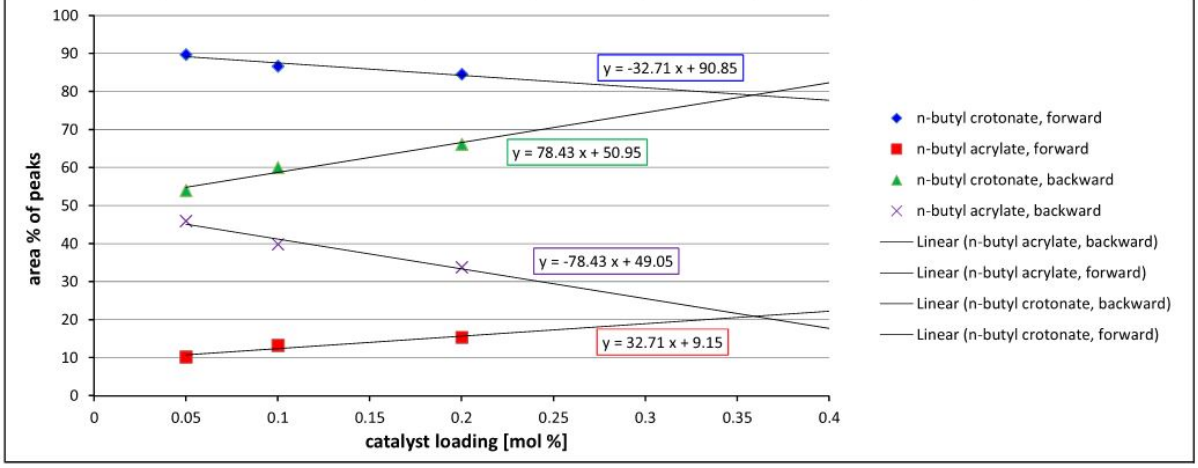


Figure 15. Results of Metathesis equilibrium studies with *n*-butyl crotonate

The equilibrium composition can be easily calculated from this data. Thus, the equilibrium state of our metathesis reaction contains 57.60 mol % of *n*-butyl crotonate and 42.40 mol % of *n*-butyl acrylate. This near 3/2 *n*-butyl crotonate to *n*-butyl acrylate ratio represents the maximum achievable amount of *n*-butyl acrylate via the reaction of *n*-butyl crotonate with ethylene. In other words, in all cases separation of the produced *n*-butyl acrylate from the reaction mixture containing an excess of *n*-butyl crotonate will be necessary. Interestingly, in comparison to the equilibrium state of the ethyl ester metathesis reaction (39.5 mol % ethyl acrylate), the equilibrium state of the *n*-butyl ester metathesis reaction (42.4 mol % *n*-butyl acrylate) is shifted by a small amount (2.9 mol %) towards the product side, indicative of a more hydrophobic environment in the *n*-butyl ester system.

Comparing the data previously acquired to determine the equilibrium state in the ethyl ester system with the data just acquired to determine the equilibrium state in the *n*-butyl ester system, one notices that a near identical equilibrium state is approached in both systems. However, in the *n*-butyl ester system more reactions occur prior to the catalyst losing activity, *i.e.*, the equilibrium is reached at an apparent catalyst loading of 0.359 mol % whereas in the ethyl ester system the equilibrium is reached at an apparent catalyst loading of 0.567 mol %. It is thus of interest to determine the time course of the ethylene

metathesis, *i.e.*, for how long conversion to product occurs before the catalyst loses activity (decomposes).

