Amyris, Inc.
Integrated Biorefinery
Project Summary

December 28, 2009 – June 30, 2013
Final Report – Public Version

Amyris IBR Final Report

Award No. EE0002869

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Glossary of Terms

ACF – aerobic constant feed
AFRL – Air Force Research Laboratory
ASME – American Society of Mechanical Engineers
ASTM – American Society for Testing and Materials
BAAQMD – Bay Area Air Quality Management District
BHP – boiler horsepower
BSM – basal salts media
BTU – British thermal unit
CARB – California Air Resources Board
CAS – Chemical Abstract Service
CCB – concentrated-cell-broth
CEQA – California Environmental Quality Act
cFAD – condensed fill and draw
CFD – computational fluid dynamics
CFSC – constant feed with spike checks
CIP – clean-in-place
CLSI – Clinical Laboratory Standards Institute
CMO – contract manufacturing organization
COD – chemical oxygen demand
CODF – chemical oxygen demand filtered
cu ft – cubic feet
DMM – dimethyl maleate
dO2 – dissolved oxygen
DOT – Department of Transportation
DSI – direct steam injection
EA – environmental assessment
EC – European Commission
ECHA – European Chemicals Agency
EIR – Environmental Impact Report
EIS – Environmental Impact Statement
EPA – Environmental Protection Agency
ETMA – Emeryville Transportation Management Association
FAD – fill and draw
FDU – flash distillation unit
FM – factory mutual
GC – gas chromatograph
GC-FID – gas chromatography with flame ionization detector
gal – gallon
GLSP – good large scalable practices
GMM – genetically modified microbe
GMO – genetically modified organism

GPM – gallons per minute
HMF – hydroxyl-methyl furfural
HPBL – human peripheral blood lymphocytes
HTST – high-temperature short-time
ICP – inductively coupled plasma
IPC – in-process control
IUPAC – International Union of Pure and Applied Chemistry
kg - kilograms
kW – kilowatts
kWh – kilowatt-hour
L/L – liquid-liquid
L/S – liquid-solid
lbs – pounds
LCA – life cycle analysis
LMH – $L/m^2/hr$
LOEC – lowest-observed-effect-concentration
LoQ – limit of quantitation
MAPF – micro aerobic pulse feed
MAPFx – adaptive micro aerobic pulse feed
MATC – maximum acceptable toxicant concentration
MBH – thousands of BTUs per hour
MCAN – microbial commercial activity notice
mEq – milliequivalents
MSDS – material safety data sheet
mT – metric tons
NASA – National Aeronautical and Space Administration
NEPA – National Environmental Policy Act
NFPA – National Fire Protection Association
NIH – National Institutes of Health
NOEC – no-observed-effect concentration
NOx – oxides of nitrogen
OCSPP – Office of Chemical Safety and Pollution Prevention
OEM – original equipment manufacturer
OECD – Organisation for Economic Co-operation and Development
OLR – organic loading rate
OPPTS – Office of Prevention, Pesticides & Toxic Substances
OSHA – Occupational Safety and Health Administration
OTFF – Old Town Fuel and Fiber
OTR – oxygen transfer rate
OUR – oxygen uptake rate
PCV – packed cell volume
PM – particulate matter
PMN – pre manufacture notice
PMP – project management plan

PPE – personal protective equipment
psig – pounds per square inch [gauge]
PVC – polyvinyl chloride
QC – quality control
RCRA – Resource Conservation and Recovery Act
REACh – Registration, Evaluation, Authorisation and Restriction of Chemicals
RFS – Renewable Fuel Standard
RODI – reverse osmosis/de-ionized
RSB – Roundtable for Sustainable Biofuels
RSD – relative standard deviation
SAT – site acceptance testing
SCFM – standard cubic feet per minute
SIM – selected ion monitoring
SLPH - standard liters per hour
SMR – steam methane reforming
SOP – standard operating procedure
SSIBR – sweet sorghum integrated bio-refinery
TFF – tangential cross flow filtration
TRS – total reducing sugar
TSDF – treatment, storage, and disposal facility
TSCA – Toxic Substance Control Act
TSS – total suspended solids
UASB – upflow anaerobic sludge blanket
ULSD – ultra low sulfur diesel
VOCs – volatile organic compounds
WAF – water accommodated fraction
WFE – wiped film evaporator

Summary

Scale-up and Mobilization of Renewable Diesel and Chemical Production from Common Intermediate using US-based Fermentable Sugar Feedstocks

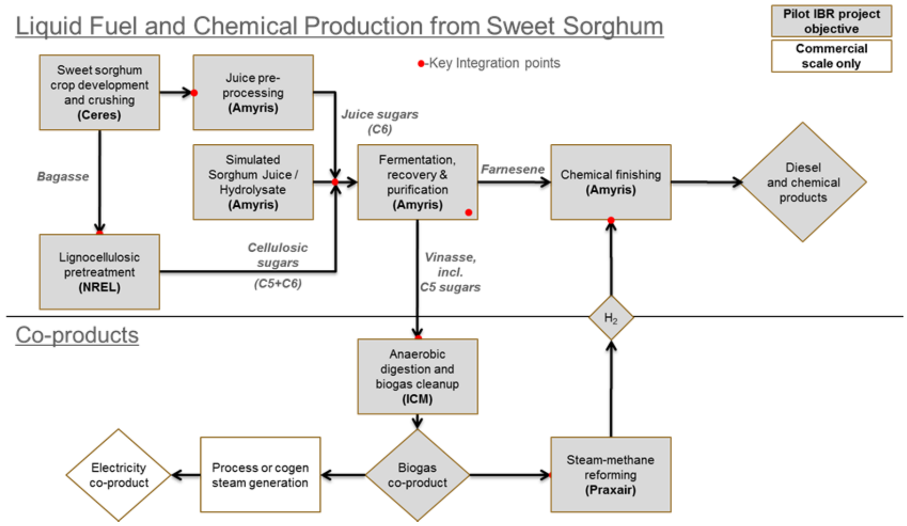
Award Number:	EE0002869
Name of Recipient:	Amyris, Inc.
Project Title:	Scale-up and Mobilization of Renewable Diesel and Chemical Production from Common Intermediate using US-based Fermentable Sugar Feedstocks
Principal Investigator:	Joel Cherry
Project Director:	David Gray
Project Partners:	Ceres, ICM, NREL, Praxair
Report Date:	September 28, 2013
Project Period:	January 10, 2010 – June 30, 2013

Executive Summary

The intent of this project was to demonstrate the production of No Compromise® renewable diesel fuel and petrochemical substitutes using high-impact biomass feedstocks. Amyris currently produces an isoprenoid called farnesene, a 15-carbon branched-chain alkene, using fermentation of glucose or sucrose syrups derived from corn, sugar beet, or sugarcane. The farnesene is then hydrogenated to produce Amyris renewable diesel, or otherwise chemically converted to produce a variety of products such as lubricants, plasticizers, or polymers. In the Amyris Integrated Biorefinery Project, we successfully demonstrated that the fermentation process could be adapted to utilize glucose derived from high-impact feedstocks to produce farnesene, and the resulting farnesene could then be hydrogenated to farnesane for use as a diesel fuel, or chemically converted to a plasticizer for use in the plastics industry. Both products were indistinguishable from those produced from sucrose or glucose syrups in testing both in-house and customer trials. The work performed in this project provided valuable data for robust ongoing techno-economic analysis (TEA), regulatory approvals, and a conceptual design for demonstration- and commercial-scale manufacturing facilities. The project finished within budget and achieved all major objectives with the exception of initiating commercial operations in the United States. Completion of the project was delayed by one year due to circumstances beyond Amyris's control including delays in equipment, feedstock delivery and processing, and associated risk mitigation activities to address the delays. Even with the delays, Amyris was able to manage the project to successful completion as determined by the DOE.

The key to the success of the IBR project was the collaboration with project partners as shown in Figure 1 below.

Figure 1: Integration of activity within the Amyris IBR Program



Process objectives and definition of project generation are shown below in Figure 2.

Figure 2: Objectives for Amyris IBR Project

Amyris IBR process objectives

	0th gen (current)	1st gen	2nd gen	3rd gen
Juice C6 sugars	Fermented	Fermented	Fermented	Fermented
Cellulosic C6 sugars	Electricity	Fermented	Fermented	Fermented
Cellulosic C5 sugars	Electricity	Biogas (Electricity)	Hydrogen (Product)	Fermented
Lignin	Electricity	Electricity	Electricity	Electricity
Technology required	Sorghum fermentation	Pretreatment & anaerobic digestion	Steam methane reforming of biogas	Strain to utilize C5 sugars
Est. fuel yield	600 gal/acre	1200 gal/acre	1200 gal/acre	1500 gal/acre

Potential technical evolution for Amyris Diesel production in US using sweet sorghum

Task A: Pilot plant upgrades and operations

The Amyris pilot plant launched initial operations in October 2008, housing two 300L fermentors along with associated process equipment, for evaluating Amyris yeast strains and process technology at a scale that provided manufacturing relevant information. In December 2009, Amyris was awarded an IBR cooperative agreement for the purpose of upgrading the pilot plant facility to double the fermentation capacity and enable evaluation of domestic feedstocks for the production of advanced renewable diesel fuel.

Budget period 1 of the project was focused on refining scope, specifying additional equipment requirements, and obtaining permits for the plant expansion. During this design phase, the Amyris pilot plant continued to operate at its existing capacity using defined media and real-world feedstocks, such as cane syrup and molasses.

The second budget period was focused on the expansion of the pilot plant and was completed on time and without interrupting the ongoing operations. The utility connections for new equipment were installed to enable rapid start-up after delivery of new equipment. The two additional fermentors were operational by February 2011, and the flash distillation and chemistry skids followed in the later part of 2011. Batches of sorghum syrup and lignocellulosic derived sugars were shipped to the plant and tested both before and after the completion of the expansion. The sorghum syrup performance test was completed in February 2012, and the lignocellulosic performance testing was completed in April 2013.

Task B: Scale-up and economic analysis of Amyris diesel production from sweet sorghum

This task focused on the production of biodiesel from lignocellulosic feedstocks and the evaluation of sweet sorghum-derived sugars as a fermentation feedstock for Amyris's yeast-based farnesene production process. Ceres performed an on-farm trial of sweet sorghum across four geographies and harvested both juice and sorghum bagasse throughout the growing seasons to determine the agronomic performance of sweet sorghum in the various geographies. In addition, sweet sorghum syrup was provided for testing at the Amyris pilot plant facility, and sorghum bagasse samples were supplied to NREL (Golden, Colorado) for evaluation and biochemical conversion into fermentable sugar. The agronomic data showed the potential to grow sweet sorghum at similar yields to sugarcane.

At Amyris, both sorghum syrup and lignocellulosic sugar feedstocks were evaluated in lab and pilot scale fermentations. The lab scale evaluations were able to identify some sugar field harvesting treatments that could negatively impact fermentation performance. Lab fermentations demonstrated that improved process yield and productivity could be achieved through fermentation strain development, fermentation process development, and feedstock choices. Sorghum syrup performed very similar to previous tests conducted using sugarcane syrup, with yield and productivity values being indistinguishable, however, the titer observed was lower due to the more dilute sugar content in the sorghum syrup.

The development of the sorghum bagasse biochemical conversion process identified the need to adjust the processing of sorghum to reduce residual sugar in the bagasse. When attempting to obtain sugars from bagasse like materials, special operational attention for feeding the biomass to the pretreatment reactor was required to avoid seizure and breaking of the scroll delivery system. The strategy selected to produce bagasse derived sugars was to operate the pretreatment reactor at a high solids loading for acid or non-acid modes. Enzymatic digestion was performed at a high loading and was not optimized for this project. The sorghum bagasse sugars were evaluated in the lab fermentors and it was noted that the sugar obtained from the high temperature pretreatments and lengthy post evaporation processing stages contained some inhibitors that caused fermentations to stall. After investigating, it was determined that the furfural, hydroxyl methyl furfural, or acetate levels in the feedstock were not responsible for this inhibition. More importantly, it was further determined that the aerobic fermentation method used is capable of assimilating those components and are hence not accumulated throughout the fed batch duration. Dilution of the sorghum bagasse sugar feeds reversed this inhibition in the lab fermentor tests. The typical inhibitors such as furfural and acetate were not at levels that would cause inhibition and therefore it is probable that the atypical inhibitor(s) originated from the lignin material during pretreatment and may be a phenolic component. We were not able to identify the specific inhibitor.

Wood chip and wheat straw lignocellulosic sugars were evaluated in the Pilot Plant. The wheat straw sugar concentration was much lower than either the sorghum bagasse or the wood chip material and reduced the yield and productivity of the fermentation relative to a standard 650g/L dextrose feed fermentation. The wood chip derived sugar was very clean and performed well in the pilot scale process, showing yield and productivity closer to the results obtained with sorghum syrup or dextrose fed fermentations. The crude farnesene obtained from the pilot scale fermentations was successfully converted to farnesane and exceeded the quality specifications for purity (>93% by weight). The wood chip derived sugar feedstock performed well in fermentation and was slightly better than wheat straw in the recovery yields. The supplementation of the wood chip feedstock with sorghum syrup (30% volume wood chip, 70% volume sorghum syrup) performed well in fermentation and recovery. The process performance evaluations were completed using sorghum syrup, wood chip lignocellulosic sugar, wheat straw lignocellulosic sugar, and a blend of wood chip sugar and sorghum syrup. Unit operations from sugar sterilization through to hydrogenation of distilled farnesene to produce the fuel product farnesane were examined. Improved strains, process strategies, and feedstock all influence the yield, productivity and titer in fermentation. Fermentation productivity and titer improvements provide advantages in downstream product recovery. The strain used in the IBR program does not consume the C5 sugar.

Task C: Development and scale-up of value-added products from diesel fermentation intermediate

The purpose of this task was to develop a chemical product which would make use of the same fermentation technology developed for our fuels production. Initially, Task C explored multiple candidate chemical products including lubricants, surfactants, and polymers. After thorough analysis of the various candidate chemicals, plasticizers were selected as the product to move forward with a full analysis.

A majority of the scope of work presented in previous sections resulted from evaluations of the Diels Alder reaction to produce the Plasticizer candidate molecule 4-(4, 8-dimethylnonyl)cyclohexane-1,2-dicarboxylic acid dimethyl ester from fermentation derived Amyris farnesene. Because of the lack of volume efficiency due to solvent dilution and the long cycle time, process development was concentrated on the Diels-Alder reaction. This was seen as an area of the process that could provide the greatest return on resource investment with regard to reducing cost. Eliminating the solvent dilution for this reaction eliminated the distillation necessary to remove the solvent and eliminated the solvent waste stream. Understanding the relationship of temperature and farnesene addition rate to the rate of converting farnesene to product rather than side-products were key variables that could be optimized to produce product within specification without further processing. Further optimization of these key variables also led to a reduction in cycle time which can significantly reduce cost on a manufacturing scale. The initial process development objective was to produce Flexasane 1011, the hydrogenated product. Testing showed both Flexasene 1010 (non-hydrogenated molecule) and Flexasane 1011 (hydrogenated molecule), as plasticizers for PVC products, were comparable so the target candidate became Flexasene 1010 and the hydrogenation step was eliminated. This was the product that was scaled to 1.5 mT at a contract manufacturing organization (CMO) proving scalability and the feasibility of a scalable process from a diesel fermentation intermediate to produce a higher value product.

The optimized process is an undiluted, single vessel Diels-Alder reaction with farnesene and dimethyl maleate (DMM) at 180 °C to produce product that meets specifications. This process was used to test the performance of alternative feedstock sources such as sorghum syrup, wheat straw and woodchip. The results of using farnesene from these alternative feedstocks in the optimized process show that there is no significant difference with regard to yield and quality of product. These results indicate feedstock flexibility with regard to the farnesene produced as a diesel fermentation intermediate for chemical processing. The economic feasibility and analysis of this optimized process was included in the TEA assessment.

This developed process (detailed in Task C) demonstrated that the Amyris yeast platform enabled production of an alternative chemical feedstock, in this case a plasticizer molecule. The process that was developed scaled successfully, demonstrating that Amyris's bio-derived diesel intermediate provides another path for renewable products in addition to advanced biofuel products.

Task D: Regulatory and end-user process and product acceptance

Regulatory and end-user acceptance of Amyris diesel and chemical products is a crucial step in the path to market acceptance, thus Task D was solely focused on this activity. With the exception of lifecycle analysis (LCA) certification and commercial approvals, all diesel fuel and chemical products objectives were achieved. During the course of the project, Amyris acquired extensive physical-chemical properties and toxicological data with production representative materials, secured significant OEM data contributions, engaged US military testing, and achieved both commercial and military end-user validations. The project also achieved significant progress towards ASTM validation for farnesane as an aviation turbine fuel blending component. DOE funding and collaboration provided the necessary confidence for acquiring OEM, military, and end-user resource commitments. Such commitments resulted in new chemical substance registrations (US and Europe), further federal and California regulatory approvals, global OEM validation and diesel engine warranty coverage, US military renewable (and high purity) fuel opportunities, and pipeline distribution product code and specifications. These achievements are essential tools for ensuring successful commercial introduction, contributing to US energy independence (diesel and aviation turbine fuels), and developing of new market opportunities (e.g. US military fuels, aviation turbine fuel and specialty chemicals).

Task E: Project Management and Reporting

Tasks A through D formed the basis of a detailed project management plan (PMP) with defined sub-tasks, deliverables and decision points, each associated with a timeline. The project was tracked using the Earned Value Management System with % completion of each task as a performance-to-plan metric. Budgetary, feedstock supply, and technical risk were all addressed in the planning process.

Major accomplishments

Task A: Pilot plant upgrades and operations

Amyris completed an expansion of its pilot plant facility in Emeryville, California to facilitate fermentation of sugar streams resulting from processing of biomass at a rate of one ton per day. In addition, a chemical skid was installed to allow pilot-scale demonstration of the conversion of farnesene, the primary fermentation product, to diesel and a plasticizer molecule. All work on the planning, permitting, facility modification, and installation was initiated in January of 2010 and completed in September of 2012.

The National Environmental Policy Act (NEPA) documentation for budget phases 1 and 2 was completed in February 2010. A Risk Management Plan was developed and finalized in April 2010. During the pre-construction phase, existing pilot plant operations were up and running to evaluate defined and cane syrup feedstocks from fermentation through to farnesane. Specifications for the anticipated new equipment were finalized and equipment ordered with the objective of minimizing down time while taking into account the delivery lead time. As anticipated, there was little or no downtime of ongoing pilot operations during upgrade design and installation.

The expansion of the pilot plant capacity and capabilities required construction to modify existing space and some remodeling of an adjacent space to house expanded media sterilization equipment. The ordering of new equipment was completed in November 2010. The expansion area construction work was completed on time in July 2010, enabling movement of the high-temperature short-time (HTST) sterilizer out of the fermentation hall and into the dedicated expansion area by September 2010. The remaining fermentation laboratory construction was completed in September 2010. The completion of the chemistry area, which mainly involved constructing enclosures around existing open space, was deferred until the arrival of the distillation and chemistry skids to facilitate customization. Following this approach, the chemistry construction completed in August 2011. Commissioning of the two additional fermentors was completed in February 2011 after resolution of site acceptance testing (SAT) identified modifications to the exhaust filters and side wall ports. The chemistry skids were in service December 2011. The new direct steam sterilizer (Barnum) was operational in September 2012.

As a test of the expanded facility, sorghum syrup was evaluated at pilot scale in 2011 across all unit operations. A performance protocol was completed in February 2012, evaluating the unit operations from sorghum sugar treatment through to production of biodiesel farnesane and plasticizer molecule flexasane. A performance evaluation of the lignocellulosic feedstock based process unit operations was completed in April 2012.

Task B: Scale-up and economic analysis of Amyris diesel production from sweet sorghum

Sweet sorghum hybrids were evaluated by partner Ceres Inc. in four geographies to evaluate the crop productivity. The south east plantings (Florida, Tennessee, and Alabama) provided consistent yields across the planting windows while the Hawaii plantings showed the yield decreasing with later plantings. One Hybrid (3) appeared to produce better consistency of yield in the staged Hawaii planting test. Larger scale planting and syrup supply was completed at the Florida and Tennessee locations. The 2010 Tennessee yield and supply was the highest of all geographies and served as the source of test sorghum for Pilot Plant fermentation runs. Bagasse production was successful with high yields being obtained in the Tennessee 2010 field trial, supplying approximately 6 dry mT of bagasse to NREL for evaluation and biochemical conversion into hydrolysate for fermentation testing.

Pilot scale fermentations were operated using defined and complex feedstocks. Sorghum syrup evaluations occurred during 2010 and 2011. Lab evaluation of bagasse and other lignocellulosic feedstocks was followed by pilot scale evaluation of wheat straw and wood chip-derived lignocellulosic sugars. The fermentation strain and methods employed enabled the consumption of common by-products of lignocellulosic pretreatment such as acetate. Yield, productivity, titer measurement, and organic acid analytical procedures were established in-house. Less frequently required specialized assays, such as metal ICP (inductively coupled plasma), were outsourced.

Vinasse samples obtained from pilot plant processes were delivered to the project contactor ICM for evaluation as substrates for anaerobic digestion. Samples tested were derived from a cane syrup and wood chip feedstock process.

The crude farnesene product was recovered from pilot scale fermentations using a solid liquid centrifugation step, a de-emulsification step, and a liquid –liquid centrifugation to produce crude farnesene. The crude farnesene was flash distilled and then hydrogenated to the final fuel product farnesane. Farnesane for regulatory and engine performance testing was produced at a contract manufacturing facility (60,000 liter scale) using the processes and procedures developed at pilot scale.

A comprehensive technoeconomic analysis was undertaken utilizing model input parameters, such as feedstock composition, process yields, raw material unit costs, and purchased capital cost information collated from various partners to illustrate the production costs associated with the proposed sweet sorghum IBR. The combined information was synthesized in a thermodynamic process simulation tool (ASPEN Plus) and production costs and sensitivity effects were calculated. The Nth plant production costs reveal the potential for profitable and cost competitive renewable diesel pricing.

Task C: Development and scale-up of value-added products from diesel fermentation intermediate

Process development to perform the conversion of farnesene to the target plasticizer molecule, flexasene, was completed at both lab and demonstration scale. Laboratory scale process development was performed in the Amyris chemistry labs and successfully transferred to pilot scale using the installed chemical skid at the Amyris pilot plant. The pilot plant run produced kilogram quantities of the desired product. Material from this production was characterized, and provided the basis for determining product specifications. Two batches of 1.5 mT each were produced during a Flexasene 1010 campaign at a CMO after transferring the process from pilot scale runs. The campaign was successful, producing product that met specifications without further processing. The technoeconomics were derived from the optimized reaction to meet the piloting goals.

Task D: Regulatory and end-user process and product acceptance

The project collected significant data in terms of quality and quantity required to support further fuel registrations, new chemical registrations, end-user acceptance, and Original Equipment Manufacturer (OEM) validation of diesel and jet fuel. Operational data was also collected supporting efforts to obtain engine OEM commercial warranty coverage. Data collection was on neat (100%) Amyris Diesel as required by the OEMs. The project acquired extensive data verifying real-world fuel storage, transport, distribution, and application.

The project achieved a completed primary LCA for diesel fuel under the Environmental Protection Agency (EPA) Renewable Fuel Standard (RFS) II and California Air Resources Board (CARB) protocols. The file is ready for submission to EPA for certification. Assessment activities are ongoing with the international Roundtable for Sustainable Biofuels (RSB). RSB is a recognized global sustainability auditing and certification body. The purpose of this activity is to certify by independent body the Amyris sugar pathway. The activity will be completed post IBR project timeline.

The projected acquired extensive data from OEM engagement including computational fluid dynamics (CFD) modeling, combustion and kinetic modeling, spray visualization, engine bench and vehicle chassis testing, and on-highway validation.

The project completed three (3) comprehensive US on-highway demonstrations involving major OEMs, namely Cummins, Volkswagen, and Navistar. The demonstrations included heavy-duty Kenworth trucks operated by FedEx, a medium-duty diesel-hybrid commercial truck operated by Sierra Nevada Brewery, a medium-duty regional transit bus operated by Emeryville Transportation Management Association (ETMA), and light-duty passenger vehicles provided by Volkswagen and operated by Amyris. The programs confirmed drop-in compatibility in terms of engine performance, vehicle reliability, fuel economy, fueling, fuel storage, and emissions (exhaust and evaporative) levels.

A key to successful commercialization of farnesene as a diesel fuel and farnesene as a chemical feedstock is regulatory approval under U.S.Toxic Substance Control Act (TSCA) and Europe's Registration, Evaluation, Authorisation and Restriction of Chemicals (REACh), and CARB. The project acquired data justifying a substantially similar finding for farnesene as a listed TSCA chemical and data supporting a farnesane Pre Manufacture Notice (PMN) application. The farnesane PMN application is progressing through the final phase of agency review with approval expected near-term. Under the European Commission (EC) REACh directive, the two chemical substances (farnesene and farnesane) were successfully registered. CARB has concluded, based on submitted farnesane data that no further multi-media testing is required. The farnesane data is now supporting evidence justifying a CARB proposed regulation classifying renewable diesels (those compliant with American Society for Testing and Materials (ASTM) D975 specifications) as ‘diesel’. CARB has approved Amyris renewable diesel fuel registration up to 35% blends, equivalent to the approval obtain from the U.S. EPA previous to this project.

In addition to the regulatory approvals, significant progress was made in user acceptance from pipeline operators for fuel transport, fuel storage, and environmental testing of farnesene for persistence in the environment. Through data sharing and manufacturing process explanation, the project acquired the first US pipeline renewable diesel product code and corresponding product specifications listing. Farnesane as both a diesel fuel and as an aviation turbine fuel blending component was assessed for long-term thermal and oxidation stability, and the effectiveness of commercial oxidation stabilizing additives on farnesane was investigated. The product was found to maintain storage stability equal to or better than fossil-derived fuels. New chemical substances are required to be registered in the US under the TSCA and in Europe under the REACh directive. The project specifically focused on acquiring enhanced biodegradation data, eco-toxicity studies, and assessment of physical-chemical properties within production variation. EPA and European Chemicals Agency (ECHA) guidance for low soluble test materials like farnesane required development of alternate procedures while maintaining the test protocol limits. For example, the farnesane and farnesene enhanced biodegradation testing was extended to 60 days to demonstrate degradation >50% (measured biodegradation was 60%). As a result, both farnesene and farnesane can be classified favorably as non-persistence.

Outstanding Items:

Commercial production process approvals and acceptance was not pursued in this project. The establishment of state, local and NEPA requirements for such a plant would require selection of a potential US site for Amyris using biomass fedstocks. In addition developing a permitting dossier would require selection of a site. Initiation of these activities is dependent on in-place domestic, sustainable, cost-competitive technologies for the production of biomass based sugars. This will require integration and optimization of biomass handling logistics, and production of sugar from biomass at a scale that supports economic supply. Co-location of a fermentation plant for conversion of sugar into advanced biofuels and high value co-products would be preferable. To date Amyris has scaled its technology and has ongoing commercial production in Brazil where abundant, low cost sugarcane feedstock is available.

Task A: Amyris Pilot Plant Upgrades and Operations

Task Objective: Unit operation upgrades and capacity augmentation for both fermentation and chemical processing at Amyris's existing Emeryville pilot plant form a core component of this IBR project. During the two budget periods of the project Amyris will leverage its experience in pilot plant design, construction and operation to rapidly execute with minimal incremental permitting required. In particular, Amyris anticipates little or no downtime of ongoing pilot operations during upgrade design and installation.