Time Course of the Ethylene Metathesis

In order to determine how long the reaction proceeds before the catalyst decomposes, time courses of the metathesis reactions with ethylene of both ethyl crotonate and *n*-butyl crotonate were investigated via a series of discrete/independent experiments. The alkyl crotonate containing a known amount of metathesis catalyst, 0.05 mol % HB, was stirred at room temperature and exposed for a given amount of time to a constant ethylene pressure (160 psi). The Parr reactor was then immediately opened and the composition of the reaction mixture was determined by HPLC. The results are presented in Figure 16. It was found that the ethylene metathesis with ethyl crotonate ceases within just 20 min, whereas the corresponding reaction with *n*-butyl crotonate occurs for up to 3 h. Ceasing of product formation, while the equilibrium has not been reached, is indicative of the catalyst having undergone decomposition. Since the HB catalyst is more stable in *n*-butyl crotonate than in ethyl crotonate, it can catalyze more reaction cycles in *n*-butyl crotonate (TON = 433 under our reaction conditions) than in ethyl crotonate (TON = 267) before it decomposes. The higher catalyst stability in *n*-butyl crotonate as the solvent, as compared to ethyl crotonate, is also evident in the equilibrium determination experiments previously discussed. In comparison, since methyl oleate is much more hydrophobic than *n*-butyl crotonate, even longer catalyst life times are realized in it, allowing the achievement of even higher ethylene metathesis TONs.[29,30,31,32]

Several additional metathesis catalysts were obtained towards the end of this Task in Q4, 2013 and screened using the optimum conditions established. Using *n*-butyl crotonate as the ethenolysis substrate, the temperature of the reaction in the presence of the maximum possible ethylene pressure (255 psi, as determined by our ethylene tank) was varied. At the reaction temperature achieving the maximum TON, the comparative ethenolyses of ethyl crotonate and ethyl cinnamate were then performed. TON's over 500 were achieved when using the n-butyl crotonate ester.

Key Accomplishments/Learnings and Publication of the Metathesis Work

- TONs increased by 2 orders of magnitude as compared to the literature.
- Maximum TONs in the 500s are observed for the ethenolysis of *n*-butyl crotonate, in the 300s for ethyl crotonate, and in the 100s for ethyl cinnamate.
- This is an equilibrium reaction.
- A commercial process must include a separation step.
- The TON limiting factor is the stability of the catalyst.

Metabolix clearly demonstrated the conversion of crotonic acid to acrylic acid via metathesis. The metathesis chemistry findings were published in a peer reviewed journal.[33]

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[29] *Organometallics* **2004**, *23*, 2027-2047. In this paper, the half-life time of Grubbs I catalyst in methyl oleate was determined to be just 10 min.

[30] *Organometallics* **2004**, *23*, 4824-4827.

[31] US 20100145086

[32] US 20120289729

[33] Schweitzer, D. and K.D. Snell, *Acrylates via metathesis of crotonates.* Org Process Res Dev, 2015. **19**, 715.

	Notebook							
	Time [h]							
	0.04	0.08	0.17	0.33	0.67	1.33	2.67	5.33
Ethyl Crotonate Reaction	DS06-19	DS06-08	DS06-09	DS06-10	DS06-11	DS06-12	DS06-13	DS06-14
n -Butyl Crotonate Reaction	DS06-23	DS06-15	DS06-16	DS06-17	DS06-18	DS06-21	DS06-20	DS06-22

Time Course of the Metathesis with Ethylene

		Time [h]							
		0.04	0.08	0.17	0.33	0.67	1.33	2.67	5.33
Ethyl Crotonate Reaction	Ethyl Acrylate [a %]	3.5	4.2	5.0	5.7	5.7	6.0	5.8	5.8
	Ethyl Crotonate [a %]	96.5	95.8	95.0	94.3	94.3	94.0	94.2	94.2
	TON	165	196	229	260	259	275	267	265
	% Conversion Ethyl Reaction	8.2	9.8	11.5	13.0	12.9	13.7	13.3	13.2
n -Butyl Crotonate Reaction	*n* -Butyl Acrylate [a %]	3.4	4.8	6.1	7.4	7.9	8.3	9.0	8.8
	n -Butyl Crotonate [a %]	96.6	95.2	93.9	92.6	92.1	91.7	91.0	91.2
	TON	180	247	306	365	387	403	433	425
	% Conversion *n* -Butyl Reaction	9.0	12.4	15.3	18.2	19.3	20.2	21.7	21.2