Subtask A.1. Amyris pilot plant upgrade design and approvals

- *Completed September, 2011*

Subtask A.2. Amyris pilot plant upgrade installation

- *Completed September, 2010*

Subtask A.3. Post-upgrade Amyris pilot plant operations

- *Completed June, 2013*

Subtask A.4. Decision to install additional feedstream clarification unit ops (e.g. TFF)

- *Completed September, 2011 – additional unit ops will NOT be installed based on feasibility data-in-hand that current feedstream clarification process sufficient*

Subtask A.5. Decision to install additional product recovery unit ops (e.g. falling film distillation)

- *Completed September, 2011 – additional unit ops will NOT be installed based on feasibility data-in-hand that current recovery process sufficient*

Accomplishments Against Goals

Subtask A.1. Amyris pilot plant upgrade design and approvals

Amyris leveraged its experience in pilot plant design, construction and operation to execute with minimal incremental permitting and cost. The NEPA documentation for budget phases 1 and 2 was completed in February 2010. A Risk Management Plan was developed and finalized in April 2010. During the pre-construction phase, existing pilot plant operations were up and running to evaluate defined and cane syrup feedstocks from fermentation through to farnesane. Specifications for the anticipated new equipment were finalized and equipment ordered with the objective of minimizing down time while taking into account the delivery lead time. As anticipated, there was little or no downtime of ongoing pilot operations during upgrade design and installation.

Subtask A.2. Amyris pilot plant upgrade installation

The ordering of new equipment was completed in November 2010. The expansion area construction work was completed on time in July 2010 which enabled movement of the HTST sterilizer out of the fermentation hall and into the dedicated expansion area by September 2010. The remaining fermentation laboratory construction was completed in September 2010. The completion of the chemistry area, which mainly involved constructing enclosures around existing open space, was deferred until the arrival of the distillation and chemistry skids to facilitate customization. Following this approach, the chemistry construction completed in August 2011. Commissioning of the two additional fermentors was completed in February 2011 after resolution of SAT identified modifications to the exhaust filters and side wall ports. The chemistry skids were in service December 2011. The new direct steam sterilizer (Barnum) was operational in September 2012.

Subtask A.3. Post-upgrade Amyris pilot plant operations

Sorghum syrup was evaluated at pilot scale in 2011 across all unit operations. A performance protocol was completed in February 2012 which evaluated the unit operations from sorghum sugar treatment through to production of biodiesel farnesane and plasticizer molecule flexasane. A performance evaluation of the lignocellulosic feedstock based process unit operations was completed in April 2012.

Subtask A.4. Decision to install additional feedstream clarification unit ops (e.g. TFF)

Early evaluations using tangential cross flow filtration (TFF) for clarification of syrups showed that the performance of that method was unsuitable as an option for large-scale processing. The high viscosity and density of syrups leads to a low flux rate through the filter which limits the overall productivity of the operation. In addition, high solids in feedstocks contributed to a further reduction in the filtration flux. It was decided to not pursue filtration as a sugar treatment method.

Subtask A.5. Decision to install additional product recovery unit ops (e.g. falling film distillation)

From the perspective of evaporator scale up decisions it was decided to incorporate a flash distillation unit (FDU) rather than a falling film unit.

Project Activities

Subtask A.1 Amyris pilot plant upgrade design and approvals

All construction and operation activities were performed in accordance with applicable federal, state, and local regulations, codes, standards, guidelines, policies and laws. Detail on the extension of these activities for the expansion of the pilot plant is provided below.

Permitting Activity

In February 2010, following the receipt of the December 2009 IBR Award, Amyris leased an additional 5972 ft^2 of warehouse space adjoining the pilot plant. While much of the permitting obtained for the original space was sufficient to cover the proposed changes, some additional review and approval was required and obtained to allow for the construction and the operation of four 300L fermentors (Table A.1).

Table A.1: Summary of Permits and Approvals for Construction and Operation of the Pilot Plant

Permit/Approval	Responsible Agency	Status	Comments
FEDERAL			
Environmental Assessment (EA) or an Environmental Impact Statement (EIS) under NEPA	DOE, as Lead Agency	Prepared prior to application	Required in accordance with NEPA in projects where a federal agency (i.e., DOE) is proposing to provide financial assistance to a project that could have an impact on the environment.
Approval under Reporting Requirements and Review Processes for Microorganisms	United States Environmental Protection Agency (USEPA)	Reviewed/accepted between Amyris and DOE	Required under Toxic Substances Control Act regulation 40 CFR 725 for manufacture or import of genetically modified microorganisms (GMO). Amyris GMO is eligible for approval under a Tier I exemption (A Tier II exemption or full Microbial Commercial Activities Notice may be required for higher scale operation and is determined on a case by case basis)
Hazardous Waste Generator Identification Number CAR000194928	USEPA	Existing for current facility and applicable to upgraded facility	Required for management and disposal of certain solvents, cleaning products, spent cleaning rags, etc. The waste contractor operates using a manifest system
STATE			
Environmental Impact Report (EIR) under California Environmental Quality Act (CEQA)	City of Emeryville	Reviewed by Emeryville City and accepted (Report ESA/D208071 March 2008)	As required in accordance with CEQA because certain agencies in the State of California (i.e. City of Emeryville) have approval authority for the proposed project. The City completed an environmental checklist in 2008 for the existing facility which resulted in a Negative Declaration. The City notified Amyris that it will not require another environmental checklist for the proposed expansion.

Permit/Approval	Responsible Agency	Status	Comments
LOCAL			
Air Permit to Operate	Bay Area Air Quality Management District (BAAQMD)	Plant No. 19112 Currently permitted through 10/1/2013	Amyris determined the amounts of volatile organic compounds (VOCs) emitted from pilot plant were low (~ 4 lbs/day) and well below the threshold for permitting with local authorities (~10 lbs/day issued by BAAQMD). Also operations involving use of solvents/fuels/or gases are carried out within the chemical hoods. Filters are included on the fermentation equipment to prevent the venting of microbial products into the surrounding air.
Hazardous Materials Business Plan and Permit to Operate	Alameda County Department of Environmental Health	Plan for Existing Facility Submitted December 2008, Permit renewed in 10/31/2010	Required for the storage of certain quantities of hazardous materials and hazardous wastes. The plan is to be updated every three years or within 30 days of changing the inventory of hazardous materials stored at the facility.
Conditional Use Permit (Modified Approval)	City of Emeryville Planning Department	Modified Approval 2/17/2010	Allows for the conversion of 16,372 square feet of industrial manufacturing from Custom Manufacturing to High Technology and the installation of four 300 liter fermentor vessels.
Building Permits, Occupancy Permits, Fire Permits, etc	City of Emeryville	Building permits were approved and issued	Required to ensure that building plans conform with applicable Municipal Codes and Conditional Use Permit.
Construction Permit for Sewer Connection	East Bay Municipal Utility District	obtained prior to construction	Required for new interconnection to sewer system.

Basis of Expansion

The basis for increasing the pilot plant capacity was to enable simultaneous investigation of a number of different domestic sugar feed stocks, and to provide a venue for integrated processing capability from a number of different sugar sources to biodiesel. In order to accommodate the new equipment and operations on the plant floor while maintaining a safe working environment, additional space was required. The additional 5972 ft^2 of warehouse space was used to harbor equipment, create an area for processing syrups with the HTST and TFF, and build a new 280 ft^2 cold room to house the additional feedstock from our sub-contractors on the project.

From March 2010 through July the design and permitting activities were completed for the 5 phases of the new expansion. The 5 construction phases were:

1. Expansion of the warehouse space, adding larger cold room, and tunnel (March -July 2010)
2. Installation of underground drains and troughs (July-Aug 2010)
3. Installation of mechanical, electrical, and plumbing (MEP) in the HTST room (Aug-Sept 2010)
4. Installation and commissioning of Fermentation MEP (Aug-Sept 2010)
5. Installation and commissioning of two new 300L fermentors (Nov-Dec 2010)

Pilot Plant Utilities

No major changes to utilities were required for the general expansion project through 2010. The later installation (May 2012) of the Barnum direct steam injection (DSI) sterilizer required purchase and installation of an additional boiler that could provide steam at greater than 100 psig (pounds per square inch [gauge])and 300 lbs/hr, a higher pressure than the original plant systems. This was required to provide steam to the Pick steam injection system in the Barnum DSI HTST unit. The plant utility systems are as follows:

Plant Steam is used for indirect process heating. Steam is generated from four 9.5 BHP (boiler horsepower) California Special Boilers which supply plant steam to the fermentor process tanks, clean steam generator, autoclave and other steam drops in the pilot plant. The boilers were designed to meet California Title 8 requirements for boilers that are not required to have annual inspection and do not require a permit to operate. A typical boiler feed water skid protects the boiler by ridding the feed water of oxygen and inhibiting scale. The four boilers have FM (Factory Mutual) approved gas trains and on/off firing control. The boilers deliver 85 psig steam to the main supply header to support the clean steam generator. Other loads are reduced to 60 psig.

Media Sterilizer Steam is supplied from a Valin-Chromalox CHPES Electrosteam boiler. The system provides steam at a regulated pressure of 100-110 psig at approximately 310 lbs/hr. The supply water is not chemically treated.

Compressed Air System provides 120 SCFM (standard cubic feet per minute) of clean dry air at -40 °F dew point and 90 psig for sparging within the fermentors and other process uses. Instrument air for the pilot plant is taken upstream of the compressed air final filtration. The compressed air system is composed of typical compressed air equipment with compressed air headers and instrument air drops provided where needed.

Sanitary Waste and Vent System is provided for the showers and restrooms, kitchens and sinks and for the floor drains within the mechanical equipment room.

Domestic Water System services the Facility for domestic water needs, sinks, and emergency eyewash/shower stations. Domestic Hot and Cold water systems were designed in accordance with the 2007 California Plumbing Code. Domestic water pressure is assumed to be in the range of 70 psig. Domestic water is supplied by the East Bay Municipal Water District.

Industrial Water System is fed from a backflow preventer in the mechanical room. Industrial water service provides makeup water for the purified water skid, process chilled water, heating hot water system and steam boiler feed water. Industrial cold water is also provided for lab/fume hood, sinks, wash down area, glass washer and autoclave. It is used for quenching clean steam condensate prior to its release to the drain.

A 75 MBH (thousands of BTUs (British thermal units) per hour) gas fired water heater and return system located in the mechanical room provides Industrial hot water.

Natural Gas Distribution System provides 2 psig fuel for the plant steam boilers, heating hot water boiler, and industrial and domestic hot water boilers. A pressure supply regulator reduces the pressure to 14" water column for all gas using equipment.

Nitrogen System is composed of a high pressure nitrogen system (725 psig) and a low pressure nitrogen system (90psig) fed by six compressed nitrogen cylinders. Low Pressure Nitrogen is supplied to the Fermentation Room for the centrifuges and to the fume hoods in the Chemical Final Processing and Purification rooms. The system is provided with a 0.2 micron filter for all loads. The High Pressure Nitrogen is supplied by compressed gas cylinders to the fume hood in the Chemical Final Processing Room for the Hydrogenation Reactor.

Hydrogen System is supplied by two compressed six pack gas cylinders using a manifold to a source panel in the H2/H3 room to a single drop within the fume hood of the Chemical Final Processing Room. Hydrogen is used for the Hydrogenation Reactor at 740 SLPH (standard liters per hour) and735 psig. An emergency shutdown system will be employed to shut off Hydrogen flow in the event of excess flow. Nitrogen can be used to purge the system.

Vacuum system producing 45 ACFM –actual cubic feet per minute at 28 inches mercury is provided for general lab usage.

Process Chilled Water System provides 10-12 °C chilled water for cooling of the fermentors, media tanks, and other process equipment. The system consists of a chiller (roof) duplex primary pump, air separator, expansion tank, surge tank, and chemical pot feeder.

Clean Steam System is provided for process sterilization at a rate of 400 lbs/hr at 40 psig. Clean steam will be distributed to the autoclaves and process equipment with point of use pressure regulators. Clean steam condensate is currently sent to the Process Waste System. The clean steam condensate is returned to the plant steam system to reduce the possibility of contaminating the clean steam generator should there be a seal failure.

Reverse Osmosis/De-Ionized (RODI) Water System provides water for clean-in-place (CIP), Clean Steam Generator feed, reagent water and general cleaning within the production envelope. RODI water is supplied to storage tanks, pretreatment skid, and RO water skid. The design of the RODI System follows ASTM and Clinical Laboratory Standards Institute (CLSI) guidelines for reagent waters, as well as recognized industry practices to minimize bacteria formation and growth and provide for system drain-ability and sanitization. RODI generation is 6 GPM (gallons per minute) with a 600 gallon storage tank and continuously re-circulating system.

Process Waste System – All drains in the pilot plant area are directed to the process waste system. The process waste system neutralizes liquid waste to within the acceptable facility liquid pH discharge specification (6.0<pH>9.0) prior to discharge into the local municipal sewer system. Facility design and operation are designed to minimize release of viable microorganisms in accordance with National Institutes of Health (NIH) guidelines. Viable microorganisms are heat inactivated prior to disposal by a licensed waste disposal company. The use of submicron to 1.2 micron nominally rated gas filters on the exhaust gas lines of the fermentors prevents release of microorganisms into the air.

Power is provided by Pacific Gas and Electric. An emergency diesel generator provides back up power to provide an orderly shutdown, if required, for approximately 12 hours.

Environmental Impact-NEPA Documentation

Prior to the original construction of the Amyris Emeryville pilot plant, a study (ESA/C20871 March 2008) was carried out by the city of Emeryville following CEQA Regulations and a Negative Declaration was made. This document was provided to the DOE and contained information on a wide range of environmental areas of interest. These areas were re-evaluated by Amyris under the context of the pilot plant expansion project scope. From Amyris's review, no extraordinary circumstances or significant effects to the environment (as identified in the EF-1, Section II A, B or C of the CEQA regulations) were evident with the proposed increase in capacity.

During both budget periods, Amyris collaborated with selected vendors and partners to provide services that include feedstock growth, harvesting, processing, analysis, pretreatment, and scale-up capabilities. Environmental consideration of these vendors was also evaluated and communicated to DOE at that time.

NEPA considerations for contract activities

Baseline Conditions

The pre-existing pilot plant facility was designed to scale-up laboratory processes to the 300L fermentation scale, using two 300L fermentors with a total working volume capacity of 500L (130 gallons). The facility was built in 2008 as a lease-hold improvement of an existing warehouse, formerly used as a printing shop, in a light industrial section of Emeryville California. The property borders a parking lot, a greenway, a street and businesses. The area is classified as a mixed use zone with a mix of residences, businesses, and light industry. The property is owned by Emery Station Triangle, and affiliate of Wareham Properties. At the time of the award, Amyris was 16 months into a 10-year lease for the property and had been operating the pilot plant without issues or complaint from area residents. A picture of the facility from the street entrance is shown in Figure A.1, and a view of the interior fermentation hall is shown in Figure A.2.