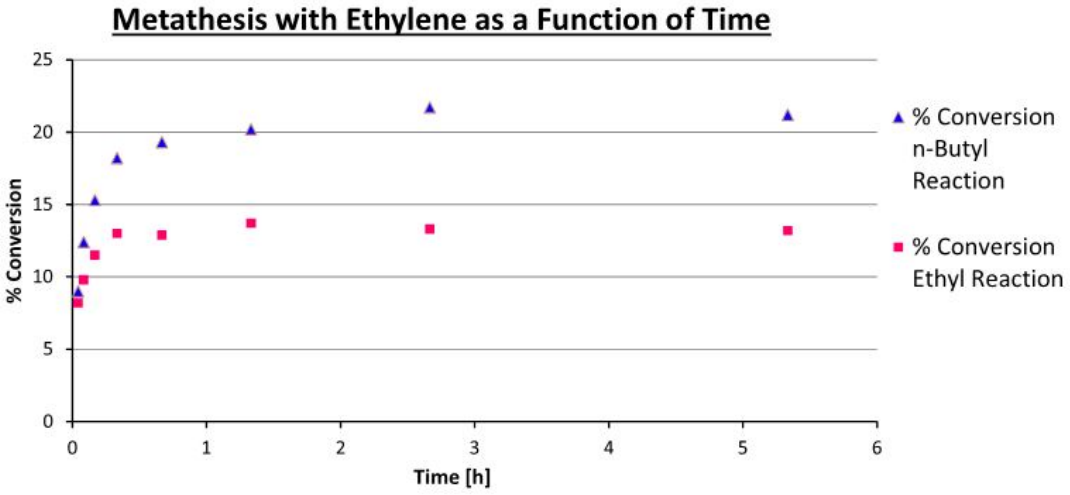

Figure 16. Results of Metathesis Using Ethyl and Butyl Derivatives

Metabolix does not have the in-house expertise to carry out sophisticated metathesis catalyst development and so this Task was considered complete. When the company achieves high levels of PHB in switchgrass resulting in the ability to produce low cost crotonic acid, we will then be in a position to collaborate with other company's having the necessary expertise.

Task C. Lifecycle Analysis of the REFABB Integrated Biorefinery

The life cycle analysis (LCA) was completed by Bruce Dale's group at Michigan State University and a final report was received. This study was based on the production of *n*-butanol from crotonic acid which we clearly demonstrated in Task B.2.1 *Develop technology to convert crotonic acid, Testing of hydrogenation catalysts.* The goal of this life cycle assessment (LCA) study was to estimate cradle to gate greenhouse gas (GHG) emissions associated with *n*-butanol derived from P3HB in genetically-engineered

switchgrass. The functional unit is defined as one kg of n-butanol. The system boundary is illustrated in Figure 17, including genetically-engineered switchgrass production in the United States, baled switchgrass storage, transportation of baled switchgrass, the thermolysis/crotonic acid recovery facility, the co-product upgrading system, avoided systems for co-products, transportation of crotonic acid, the *n*-butanol conversion facility, and upstream processes. The co-product upgrading system is the hydroprocessing facility for thermolysis oil. The upstream processes are material and fuel production systems (e.g., nitrogen fertilizer, diesel, natural gas, electricity, etc.). The avoided systems are coal displaced by biochar in a coal-fired power plant (including both coal production and combustion), and petroleum-based liquid fuel displaced by renewable liquid fuel derived from thermolysis oil (including production and use). The allocations between crotonic acid and co-products (i.e., biochar and thermolysis oil) in the thermolysis/ crotonic acid recovery facility are done by the "displacement" method[34], which includes all product systems displaced by co-products (avoided systems) in the system boundary.

The process is currently being developed, and only mass balance information is available at this time. Energy consumption in the thermolysis/crotonic acid recovery and the *n*-butanol conversion facilities is estimated based on literature and Aspen Plus simulations. Agronomic inputs and fuel consumption in switchgrass production are obtained from literature. Life cycle inventory data for the upstream processes are obtained from the GREET model[34] and LCA databases.[35,36]

Greenhouse gas (GHG) emissions include carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), and CO_2 converted from carbon monoxide and VOCs (volatile organic compounds) in the atmosphere. Carbon dioxide emissions associated with soil carbon sequestration are included in the GHG calculations. Soil N_2O emissions during switchgrass production, which are estimated based on the GREET model,[34] are also included. Carbon dioxide releases from urea applied during switchgrass production are also included. Greenhouse gas emissions associated with indirect land use change (iLUC) are not included in the GHG calculations. The US Billion-Ton Update report[37] shows that indirect land use change associated with cellulosic biomass production can be minimized by intensification of grassland-based biomass production, such as switchgrass. This system is based on switchgrass and thus indirect land use change and the hypothetical associated greenhouse gas emissions are neglected. The 100-year time horizon global warming potentials are used in the GHG calculations. The methodologies of life cycle assessment used in this project follow ISO standards.[38,39]

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[34] Argonne National Laboratory. Greenhouse Gases, Regulated Emissions, and Energy Use in Transportation (GREET) Computer Model. Argonne National Laboratory, US Dept of Energy, Argonne, IL. 2014.

[35] National Renewable Energy Laboratory, U.S. Life-Cycle Inventory (LCI) Database. https://www.lcacommons.gov/nrel/search

[36] Swiss Centre for Life Cycle Inventories, Ecoinvent database, Switzerland

[37] U.S. DOE, U.S. billion-ton update: supply for a bioenergy and bioproducts industry, ORNL/TM-2011/224, United States Department of Energy and Oak Ridge National Laboratory, Oak Ridge, TN, pp. 227 (2011).

[38] International Organization for Standardization, ISO 14040: Environmental Management – Life Cycle Assessment – Principles and Framework. 2006.

[39] International Organization for Standardization, ISO 14044: Environmental Management – Life Cycle Assessment – requirements and guidelines. 2006.

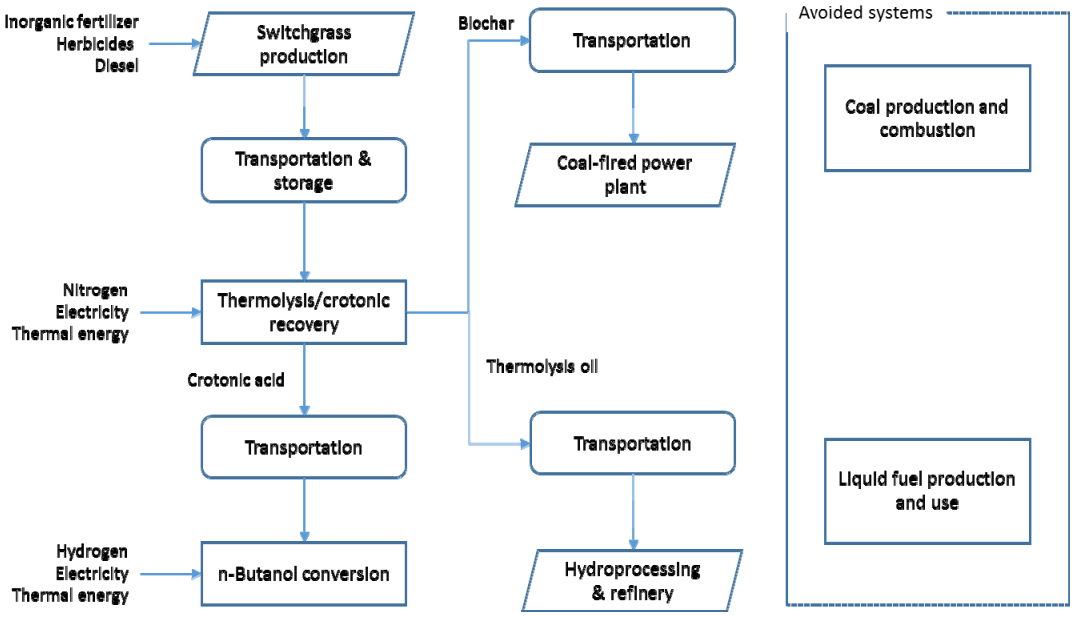

Figure 17. System boundary

Co-products (i.e., uncondensed gas, thermolysis oil and biochar) in the thermolysis/crotonic acid recovery facility can be used to provide thermal energy. Three energy scenarios were investigated. In scenario A, uncondensed process gases and natural gas are used to provide thermal energy in the thermolysis/crotonic acid recovery facility, while in scenario B uncondensed gas and thermolysis oil are used as thermal energy sources. Uncondensed gas and biochar provide thermal energy in scenario C.