Figure A.1: Pilot Plant entrance

The facility was constructed in compliance with applicable Federal, State and Local Building Codes and Regulations as a mixed-occupancy facility:

- Pilot plant floor and labs: 15224 ft^2 (B-occupancy with 2 control areas with 1 hour fire rated walls)
- Warehouse: 1145 ft^2 (S occupancy)
- Hazmat room (H2/H3/H4): 269 ft^2 (H2 occupancy with 2 hour fire rated walls)

The facility is equipped with a fully automated sprinkler system and emergency equipment including fire extinguishers, safety showers, eye washes, first aid supplies and spill kits. The pilot plant floor had sufficient space to accommodate additional 300 L fermentors with the modifications indicated above. The extra space was needed to house the processing equipment and syrup.

Figure A.2: Main fermentation area of Pilot Plant housing original two 300L bioreactors

The original project plan and construction went through a thorough design review, which included the completion of an initial study on environmental impact (ESA/C20871 March 2008) according to CEQA guidelines. The original evaluation enabled Amyris to provide a a copy of the study to DoE to assist in the NEPA review of the IBR project scope. The CEQA study of March 2008 evaluated the environmental impact of the initial project consisting of design, construction and operation of the pilot plant with two 300 L fermentors to produce, process, and finish Amyris Renewable Diesel fuel from sugarcane feedstock in the amounts of 24 gallons a week with a maximum storage quantity of 300 gallons. The environmental factors considered in this initial study included: Aesthetics, Biological Resources, Hazardous Materials, Mineral Resources, Public Services, Utilities, Agricultural Resources, Cultural Resources, Hydrology and Water Quality, Noise, Recreation, Mandatory Findings of Significance, Air Quality, Geology, Soils and Seismology, Land Use, Population and Housing, and Transportation. The review concluded with a Negative Declaration or a finding that the proposed project could not have a significant environmental impact.

Process Operations

Amyris was responsible for the optimization of sweet sorghum juice- and lignocellulosic sugar-based fermentation at lab scale in existing labs and then in its pilot plant using modified *Saccharomyces cerevisiae* yeast strains.

Amyris collaborated with NREL to develop sweet sorghum bagasse pretreatment conditions to afford additional sources of sugars for fermentation. The pretreatment process was evaluated at NREL, first at lab scale and then at pilot scale, based on feedback from Amyris regarding fermentation performance using different bagasse-derived sugar streams. The fermentation waste or vinasse was evaluated by ICM in anaerobic digestion for biogas production for use as an energy source for the chemical finishing of the fuel. Biogas cleanup procedures were modeled by Praxair for production of highly pure methane for conversion to hydrogen, a key component for Amyris product finishing, via steam-methane reforming (SMR). This was accomplished by shipping samples of the fermentation waste to ICM for anaerobic digestion studies. Praxair evaluated the outputs of the ICM biogas process to identify technical and economical parameters for the production of hydrogen by SMR

As the agricultural lead on the IBR project, Ceres managed all aspects of feedstock development and handling including land management, planting, harvesting, bagasse drying, and juice concentration to syrup. All facilities used by Ceres in this project were already constructed and operational.

Ceres shipped syrup samples to Amyris from all four varietal trials (net plots) and the larger scale plantings in Florida and Tennessee. Ceres also shipped chopped and dried bagasse to NREL for lignocellulosic biomass analysis and pre-treatment.

Ceres planted all crops in established fields that they either owned or leased. These fields were representative of farmland in the geographic zone they represent and will potentially continue to be used for agronomic purposes. As such, these fields were not subject to the Farmland Protection Policy Act. These plots had been previously used to grow a variety of crops including turfgrass, corn, and sugarcane. Crops were harvested according to recommended harvest practices for sweet sorghum. Generation of criteria air pollutants was not considered to be a factor in crop planting and management.

All pesticide used were registered with the USEPA and used according to their label requirements. All pesticide applications were made by licensed applicators. The herbicides used in the early growing season were broad spectrum herbicides that can be used with many different crops, e.g. Glyphosate, S-Metolachlor, or Atrazine. Insecticides included Zeta-cypermethrin or similar chemistry products. Copies of the labels and the records of application were maintained at the sites.

Lab Operations

No additional permitting was required to progress this part of the project. These facilities were fully permitted and had established safety protocols including an Illness Injury Prevention Plan, Chemical Hygiene Plan, Hazard Communication Plan, Ergonomics, Fire Prevention Plan and Emergency Response Plan. Procedures had been established for the management of hazardous waste and a hazardous waste Permit to Operate had been granted by Alameda County Environmental Health Department. As part of the Amyris safety program, a process safety review is carried on new procedures or equipment that have an inherent risk of physical or chemical hazards associated with them. Standard operating procedures (SOPs) were in place for existing practices and additional SOPs are written as new practices are developed. Amyris has a dedicated EH&S officer and an established safety committee. Facility audits are carried out on a quarterly basis and training in all safety programs is carried out as required by OSHA. Amyris is subject to independent audits by Insurance Loss Control Representatives, Alameda County Health Department, Emeryville Fire Department, EBMUD and BAAQMD representatives.

Lab procedures are also well established at NREL, Ceres, and ICM. These facilities have established safety programs according to OSHA regulations and no additional permitting was required to progress this phase of the project.

Amyris Pilot Plant Upgrades

The upgrades involved relocation of some utilities, addition of new drains, a new exhaust fan, and condenser on the roof. While no exterior walls needed to be modified, to access the extra warehouse space, an entry way was planned from the utility area into the existing room. Contaminated soil was discovered under the building during excavation work required to relocate drains. The soil, contaminated with oil, was removed as hazardous waste. Asbestos removal was not anticipated as it was not used in the current facility for insulation. These modifications were carried out according to California/Uniform Building Codes and new spaces were fitted with life safety systems as required. Amyris employed licensed general contractors (Dome Construction Corporation; www.domeconst.com) for structural modifications and was committed to the use of Green building materials and energy saving equipment where possible. The changes required review by the City of Emeryville and administrative changes to the conditional use permit and fire permit. The sewer tie-in required a permit from the East Bay Municipal District. The equipment installations were evaluated from a safety perspective prior to installation according to Amyris procedures for process safety review.

With respect to land use, wetlands, coastal regions, air quality districts, aesthetics, endangered species or other biological resources, and navigable air space, the proposed expansion did not include any changes that effected the original assessment of a negative declaration made in the initial CEQA study.

Proposed Operation

The additional two fermentors would potentially generate a total of 12 gallons a week of Amyris renewable diesel fuel with a proposed maximum storage amount of 300 gallons on site. The original output of the pre-existing fermentors was 12 gallons a week with the sugarcane feedstock. The combined output of the four fementors was determined as 24 gallons a week, the same output volume that was assessed under the original CEQA analysis.

To run the operation, additional staff would be needed. A total of 12-14 full time employees were considered in the initial study. After an in-depth review, it was determined that only 3-5 additional employees would be needed to operate the process under the DOE project scope. Parking to accommodate the additional personnel was sufficiently available at Amyris's neighboring R&D facility across the street thus no additional permitting was required with the city of Emeryville.

Prior to the expansion there were deliveries up to two times a week to the warehouse entrance on 59th Street where there was ample space for trucks to pull in off the Street. With the project expansion, we experienced a modest increase in truck deliveries as outlined in the logistics section. The delivery location moved from 59th Street to Hollis Street where a truck pull-out was constructed allowing for the complete removal of trucks from traffic.

Material Use, Storage, and Disposal

The components of the fermentation broth include mostly water (80+ %), miscellaneous non-hazardous media components, sugars, Amyris modified yeast strains, ethanol (~1%), and Amyris C15 intermediate (approximately 3 gallons per fermentor/per run). The Amyris fuel is classified as a class IIIB combustible material with irritant properties and the Amyris modified yeast strains are considered Risk Group 1 - no threat to human health or the environment. Amyris has carried out independent toxicity assessments on both the C15 intermediate as well as the modified yeast strains and the results have supported low toxicity of these substances.

As part of the DOE project, large shipments of sorghum juice were received from Ceres for use in the fermentation process. The shipments, often made in bulk, consisted of up to five 55 gallon drums per shipment. The syrup was stored in a large cold-room, in the 55 gallon drums, until ready for use.

The facility is built to comply with NIH recommendations for good large scalable practices (GLSP) handling and containment of genetically modified organisms (GMOs). An underground holding tank was constructed to capture any spills per US NIH Guidelines for Research Involving Recombinant DNA Molecules, 2002, Appendix K BL-1-LS. Fermentations are performed and contained within bioreactors that are equipped with condensers and filters to contain volatile organic components and GMOs.

The bulk of flammable, combustible and corrosive materials needed for the process are currently stored in a specially designed Hazmat room rated Class 1 Division 2 with two-hour fire rated walls. The room sits on a concrete slab with an underground trench for spill containment. The room is outfitted with a water mist fire suppression system and a sophisticated early warning and alarm system monitoring concentrations of potentially explosive vapors (lower explosion limit detection).

A listing of hazardous substances with estimated maximum storage amounts and location is shown below in Table A.2. A comparison of the old versus new storage requirements is shown in Table A.3.

Table A.2: Total amounts of hazardous substances in expanded facility

Substance	Max Amount Stored	Hazard class	Storage area
Amyris fuel	300 gallons (gal)	irritant, class IIIB	cold room
Diesel fuel	500 gal	combustible class II	outside generator
Diesel fuel	220 gal	combustible class II	H2/H3/H4 hazmat room
Mixed solvents: hexane, acetone, ethanol	110 gal	flammable IB	Labs and H2/H3/H4 hazmat room
Ammonium hydroxide	110 gal	corrosive/toxic	H2/H3/H4 hazmat room
Phosphoric acid	110 gal	corrosive	Warehouse (S-occupancy)
Hydrogen gas	1176 cubic feet (cu ft)	flammable gas	H2/H3/H4 hazmat room
Nitrogen gas	2736 cu ft	asphixiant	dock area (S occupancy)
Nitrogen gas	1824 cu ft	asphixiant	H2/H3/H4 hazmat room
Hazardous waste, liquid	25 gal	flammable	H2/H3/H4 hazmat room
hazardous waste, solid	240 lbs	corrosive/toxic	H2/H3/H4 hazmat room

Table A.3: Comparison of the total amounts of hazardous substance from the proposed DOE plan with the original plan

Substance	Original Plan	Proposed Plan	Storage limits (B-occupancy)
total flammable liquids (1A, 1B, 1C)	590 gal	135 gal	240 gal/control area *
total corrosives	110 gal	220 gal	500 gal/control area
total flammable gas	784 cu ft	2352 cu ft	NA (stored in Hazmat room)-4000 cu ft
total asphixiants	1368 cu ft	4560 cu ft	NA (stored in hazmat room and loading dock)
total combustible (II)”	425 gal	220 gal	240 gal/control area* but mostly stored in the Hazmat room.
Total combustible (IIIB)	300 gal	300 gal	13,200 gal/control area+

*quantities doubled based on presence of sprinkler system (may be doubled again for storage in rated cabinets).
+ quantities shall not be limited in buildings with approved automatic sprinkler systems.
“ excludes 500 gal for the back-up generator

The assessment showed that the total flammable/combustibles (IA-C, II, IIIA) were less than what was considered for the original project scope (limited to <1000 gallons).

We remained under the amounts of flammable materials that would trigger a risk management plan per CalARP (<1300 gallons of flammables).

The facility was already equipped with an H2/H3/H4 hazmat room that allowed for greater quantities of flammable storage than normal B-occupancy control areas. (sprinklered B-occupancy control areas can accommodate 240 gallons of stored flammable/combustible materials).

The facility was listed as a Small Quantity Generator with the California Department of Toxic Substance Control and under the DOE project, this classification was planned to be preserved. The volumes of hazardous waste generated per month were approximately 200 lbs/month. With IBR activities, a slight increase in hazardous waste was expected (up to 400 lbs/mo) which was still well below the limits for a Small Quantity Generator (2200 lbs/month). A Hazardous material business plan was filed with Alameda County environmental Health Department and a Permit to Operate was issued by the County for hazardous waste. Hazardous waste is contained and removed by certified waste haulers according to state and federal laws. All hazardous waste is traceable through the uniform manifest system and all records are retained.

Process Waste

The original fermentation process generated approximately 250 gallons/week of fermentation process waste-water. Under the DOE IBR award, the volume of process waste-water doubled. The waste-water is heat deactivated and trucked by licensed waste haulers as non-regulated aqueous waste to Evergreen Oil, a licensed facility for waste-water treatment as well as hazardous waste disposal. The Evergreen hazardous waste treatment, storage, and disposal facility (TSDF) is located in Newark, California, within 30 miles of the pilot plant. Prior to the pilot plant expansion, one process waste-water pick-up was carried out per month. With the volume of waste-water doubling, the disposal was also doubled. The waste is transported according to Department of Transportation (DOT) regulations and all transports are documented under a non-regulated aqueous waste manifest.

Evergreen Oil, Inc. is a California based pioneer environmental group, operating for over 22 years as a leading collector and re-refiner of used automotive oils which has grown to lead the industry in developing safe and efficient used oil recovery operations while providing other environmental services as a one-stop shop for hazardous waste management and disposal in the State of California. At this site, Evergreen operates a newly built waste water treatment plant that can accept and treat up to 55.0 million gallons annually. The plant can accept any type of non-hazardous and non-RCRA (Resource Conservation and Recovery Act) waste water, and RCRA waters with low concentrations of organics. Oily water and sludge from clarifiers and sumps, R&D wash water, and RCRA water from contaminated properties can all be treated for re-use in their state-of-the-art treatment plant. The plant utilizes the following processing methods:

- Mechanical removal of solid content up to 65% by volume
- Lamella Clarifier
- Dissolved Air Flotation

- pH Adjustment and Precipitation
- Mixed Media filtration

Under the DOE IBR award, Amyris further explored the conversion of fermentation waste to usable energy for the process. To develop this waste-to-energy process, Amyris shipped some of the process waste water to ICM for treatment in ICM's Bio-Methanators™ methane digestion system. These pre-fabricated systems remove organic contaminants from process water and turn them into methane gas. The biogas that is captured in this process can be routed to boilers as a supplemental fuel source, or it can be utilized for combined heat and power generation, substantially reducing energy costs. This solution could reduce a facility's carbon footprint and furthermore potentially allow the final water effluent to be recycled for use in the process.