The median GHG emissions of *n*-butanol derived from P3HB in genetically-engineered switchgrass are negative regardless of the thermal energy sources used in the thermolysis/crotonic acid recovery facility (see Table 8 and Figure18). GHG emissions of *n*-butanol derived from P3HB in genetically-engineered switchgrass in scenario C is higher than those in other two scenarios. This occurs because using biochar as a thermal energy source in the thermolysis/crotonic acid recovery facility greatly reduces GHG credits associated with coal displaced by biochar in a coal-fired power plant. GHG emissions of *n*-butanol within the 95% confidence interval (error bars) in scenario B are less than those of *n*-butanol derived either from corn grain or from petroleum.

Table 8. Arithmetic mean, median and standard deviation for GHG emissions of n-butanol (kg CO_2 eq./kg)

	Scenario A	Scenario B	Scenario C
Median	-1.83	-3.05	-0.83
Arithmetic mean	-2.12	-3.36	-1.17
Standard Deviation	3.27	3.25	3.13

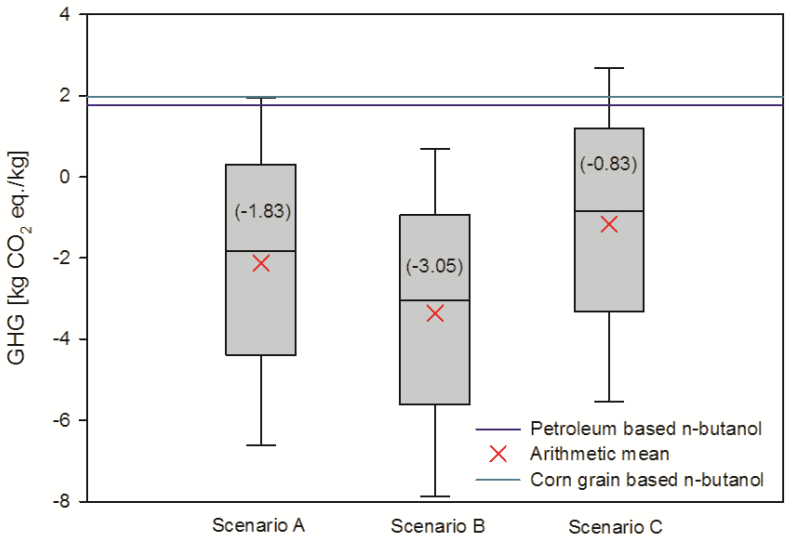

Figure 18. GHG emissions of *n*-butanol. The bottom and top of the box are the first and third quartiles, and the horizontal line inside the box is the median. The cross is the arithmetic mean. The ends of the whiskers represent 5th (lower) and 95th (upper) percentile. The values show the median with number of datasets in parentheses.

According to Monte Carlo simulations, the following factors should be considered as key GHG determinants as pilot-scale process development proceeds: 1) recycling of N_2 carrier gas, 2) the thermal energy source, 3) energy consumption for crotonic acid purification in the thermolysis/crotonic acid recovery facility. When the N_2 carrier gas is not recycled, GHG emissions of *n*-butanol are much larger than those for petroleum-based *n*-butanol. More than 47% of N_2 carrier gas (equivalent to < 1.17 kg per Mg of dry switchgrass+P3HB for make-up N_2) should be recycled so that GHG emissions of *n*-butanol derived from P3HB in genetically-engineered switchgrass are less than those of petroleum based *n*-butanol. When biochar displaces coal in a coal-fired power plant and thermolysis oil is used in producing liquid fuels, GHG credits of biochar (75 g CO_2 equivalent/MJ of biochar) are much larger than those of thermolysis oil (26 g CO_2 equivalent/MJ of thermolysis). From a global warming perspective, thermolysis oil has more impact as a thermal energy source in the thermolysis/crotonic acid recovery facility than biochar. A separation technology for thermolysis oil and crotonic acid should consume thermal energy at a rate of less than 1.83 MJ/kg of thermolysis oil and crotonic acid in scenario A, 6.27 MJ/kg in scenario B, and 1.02 MJ/kg in scenario C so that GHG emissions of *n*-butanol derived from P3HB in genetically-engineered switchgrass are less than those of petroleum based *n*-butanol.