ICM is located in Colwich, Kansas and has been in the business of building ethanol plants with this type of waste water treatment system for many years. Amyris shipped four separate waste samples to ICM for waste-to-energy processing using the methanating equipment at ICM. These runs were carried in sequence and tested initially at lab scale followed by pilot scale at the Colwich Facility. To complete the work for Amyris, no additional permitting was required on the behalf of ICM as the work that were performed was within existing facilities and within the normal scope of services at the facility.

The Environmental Management at ICM, Colwich has conducted their own EA for this site for DOE funding of separate projects (not including the Amyris project) and has concluded no significant findings. The activities or conditions found to be of special interest covered in this EA are pre-existing contamination, asbestos, criteria pollutants, and floodplains – the ICM site sits on a 100 year floodplain. On the NEPA environmental EF-1 checklist, no other areas of special interest resulted from the activities of ICM under DOE NEPA. ICM is planning on filing for an R&D exemption, with respect to air emissions, and for a building permit to carry out a pre-planned expansion unrelated to the Amyris-IBR award.

As a final component of this waste handling process, Amyris and ICM collaborated with Praxair to model proper biogas cleanup procedures to ensure effective conversion of methane to hydrogen via steam methane reforming. This is strictly a modeling exercise with no extraordinary circumstances or significant environmental impact.

Logistics

Under the scope of the Amyris IBR award, there was a need to consider transportation of materials as indicated in the SOPO. The Table A.4, shown below, summarizes the large material transportation requirements:

Table A.4: Frequency and Amount of Materials Transported

Material	From/To	Amount and Frequency
Syrup	Ceres (FL)/Amyris (CA)	180 gal every two weeks from May to December for two years (2010, 2011)
Bagasse	Ceres (FL)/NREL (CO)	Three shipments for a total of 13,200 lbs
Syrup from pretreatment	NREL (CO)/Amyris (CA)	Six shipments of 200 gal per shipment
Waste-water	Amyris (Emeryville, CA)/Evergreen (Newark, CA)	Twice a month for a total of 1600 gal/month
Waste-water	Amyris (CA)/ICM (KS)	Up to four separate shipments of 60 gal each

These extra shipments do not represent any significant increase in transportation versus the likely increases as a result of population increase in the area and as such, are consistent with the Clean Air Policies of the California Air Board. Amyris strives to minimize shipments and deliveries as much as possible by consolidating shipments and bulking materials.

The Amyris IBR project did not include import or export of toxic substances listed under the TSCA 40 CFR 700-799. Amyris applied for and was granted an R&D exemption from TSCA for the development of Amyris Renewable Diesel. The Amyris C15 intermediate is currently listed on the TSCA inventory. As *Saccharomyces cerevisiae* is listed (under TSCA Tier I) as a well characterized GRAS (generally recognized as safe) micro-organism, Amyris qualified for an exemption from filing an MCAN (microbial commercial activity notice).

Air Quality

The only air contaminant of concern that pertains to Bay Area air quality plans is the ozone precursor, volatile organic components (VOCs). Based on air emissions modeling as summarized in the initial CEQA study, the estimated worst case emissions (failure of condenser) of VOCs were determined to be approximately 1.6 lbs/day. With equipment functioning as designed, the emissions of VOCs were calculated to be 10 fold lower or approximately 0.16 lbs/day. Even with the expansion of the pilot plant and doubling of fermentation capacity, the emissions would not be more than double this amount depending on the frequency of fermentation runs. A Research and Development exemption from BAAQMD is in effect for air emissions under 10 lbs/day.

A backup generator is situated on the site and tested for brief periods monthly. An air permit has been acquired for this generator and this permit is not impacted by the scope of this project.

The Bay Area is designated as a marginal non-attainment area with respect to ozone only. According to the initial CEQA study, emissions of 80 lbs or more per day would be considered as a trigger for impact according to the 2005 Bay Area Ozone Strategy document. We were well below that limit at 1.6-2.0 lbs/day

Water Use

Water discharge rates from the pilot plant range from 1000-2000 gallons per day. The effluent includes tank rinses, steam condensate, lab sink and diswasher use, and RODI system use. The process or fermetation wastewater taken directly from the tanks is not committed to the drain but trucked to a wastewater treatment plant as described above in the Process Waste section.

The water discharge that is committed to drain passes through a water treatment system prior to entering the sewer. The water treatment system performs a simple pH adjustment to ensure that the water remains within acceptable ranges for the local sewer authority, East Bay Municipal Utilities District. The water discharge has been analyzed for chemical oxygen demand filtered (CODF), total suspended solids (TSS), and metals, with the resulting values well under the limits of concern for EBMUD. A wastewater discharge permit was not required for this effluent. With the expanded facility the amount of effluent increased due to additional tank cleaning and modest increase in staff, by approximately 50%. EBMUD did not experience any difficulties in accommodating this increase at their plant. As part of its commitment to the environment, Amryis continues to explore alternative water recycling and conservation measures.

Noise

The City of Emeryville has strict limits on environmental noise. For a mixed use zone the acceptable noise limits range from 50-60 dB. Amyris has met these conditions for the original pilot plant as determined from actual measurements at the property line.

For the addition of another exhaust fan and condenser under the DOE IBR award, Amyris commissioned sound consultants to re-calculate the contribution to the surrounding envrionment from the additional exhaust fan and condenser on the roof. As a result of these calcuations, the new equipment was found to not significantly alter the noise level at noise sensitive recievers in the immediate area, and to conform to city noise limits. No additional permitting was required.

Presence of Extraordinary Environmental Consequences

The effects on the environment resulting from the Amyris IBR expansion as outlined in the project SOPO were anticipated to be minimal. The presence of extraordinary consequences to endangered species, archeological areas, Class 1 Air Quality Districts, and other sensitive ecological systems are not evident. The project involved activities carried out in existing structures or previously cropped fields in areas that have been developed for this type of use for many years. Modest increases in existing material inputs and outputs are a factor in this project but all sites involved have established mechanisms for safe transport, handling, containment and disposal of process materials, emissions and wastes.

EHS Procedures and Documentation

Amyris

Amyris has established safety procedures in compliance with federal, state and city standards. These are summarized in the following bullet points.

i. Amyris safety procedures incorporate the following features
    - SOPs and batch records have been developed for our bio-processing operations
    - Process hazard analyses are carried out prior to initiation of work projects involving hazards, physical and or chemical
    - Chemical hygiene plans and an injury illness prevention program per Occupational Safety and Health Administration (OSHA) regulations
    - Safety training programs carried out following OSHA regulations
    - Established evacuation procedures
    - Active safety committee with quarterly lab and facility inspections and a First Responder team
    - NIH proposed guidelines for GLSP handling of GMO RG 1 organisms followed

ii. Amyris has an EHS organization that monitors safety compliance
    - Internal EHS director and safety committee monitors operations according to OSHA and Cal OSHA standards
    - Handling of hazardous waste is audited by the Department of Environmental Health
    - Air quality permits are monitored by officials from BAAQMD
    - Audited annually by the loss control division of our insurance company

iii. Amyris policies for chemical handling include
    - All flammable chemicals are stored according to California Building and Fire Code and National Fire Protection Association (NFPA) 45 recommendations in flammable cabinets, and/or fire rated storage areas (H2/H3)
    - Combustible fuel products are stored at 4 °C in a cold room in DOT rated cans
    - Flammable gases are restricted to a Hazmat storage area (H2/H3 room) with 2 hour fire rating, alarms, and mist system in-place
    - All compressed gases are attached with a minimum of two heavy gauge chains

iv. Amyris has safety equipment in place for the facilities
    - Chemical fume hoods are in-place with sprinklers. Hoods are calibrated yearly
    - A LEL detection system is in-place for monitoring hydrogen use in the lab area. These are calibrated every six months

v. Permits in place
    - BAAQMD permit to operate an emergency generator
    - Department of Environmental Health Permit to Operate (hazardous waste)
    - Occupancy permit and Fire Permit with the City of Emeryville and Fire Department
    - Occupancy permit with the city of Emeryville
    - Updated Hazardous Material Business Plan for Alameda County Environmental Health Department

vi. Liquid effluent handling
    - The liquid effluent is heat treated to deactivate the micro-organisms and pumped into large carboys and transported to a water reclamation site where the water is purified and recycled for use
    - The effluent is transported using a certified waste hauling company according to DOT regulations
    - Records are maintained of this disposal process

vii. Toxic waste handling
    - Waste is segregated from regular non-hazardous waste and stored in labeled DOT rated containers according to state and federal regulations
    - Valid EPA ID number for the generator site
    - All waste is manifested and removed from the facility using certified hazardous waste haulers

viii. Air quality management
- The primary source of air contaminants are potentially from VOC emissions (solvent use/fuel). Estimates for the potential amounts of VOCs emitted from these projects awere low (approximately 0.32 lbs/day) and well below the threshold for permitting with local authorities (approximately 10 lbs/day with BAAQMD)
- Operations involving use of solvents/fuels/or gases are carried out within the chemical hoods. Condensers and filters are included on the fermentation equipment to prevent the venting of volatiles and microbial products into the surrounding air

ix. Use of GMOs
- Our processes involve the use of genetically modified non-pathogenic common lab strains of Baker's yeast (*Saccharomyces cerevisiae*). This organism is considered RG-1 and handled using Biosafety Level-1, Good Large Scale Practices, according to NIH Guidelines for Research Involving Recombinant DNA Molecules, April 2002. This organism is currently listed on the TSCA inventory.

CERES

Data provided below from Ceres.

Ceres has a comprehensive Health and Safety Program that includes a related Chemical Hygiene Plan, Injury and Illness Prevention Plan, Hazard Communication Plan, Fire Prevention Plan, and Ergonomic Program. In addition, Ceres also has specific safety procedures to prevent hazards and injury/accidents at work. Personal protective equipment (PPE) is available to all employees and required when working with hazardous materials. Hazardous chemicals are to be handled safely under a chemical fume hood. New employees are given safety orientation with copies of the safety programs before they start working in the laboratory. All SOPs indicate safety precautions while performing any laboratory and field experiment.

Ceres is committed to full compliance with laws and regulations set by OSHA, EPA, NFPA and local agencies like the Certified Unified Program Agency of Ventura County, Ventura County Fire Department, Ventura County Air Pollution Control, and the City of Thousand Oaks. Ceres employs a Safety Compliance and Laboratory Operations Manager who implements and monitors the health and safety program. Ceres also has a Health and Safety Committee that includes the Safety Compliance and Laboratory Operations Manager, Fire Safety Officer, Chemical Hygiene Officer, Respiratory Protection Officer, and First Aid Staff. They meet quarterly and discuss the following: progress and goals, injury/accident report, laboratory safety, inspections, hazardous waste disposal, first aid, ergonomics, and general health and safety. Laboratory inspections are conducted quarterly by Ceres safety officers. A third party safety audit is conducted at the Thousand Oaks and College Station research facilities at least once a year.

The Chemical Hygiene Plan abides by the Cal/OSHA Standard – Title 8, CCR 5191 and 5155 and labeling, storage, handling, and disposal are in accordance with these regulations. SOPs are established for Chemical Storage, Chemical Handling, and Chemical Waste Disposal. Material Safety Data Sheets (MSDSs) are filed electronically with a corresponding hard copy located in a filing cabinet in the hallway outside of the main lab area. All employees are aware of and have access to the hard copies. All laboratory personnel are trained at least annually, with frequency determined by personnel exposure level, in safe handling, storage and disposal of chemicals. Hazardous chemical stock solutions are labeled properly with NFPA hazard identification and target organs. All chemicals are stored separately from their corresponding incompatible chemical(s). Chemical Waste Disposal is managed by an external contractor which collects waste once a month. Ceres has a Chemical Spill Responder team that properly handles all hazardous waste spills. Compressed gases and cold liquids (Nitrogen) are double chained to the wall and handled properly. Ceres has a permit for cryogen use issued by the Ventura County Fire Protection.

Ceres has the following safety equipment in place for their facilities:

- All work with hazardous chemicals is performed under chemical fume hoods located in laboratories and certified annually
- Laboratories are equipped with safety showers and eyewashes which are inspected at least once a month
- The entire building has a fire alarm, certified smoke detection and sprinkler systems for fire prevention per state and local building and safety ordinances

- Ceres facility is also equipped with portable fire extinguishers and fire blankets. Ceres employees are trained on how to use a portable fire extinguisher. Fire extinguishers are inspected at least once a month under the annual maintenance program.
- PPE such as safety glasses, lab coats, ear plugs, ear muffs, gloves (latex, nitrile, neoprene, vinyl, work gloves), and other PPEs (harness, back brace, steel toe boots, rubber boots, etc) are available to employees as required for their work activities.

Ceres has the following permits:

- Consolidated Permit To Operate (for generating hazardous waste) – by the Certified Unified Program Agency.
- Uniform Fire Code Permit (for cryogen use) – by the Ventura County Fire Protection.
- Uniform Fire Code Permit (for flammable/combustible use) – by the Ventura County Fire Protection.

Liquid effluent is handled via city sewer. No chemicals are disposed of in laboratory sinks. Non-hazardous materials are allowed to be disposed of only into designated laboratory sinks that are connected to a collection tank. The non-hazardous liquid collected in the tank is disposed of properly via Veolia Environmental Services.

All laboratory personnel are trained on how to handle, store and dispose of toxic waste. All toxic waste are labeled properly, stored separately in appropriate cabinets, and disposed of properly every 90 days through Veolia Environmental Services. Hazardous manifests are documented and copies are sent to California Department of Toxic Substance Control. No air pollutants were generated and no GMOs were used in the course of this project.