Since chemical processes in the *n*-butanol production system are not fully developed at pilot scale, most process information is estimated based on literature and Aspen Plus simulations. When the process is fully developed, the process information may be quite different from the information used in this study. LCA results in this study are therefore preliminary and can be used as guidelines for process improvement efforts. At this point, the preliminary LCA results would be appropriate for internal use, but probably not for

external uses such as communications with the third parties (e.g., consumers, NGO, etc.). If the results are used for external communications, the limitations and uncertainties should be fully disclosed.

The important findings from this LCA study are:

- Based on the best available information at this point, GHG emissions of *n*-butanol derived from P3HB in genetically-engineered switchgrass are less than those of *n*-butanol derived either from corn grain or from petroleum.
- Make-up nitrogen carrier gas should be less than 1.17 kg per Mg of dry switchgrass+P3HB so that GHG emissions of *n*-butanol derived from P3HB in genetically-engineered switchgrass are less than those of petroleum based *n*-butanol.
- From a global warming perspective, thermolysis oil is more appropriate as a thermal energy source in the thermolysis/crotonic acid recovery facility than is biochar.
- A separation technology for thermolysis oil and crotonic acid should consume thermal energy at a rate of less than 1.83 MJ/kg of thermolysis oil and crotonic acid in scenario A, 6.27 MJ/kg in scenario B, and 1.02 MJ/kg in scenario C.

4. Products Developed Under Award

a. Publications

1. "Acrylates via metathesis of crotonates." Schweitzer, D., Snell, K. D., **2015**, *Organic Process Research and Development*, 19, 715. This article is part of the Sustainable Chemistry special issue.

2. "Bio-based *n*-butanol prepared from poly-3-hydroxybutyrate: optimization of the reduction of *n*-butyl crotonate to n-butanol." Schweitzer, D., Mullen, C. A., Boateng, A. A., Snell, K. D., **2015**, *Organic Process Research and Development*, 19, 710. This article is part of the Sustainable Chemistry special issue.

3. "Transgene autoexcision in switchgrass pollen mediated by the Bxb1 recombinase." Somleva, M. N., Xu, C. A., Ryan, K. P., Thilmony, R., Peoples, O., Snell, K. D., Thomson, J., **2014**, *BMC Biotechnology*, 14, 79.

4. "Mild Pyrolysis of P3HB/Switchgrass Blends for the Production of Bio-oil Enriched with Crotonic Acid." Mullen, C. A., Boateng, A. A., Schweitzer, D., Sparks, K., and Snell, K. **2014,** *Journal of Analytcial and Applied Pyrolysis,* 107, 40-45.

b. Web site or other Internet sites that reflect the results of this project

1. Write up describing technology in MIT Technology Review https://www.technologyreview.com/s/515486/plastic-from-grass/ "Plastic from Grass" *MIT Technology Review*, **2013**, *116 (4)*, p. 84-87.

c. Networks or collaborations fostered;

d. Technologies/Techniques;

e. Inventions/Patent Applications, licensing agreements

1. A utility patent application entitled *Transcriptional Regulation for Improved Plant Productivity* (Inventors Madana M.R. Ambavaran and Mariya Somleva; PCT WO/2014/100289) was filed and reported to DOE (S-131,972).
2. A provisional patent application entitled *In Planta Transformation of Grasses* (Inventors Kristi D. Snell, Mariya Somleva, and Aminat Ali) was filed and reported to DOE (S-130,899). This provisional application was later abandoned.

f. Other products