Project and Risk Management

With partners and feedstock scattered across the country from Hawaii to Florida, project and risk management required a high level of coordination across all parties involved from start to finish. The success of the IBR project was dependent upon Amyris's ability to virtually integrate and effectively leverage the expertise of project partners Ceres (sweet sorghum cultivation), NREL (cellulosic pretreatment), ICM (anaerobic digestion), and Praxair (steam-methane reforming).

The program was divided into five distinct tasks

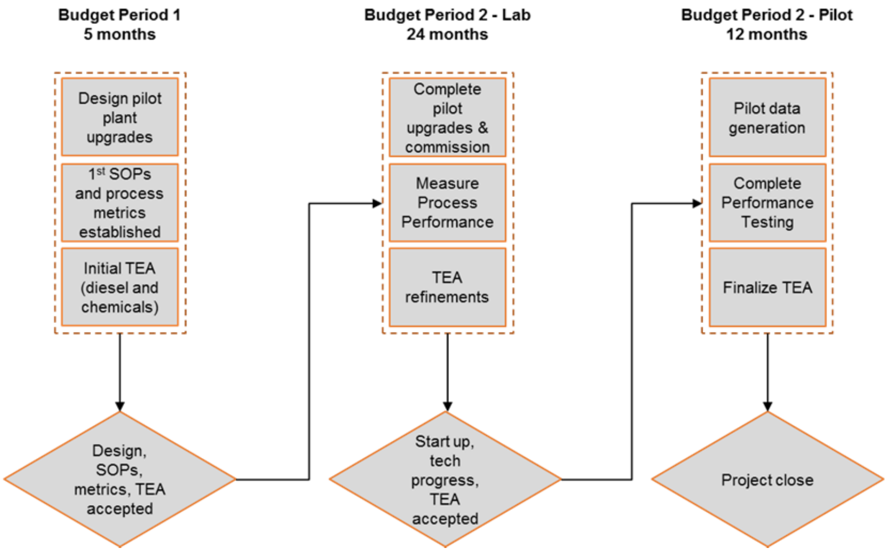
- Task A. Pilot plant upgrades and operations
- Task B. Scale-up and economic analysis of Amyris diesel production from sweet sorghum
- Task C. Development and scale up of value added products from farnesene
- Task D. Regulatory and end-user product and process acceptance
- Task E. Management

These tasks formed the basis of a detailed PMP with defined sub-tasks, deliverables and decision points, each associated with a timeline. The project was tracked using the Earned Value Management System with % completion of each task as a performance-to-plan metric. Managing the project from a technical perspective required clearly defined quantitative metrics for scale-up and ongoing process improvement for each unit operation. Amyris developed the metrics in collaboration with the directors and line managers for each technical discipline (fermentation, recovery, chemistry, process engineering, pilot plant operations, technology transfer, etc.) in order to ensure a rigorous and well thought out process.

Go/No-Go decision points focused around construction and EIRs were established early on in the project to avoid loss of significant investment or expenditure. Some stage-gating was envisaged at the end of BP1 and following the TEA as shown in

Figure A.3 to ensure that key milestones and decision points were met.

Figure A.3: Project Staging



In addition to having well defined timelines and objectives, Amyris was well aware of the principal risks to the successful completion of the IBR demonstration project. As part of its ongoing internal governance process, Amyris actively monitored all risks and developed triggers for contingencies and mitigation approaches with the DOE. These areas are summarized below.

Budgetary risk

- Amyris monitored all spending by Task and budget category on a monthly basis
- Amyris established robust governance procedures with sub-awardees and vendors on a monthly basis

Feedstock supply risk

- Amyris had alternatives to its sweet sorghum supply if Hawaii plantings or harvesting failed or a suitable site was not identified by Ceres
- Amyris established and maintained relationships with alternative sources of lignocellulosic feedstocks throughout the project in order to mitigate a potential feedstock supply risk. Initially this involved US Sugar who had agreed to provide sugarcane bagasse to develop as a corn-stover-like feedstock for Amyris diesel production, utilizing agricultural waste from food production. Later, Amyris was able to obtain lignocellulosic based sugars from Old Town Fuel and Fiber (OTFF) based in Maine, USA, and M&G Chemtex, based in Italy
- Amyris works with defined, synthetic media and can simulate real world feedstocks to hedge against seasonal and/or cyclical agricultural input unavailability
- In order to manage risk around feedstock availability, additional feedstock suppliers were considered and contacted at various stages in the project. When it became necessary to supplement the sweet sorghum feedstock from Ceres due to issues in pretreatment, Amyris was able to source wood chip and wheat straw cellulosic feedstocks for use in the project. Along with some samples from ICM, these additional feedstocks enabled Amyris to not only mitigate risk but also test a variety of feedstocks and provide a more robust data set.

Technical risk

- Most existing Amyris unit operations, as well as those proposed by Amyris, can be operated in alternative configurations or with different types of equipment or processing steps
- The Task C activity for value added products was a risk mitigation effort meant to reduce plant investment risk. This also enabled creation of a value chain approach to enable ROI earlier as a diesel product would require more development time to get to a viable manufacturing cost (N^{th} plant)

Summary of Project Performance

The project finished within budget and achieved all major objectives with the exception of initiating commercial operations in the United States. Completion of the project was delayed by one year due to delays in equipment, feedstock delivery and processing, and all associated risk mitigation activities to address the delays. Even with the delays, Amyris was able to manage the project to successful completion as determined by the DOE.

Detailed issues leading to delays:

- Issues with the two new 300L fermentors including a faulty exhaust design which did not allow for a double 'O'-ring exhaust filter to be fit into the housing, top dish nozzle modifications, and a probe port that required correction by the manufacturer – Sartorius Corporation all led to delayed start-up of the fermentors
- Significant modifications to a flash distillation skid unit from Continental Technologies following an operational and safety hazard review at Amyris
- Technical difficulties at NREL in processing sorghum bagasse material using their scroll delivery pretreatment reactor reduced the quantity of sugar provided and delayed initiation of the testing of material at Amyris. As a consequence DoE approved two six month "no-cost" extensions to enable completion of the lignocellosic sugar testing. This work was completed using recommended alternative suppliers of lignocellulosic sugars (M&G, OTFF).

The supply of lignocellulosic feed based fermentation vinasse to ICM was dependent on completion of lignocellulosic sugar based pilot scale fermentations. The ICM and final Praxair analysis work was completed in May 2013 using the no cost extension.

Project activity, spending timeline, and earned value figures are provided (Figure A.4, Figure A.5, Figure A.6, and Table A.1) below.

Figure A.4: Amyris IBR Program Gantt Summary (level 2 Tasks)

Table A.5: Budget Period Completion Timeline

Activity	Planned	Actual
Budget Period 1 (BP1)	01/01/10 thru 05/31/10	12/28/09 thru 04/21/10
Budget Period 2 (BP2)	06/01/10 thru 06/30/12	04/22/10 thru 06/30/13
Mechanical Turnover	09/10/10	09/10/10
Start-up	10/15/10	10/15/10
Commissioning (Fermentation)	11/08/10	01/15/11
Commissioning (DSP)	03/31/11	05/15/11

Figure A.5: Spending Timeline

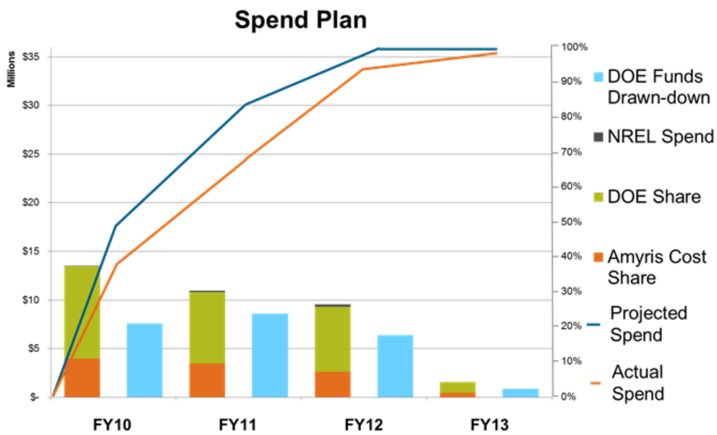
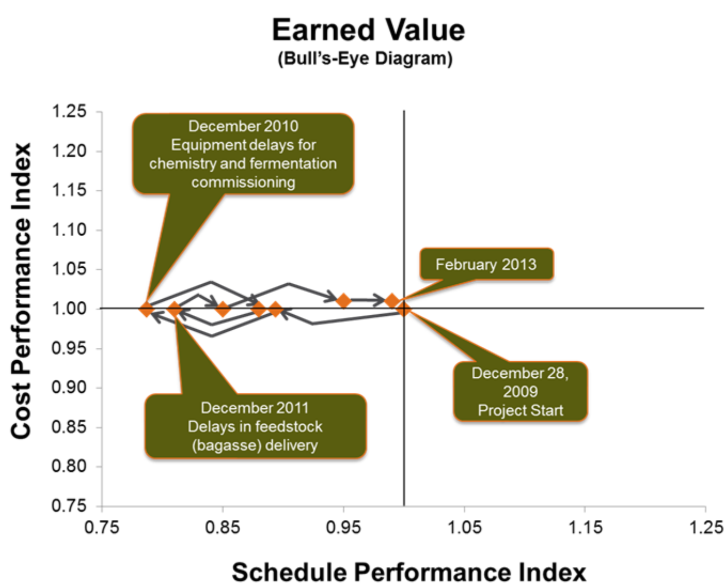


Figure A.6: Earned Value Bulls Eye Chart



Concurrent operation during facility expansion

During the design phase, Amyris's pilot plant continued to operate at original capacity using defined media and cane syrup. During the expansion, all fermentation systems, utilities, broth harvesting, chemistry purification and hydrogenation activities were ongoing. At that time the fermentation process development and strain testing were the main fermentation activities. Farnesene recovery by centrifugation, distillation purification, and hydrogenation were actively being developed.

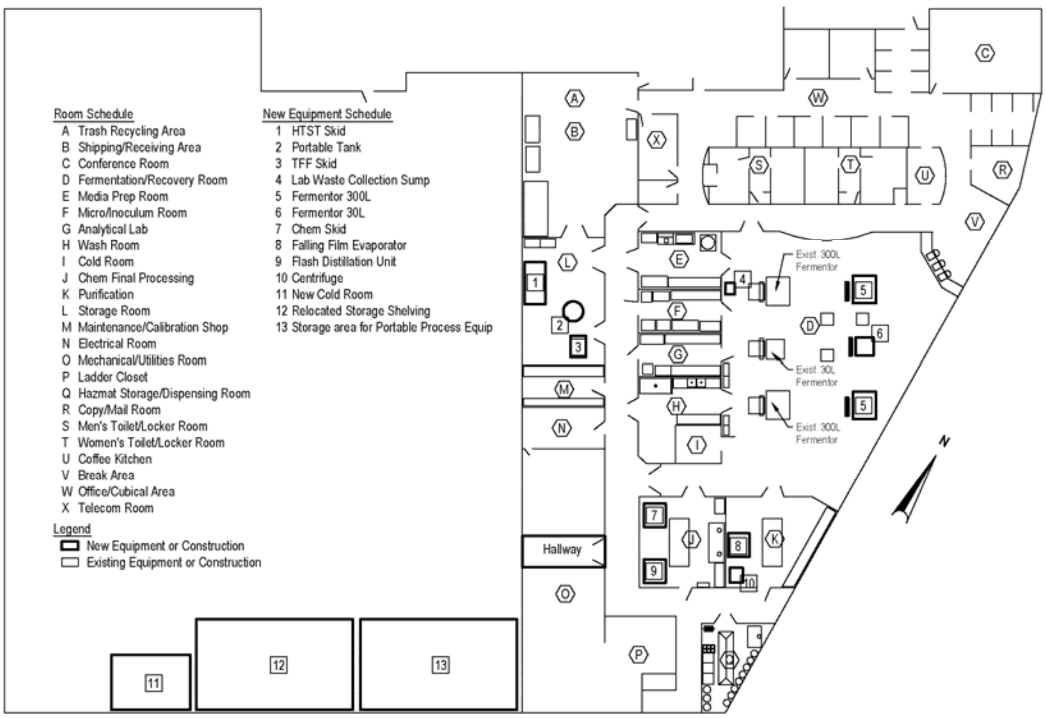
Once equipment that had been identified for the IBR expansion was specified and ordered, the team evaluated the potential facility and utility requirements needed for the new equipment. The flash distillation skid and chemistry skid required operational enclosures. The hoods/enclosures were designed and installed after the equipment was delivered as it was necessary to ensure that control valves, fittings, and connections would be accessible to operators working around the enclosed equipment. Heating and cooling requirements for the evaporator and chemistry skid would be through recirculating closed circuit systems. Clean dry air and nitrogen were plumbed to the enclosures.

Subtask A.2 Amyris pilot plant upgrade installation

The Facility was originally designed and constructed under separate design, bioengineering, and construction teams. Amyris had preliminary discussions with GL Planning & Design, Inc. (formerly Gicklhorn Lazzaroto Partners) and engaged the company for the architectural design and code compliance for the Facility. CRB Consulting Engineers were engaged for bio-engineering of the Facility, and Dome Construction was hired for design engineering and construction of the Facility. All three firms were used to build the original Pilot Plant. The floor plan of the expanded facility is shown in Figure A.7 below.

In addition to the engagement of these three construction based companies, Amyris had employees who possessed experience and skill in the areas of equipment specification, equipment operation, process development, process engineering, chemical process technology, and staff with significant biotechnology pilot plant experience.

Figure A.7: Plan of Expanded Pilot Plant



Equipment Expansion

During the course of the project, several pieces of major equipment were purchased to enable pilot scale operations of feedstock processing from fermentation to crude fuel separation to purification and hydrogenation to alternative chemistry. The equipment obtained for the expansion are shown in

Table A.6. All new equipment followed process safety review, dry commissioning, and wet commissioning testing prior to process operation.

Table A.6: Major equipment

Unit Operation	Step	Equipment Type	Supplier	Model	Notes
Fermentable Sugar Treatment	Clarification	Centrifuge	Westfalia	LAPX 4044	2-4 liters per minute (LPM)
	Screening	Vibratory Screen (400mesh)	Kason	K24 SS	
	Sterilization (HTST)	Tubular sterilizer (indirect) Direct Steam Sterilizer	Microthermics Barnum Mechanical	UHT/HTST Lab-DHEPV Custom	3LPM, 130-135°C 10LPM, 130-135°C
	Filtration	Tangential Flow Filtration	Graver	1.5-375A5P	Nominal Pore Size: 0.1micron Each module is 0.32 square meter (m^2) Membrane area with maximum of 4 cartridges is 1.28m^2
Fermentation	Inoculum prep	Fermentor (20L)	Sartorius	C plus-20	Batch and fed batch mode
	Fermentation	Fermentor (300L)	Sartorius	D-300	
Crude Fuel Separation	Liquid-Solid (L/S) Separation	Centrifuge	Alfa Laval	DX203	
	Liquid –Liquid Separation	Centrifuge	Alfa Laval	Gyrotester	
Purification	Distillation	Wiped Film Evaporator (WFE) Forced recirculation evaporator	Pope Continental	4” diameter, 1.12ft^2	0.5-1 liters per hour (LPH) 10 LPH
Hydrogenation	Hydrogenation	Fixed Bed Tubular Reactor	Parr	Custom Pilot 1L fixed bed	
Alternative Chemistry	Diels Alder Reaction	Process batch/fed batch tank	Pope	Custom 15gallon	

Fermentors

Fermentor installation was completed in December, 2010. Some delays to start up occurred during the commissioning phase between January and February of 2011 due to fabrication issues on the filter housing assembly, and the port design for sparger and top dish nozzles. A dissolved oxygen (dO2) port was refinished when a probe became lodged in the port due to a tolerance defect. The defects were corrected by Sartorius and the fermentors brought into full service February 2011. A general view of the fermentor skid is provided in Figure A.8 and

Figure A.9.

Figure A.8: Fermentor F3 in Operations after February 2011 commissioning

Figure A.9: View of the 2 Installed IBR Fermentors

Direct Steam Injection Sterilizer

The original indirect heat HTST sterilizer (Microthermics, Inc) had a maximum flow rate of 3L/min and could deliver about 800L of syrup before a full system cleaning was required. As the process run time for each batch increased, the internal surfaces of the tube heat exchanger accumulated foulants, causing sterilization issues. In order to obtain the heat transfer required to maintain sterile conditions, either the heating fluid temperature on the non-product side of the heat exchanger had to be increased, or the flow rate of sugar had to be decreased. Ultimately, these conditions made it very difficult to continue processing without risking the quality of the product or greatly increasing the operational time and effort to produce the feedstock. Given the issues with the indirect heat HTST unit, coupled with the increased demand and the need to process a greater variety of feedstock types, a recommendation to purchase the DSI HTST machine was made and accepted by the DOE.

A DSI system has a higher delivery rate, is more typical of systems utilized in a mill situation, and is able to maintain high flow rates across large batch volumes. The DSI sterilizer machine has a delivery rate of 10LPM, over three times the flow rate of the indirect HTST unit. The DSI sterilizer is able to process large sugarcane, sorghum, bagasse hydrolysates, and juice feeds whereas the indirect unit is not able to process large volumes. Similarly, in any case where there are significant solids, a DSI system is better designed to handle the effects of the solids on heat transfer surfaces.

The larger throughput of a direct steam HTST unit would require increased electrical power and increased cooling water demand. A new boiler was needed to provide the required steam rates for the steam injection media sterilization system. This unit is designed to draw a maximum of 102 kilowatts (kW) of electricity to generate > 100 psig of steam at approximately 310 lbs/hr. Figure A.10 shows an image of the Barnum direct steam HTST sterilizer.

Figure A.10: DSI HTST Unit

Flash Distillation Skid

The forced recirculation FDU skid provided an increased throughput at 10 LPH. A picture of the installation is shown in Figure A.11. The FDU provides a scale down representation of commercial scale evaporation options. The temperature operating range of the unit is 100oC-140oC with limits dictated by the tank rating. The vacuum is limited by the vacuum pump and can be operated at 2-10 Torr. The equipment was supplied by Continental Technologies, Kansas, USA.

Figure A.11: Forced Recirculation Evaporator

Chemistry Skid

The chemistry skid (Figure A.12) was purchased from Pope, Inc. The reactor enables pilot scale development of processes for the production of the alternative chemistry product (Task C), a plasticizer product. The plasticizer is produced by reacting farnesene with a suitable dienophile reactant in a Diels-Alder reaction. The reactor is capable of operating in batch mode or in a fed batch mode and has an operational volume of 15 gallons. The reactor has 18" diameter, constructed in 316L-stainless steel and is ASME (American Society of Mechanical Engineers) stamped and rated for full vacuum/50 psig at -50/250°C.

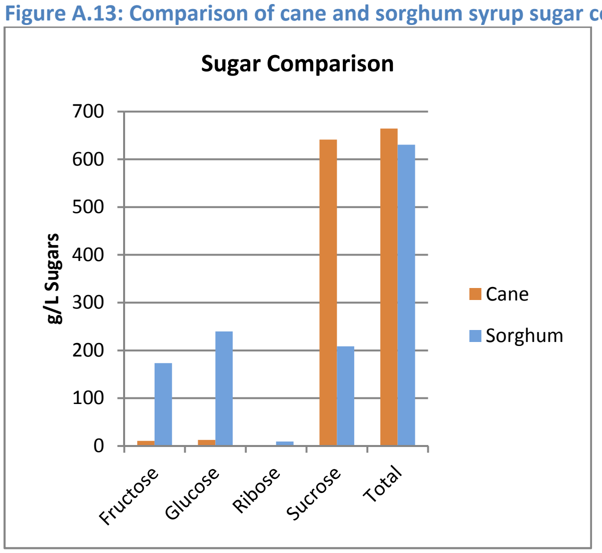
Figure A.12: Chemistry Reactor Skid (Pope)

Subtask A.3. Post-upgrade Amyris pilot plant operations

In late 2010 Amyris received shipments of Tennessee sorghum syrup from Ceres. Starting in October through November, 2010 Amyris investigated methods for clarification of the syrup. Initially, an attempt was made to utilize the TFF using 0.1 µm Graver titanium dioxide/stainless steel tubular membrane system at 50-70 ^{o}C. The high viscosity and 5% v/v solids content of the sorghum syrup reduced the filtration flux to less than 2 L/m^2/hr (LMH) and limited the supply of clarified syrup. In addition, the operation of the TFF unit at 50-70 ^{o}C and holding hot syrup for several days was not desirable and would be expected to result in degradation of the feedstock quality. The TFF/sterile filtration method was discontinued and replaced with centrifugation (DX203), followed by a Kason vibrating screen (400 mesh or 37 µm) to clarify the syrup prior to sterilization in the Microthermics indirect heating HTST sterilizer. This was the method used for heat sterilization prior to installation of the DSI sterilizer. The first sweet sorghum syrup 300L fermentation batch was completed in December 2010 using this feedstock preparation approach. After commissioning of the new fermentors we continued evaluation of the sweet sorghum syrup in 300L fermentation as well as evaluation of defined dextrose media and cane syrup in the fermentation and recovery equipment.

The Tennessee syrup was evaluated and the sugar content analyses showed that the sorghum syrup contained higher levels of the C6 sugars compared to cane syrup (Figure A.13). The sweet sorghum syrup performed similarly to cane in the 300L fermentation process.

Figure A.13: Comparison of cane and sorghum syrup sugar content



The TFF clarified/sterile filtered and centrifuge clarified Tennessee sorghum syrups were evaluated through fermentation, separation and distillation. The TFF material produced a very clean cane syrup equivalent distillate while the centrifuged material resulted in the crude farnesene exhibiting a green color. The purity of the crude farnesene, obtained from fermentation using various feedstocks, indicated that the TFF sorghum based fermentation produced similar purity to a dextrose fed fermentation. Both the cane syrup and centrifuged/screened sorghum syrup were similar but lower purity than the dextrose and filtered sorghum (see Table A.7).

Table A.7: Crude Farnesene purity as a function of feedstock used in fermentation

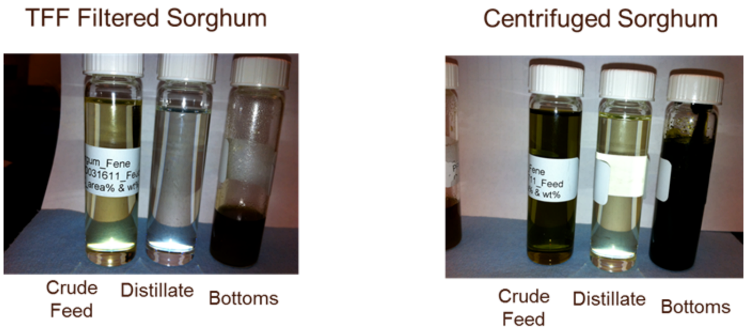
Nutrient feed	Crude farnesene purity (% w/w)
Defined Medium	96.5
Cane syrup	95.2
Sorghum syrup (TFF)	97.3
Sorghum syrup (Non-TFF)	94.3

The distilled material prepared from crude farnesene obtained from TFF/sterile filtration versus the centrifuged/screened/HTST method showed some interesting physical differences. The TFF method removed components resulting in green coloration and wax forming components after distillation. In both cases the final wt. % purity of the material was similar (see Table A.8 and Figure A.14)

Table A.8: Distilled farnesene purity as a function of fermentation feedstock used in fermentation

Process	Crude	Distilled	Hydrogenation
TFF	97.3	97.46	97.21
Cent	94.3	97.13	97.28

Figure A.14*: Appearance of distillation outputs obtained from TFF and non TFF sorghum feedstock fermentations*



By the end of 2010, data from runs at the pilot plant had established that there may be some differences in feedstock properties resulting from the different clarification approaches. The TFF process was producing a clearer feedstock with no solids which resulted in higher purity crude farnesene. Robust distillation resulted in separation of the particulate based contaminants present within the centrifuged sorghum resulting in similar purity and yield of distilled purified farnesene. The distillation produced farnesene at 97% purity, removing trace metals and high boiling point impurities.

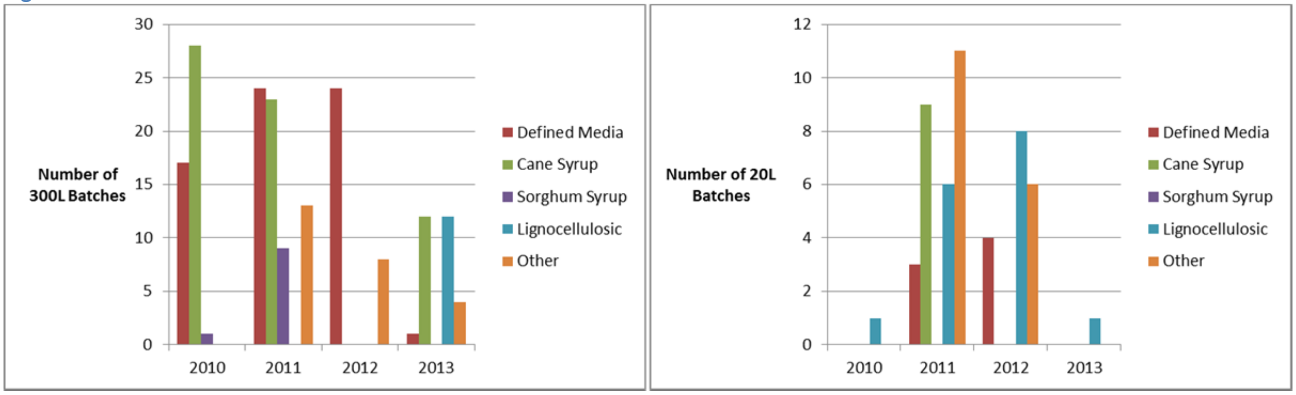
In the case of the non-TFF sorghum syrup, the persistence of feedstock components throughout the process resulted in increased amounts of cold insoluble and wax forming components which have to potential to impact on the distillation cycle. The use of TFF as a clarification method for syrups was not feasible based on the very low flux rate (volume of filtrate per unit area per hour). Following the identification that TFF was not feasible we needed to consider some alternative upstream feedstock processing methods. Some approaches considered were reduced solids in feedstock, centrifugation, and vibrating screen mesh sieves. At the stage of distillation, using the wiped film evaporator (WFE), filtration of the crude farnesene was implemented.

Some evaluation on the use of anti-foulants helped to minimize fouling by gums in distillation equipment. Use of N_2 overlay in the evaporator feeds also had a benefit in minimizing the foulant accumulation resulting from oxidative mechanisms. The Parr packed bed hydrogenation system was operational at the pilot plant. This enabled us to obtain small quantities of of farnesane for characterization, and also enabled process optimization work on production of biodiesel.

The sorghum syrup fermentation evaluations, using the Tennessee 2010 Ceres syrup occurred in late 2010 and early 2011. The fermentation activity for the 300L and 20L development batches is shown in

Figure A.15. Investigations of strains and process improvements using defined or cane syrup occurred throughout the duration of the program in the 300L fermentors. Similarly, the initial separation and purification of sorghum derived farnesene occurred in late 2010 and early 2011. Small quantities of lignocellulosic materials were obtained from M&G (Arundo donax, wheat straw), and NREL (sorghum bagasse) during 2011 and early 2012. Wheat straw and wood chip lignocellulosic sugars were tested in the 300L fermentors in early 2013. The recovery and purification of wheat straw and wood chip sugar derived farnesene was performed in conjunction with the fermentations in 2013. A performance test was completed on the overall sorghum syrup process, including pilot plant production of farnesane and plasticizer during August through November, 2011. A report on the performance evaluation was issued to the DOE in February, 2012. Performance analysis of lignocellulosic sugar based processes from fermentation through to farnesane and plasticizers was completed from January through April, 2013. A report on that work was issued in July, 2013.

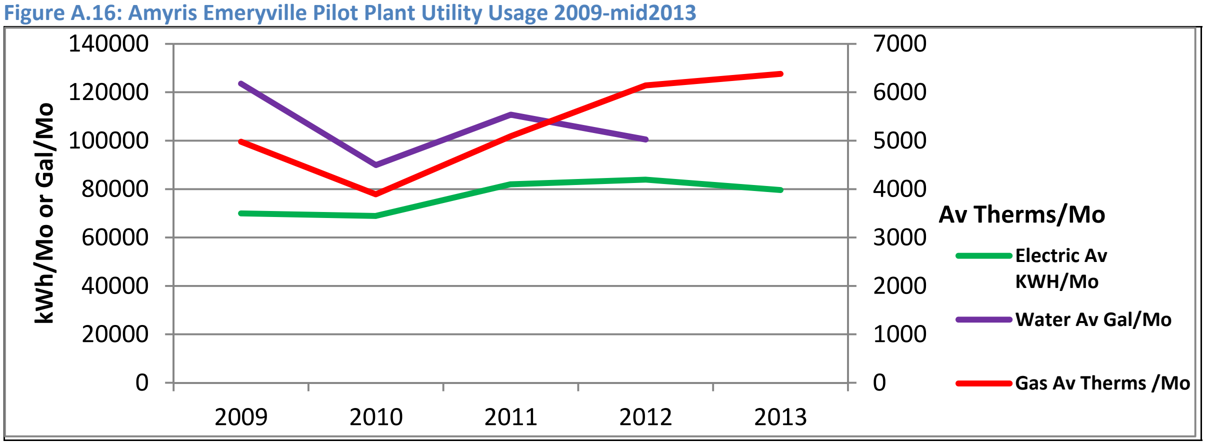
Figure A.15: Fermentation Pilot Plant Fermentation Activities 2010-2013



The Pilot Plant key utility consumption was monitored for the duration of the operations. There was no indication of a step change in consumption following the expansion and installation of the new equipment in 2011. Gas consumption, which is the source of energy for the plant steam boilers, was the only utility showing any significant increase from 2010 through 2013. This may be a result of increased piping, condensate generation, and an increase in sterilization cycles.

It is reasonable to assume that the bulk utilization rates in a relatively small pilot plant would be less sensitive to activity, and difficult to correlate to a utility consumption rate for a commercial scale plant. The utility usage is shown in Figure A.16 below.

Figure A.16: Amyris Emeryville Pilot Plant Utility Usage 2009-mid2013



The indirect HTST unit was used for sugar sterilization until November 2012. After that point, the Barnum direct steam sterilizer was used to prepare sterile sugar feedstock. The feedstock preparation data is provided in **Error! Reference source not found.** below.

Nameplate capacity

The nameplate capacity was evaluated in terms of the capacity of the plant to handle dry tons of biomass. The pilot plant does not operate a biochemical conversion process to convert biomass to sugar. The biochemical conversion occurred at NREL and the juice concentration to syrup was managed by Ceres. However Amyris was able to determine the input of biomass by back calculating biomass based on the sugar utilization rates in the fermentation process, and the agronomy data from Ceres and NREL.

The juice sugar content of the sweet sorghum was determined by Ceres and the sugar content of the sweet sorghum bagasse was determined by NREL. From the data provided in Task B and the Nth (Optimized strain, agronomy, conversion, and process) plant data for the fermentation process, the biomass throughput of the pilot plant was determined to be greater than 1 mT/day.

Task B: Scale-up and economic analysis of Amyris diesel production from sweet sorghum

Task Objective: To complement the pilot plant upgrades and operations in Task A, Amyris and partners will focus significant effort on process optimization and integration from feedstock to final diesel product finishing processes and waste treatment.

Subtask B.1. Sweet sorghum development, syrup and bagasse production

- *Completed April, 2012*

Subtask B.2. Lignocellulosic pre-treatment development and scale-up

- *Completed January, 2013*

Subtask B.3. Fermentation scale-up (leveraging analytics vendors Herguth, Medallion, or similar providing equivalent services)

- *Completed April, 2013*

Subtask B.4. Anaerobic digestion development and hydrogen production

- *Completed June, 2013*

Subtask B.5. Recovery, purification and diesel finishing scale-up (leveraging vendors Intertek, Acorn Labs or similar providing equivalent services)

- *Completed April, 2013*

Subtask B.6. TEA and LCA for Amyris Diesel production

- *Completed June, 2013*

Accomplishments Against Goals

Subtask B.1. Sweet sorghum development, syrup and bagasse production

Hybrids in four geographies were evaluated for crop productivity. Various traits established in the hybrids were assessed to determine productivity including maturity, lodging resistance, and sugar production rate.

Maturity is the most important trait to consider when looking across geographies. There are early, midseason and long season hybrids. It is important that maturity of hybrid matches the length of the growing season. If the maturity is too late, for example, the hybrid could be subjected to frost and perish before it produces high sugar concentrations. In the IBR project all the hybrids used were midseason.

Lodging resistance will be more important in geographies where you have a threat of strong storms and high wind. Lodging resistance is measured by the thickness of the stem.

Quick sugar production (use of early hybrids) is beneficial to extended harvest seasons. It is common to use a combination of early, midseason and long season hybrids to maximize the harvest time (in conjunction with running of the mill). Also it is possible to extend the mill operations by using multiple plantings to provide a consistent supply of feedstock throughout the whole harvest season.

The south east plantings (Florida, Tennessee, and Alabama) provided consistent yields across the planting windows while the Hawaii plantings showed the yield decreasing with later plantings. One Hybrid (3) appeared to produce better consistency of yield in the staged Hawaii planting test. Larger scale planting and syrup supply was completed at the Florida and Tennessee locations. The 2010 Tennessee yield and supply was the highest -of all geographies and served as the source of test sorghum for Pilot Plant fermentation runs. There were some weather setbacks in the 2011 Tennessee field trials that led to lower yields. Bagasse production was successful with high yields being obtained in the Tennessee 2010 field trial, supplying approximately 6 dry mT of bagasse to NREL for evaluation and biochemical conversion into fermentable sugar.

Project Activities

Subtask B.1 Sweet sorghum development, syrup and bagasse production

Ceres is an integrated seed company utilizing advanced breeding and biotechnology to develop seeds with specific traits for use in crops for biofuels, biochemicals, and biopower. They were established in 1997 and are headquartered in Thousand Oaks, California. Portfolio crops include switchgrass, miscanthus, high biomass, and sweet sorghum. They also provide genes for traditional row crops. The IBR project leveraged Ceres's expertise to analyze the composition of sweet sorghum, collect and synthesize agronomic data, and produce sorghum syrup and sorghum bagasse.

Sweet sorghum development Year 1

Initial efforts were on obtaining agronomic and compositional data across different geographies with different hybrid sweet sorghum crops. Sweet sorghum can be planted in a wide variety of locations. The plantings occur once the soil temperature reaches 65^{o}F. Crops were typically planted in a staggered time frame with repeated plantings 15 to 30 days apart to prolong the harvesting phase across multiple plantings. For these trials the main tasks performed were as follows:

1. Sweet sorghum crop development research through small-scale hybrid trials across multiple geographies and planting times to provide compositional analysis and small volume samples for fermentation lab evaluation.
    a. Florida
    b. Alabama
    c. Louisiana
    d. Hawaii
2. Production of sorghum syrup and bagasse from large-scale plots. This provided >1000 gallons of syrup and approximately 6 dry tons of bagasse
    a. Florida (Arcadia and Moore Heaven)
    b. Tennessee (Whiteville)

The three hybrids were selected based on their ability to achieve high stalk concentrations of sugar. In addition the rate of sugar accumulation is a trait that can be developed for short growing season location.

The juice analysis data showed that sorghum juice exhibited greater inversion than what is typically produced by sugarcane crops. The sugar content of the juice decreased in later plantings for Hybrids 1 and 2 in Hawaii, otherwise Hybrid 3 in Hawaii and Hybrids 1-3 in all other locations appeared to be similar across planting dates. The Hawaii harvesting was around 80-90 days versus around 120 days or greater for other locations. Bagasse composition analyses for the three hybrids in Hawaii showed very little change in composition of glucan, xylan, and lignin, as a function of hybrid or planting time.

Sweet sorghum production Year 1

Sorghum juice was harvested at the Tennessee and Florida sites during 2010. At the Florida site, juice was harvested from three field strips and concentrated to provide an initial 87 gallons of 61^{o} Brix syrup to Amyris. The Florida site also produced 3.29 tons of biomass from a single hybrid. The Tennessee site then produced 1402 gallons of 63^{o} Brix syrup from a combination of the four hybrids. There was no clarification process in the field at the Tennessee location and Amyris received syrup containing up to 5% v/v solids.

Field process operations issues were identified during the first year at the Florida site. The main problems were:

- Mechanical problems related to in-field harvesting and juice extraction
- Harvesting by hand cutting and stalk pressing were time consuming and lead to a reduction in yield and quality
- Lack of large batches led to storage of juice at 40 ^{o}F and subsequent contamination
- Problematic evaporator operation and maintenance
- Operational learning curve

Further agronomy challenges were experienced in the Florida location and were based around the unique climate conditions and cultural practices. Some specific considerations for the Florida location in year 1 were with:

- Providing optimal fertilization
- Maintaining sufficient soil moisture through an underground irrigation system in sandy soils
- A prevalence of plant diseases and pests
- A steep learning curve to manage agriculture conditions in Florida

Specifically, the sandy soil and high humidity provided special challenges at the Florida location in year 1.

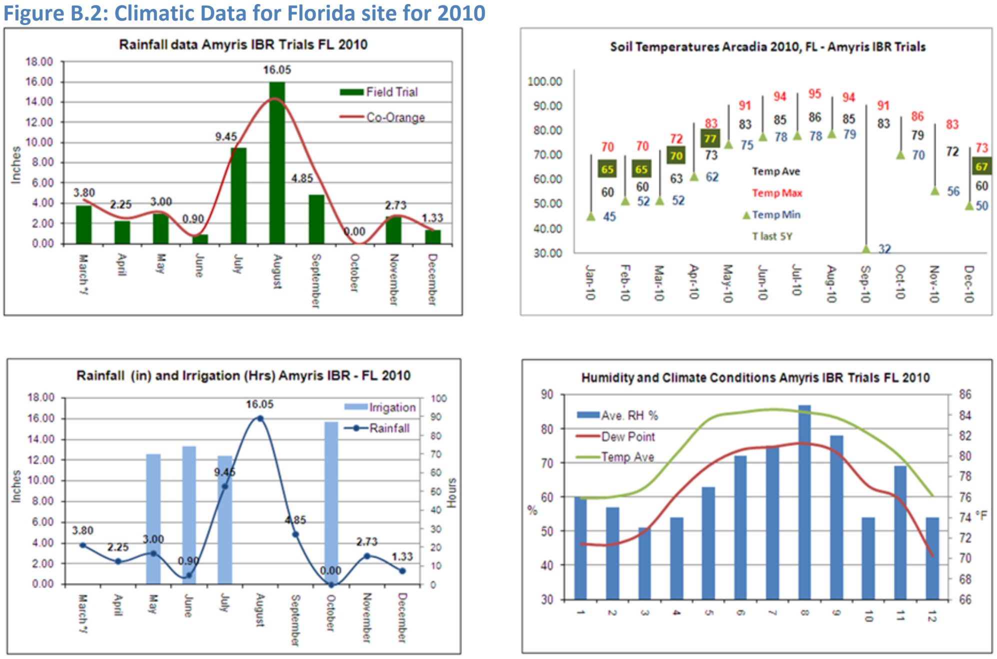
- High weed pressure increased the need to apply pre-emergent herbicide
- Multiple fertilizer applications were needed due to the rapid leaching of nutrients
- Soil was deficient in minor nutrients (Mn, Zn, Cu, Fe, B)
- Favorable environment for plant diseases and pests
- Difficulty achieving uniform irrigation

The images shown in Figure B.1 contrast the various planting sites. Climatic data for Florida in 2010, shown in

Figure B.2, underlined the need to coordinate irrigation, planting, fertilization, and pest control operations in that locale due to significant rainfall, high humidity, and high temperature which all coincided with the crop season. Figure B.3 shows the initial planting conditions as well as the improved conditions based on lessons learned.

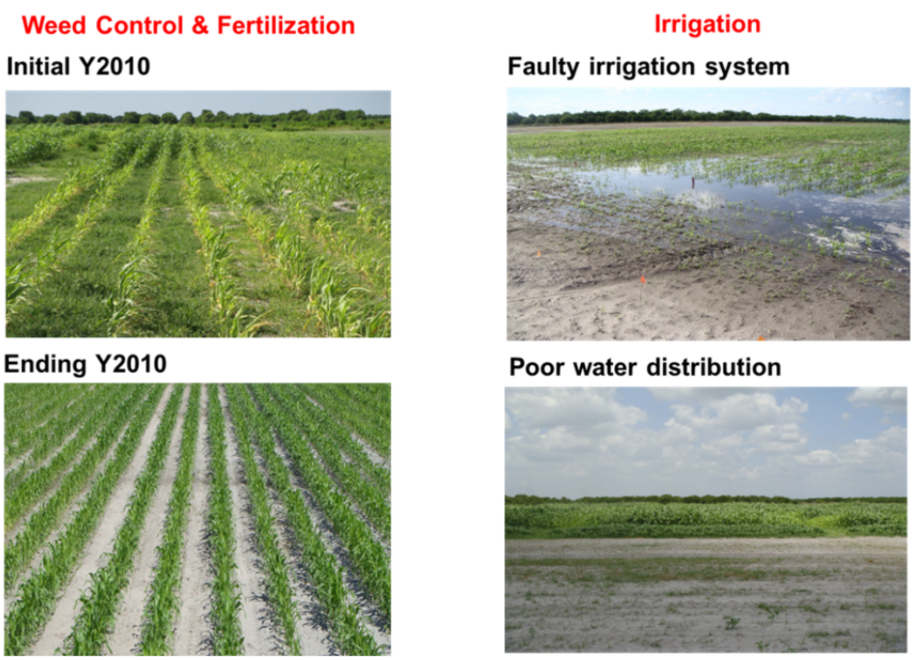
Figure B.1: Site comparison images for sweet sorghum crop

Figure B.2: Climatic Data for Florida site for 2010



Source: Ceres and Orange Grove Co. & FAWN Florida Automatic Weather Network

Figure B.3: Images showing improved field operation results at Florida site 2010



Agronomic yields were assessed at the Tennessee site in 2010. Hybrid 3 showed the highest crop density and sugar yield per acre. The juice content of the Hybrids was similar in the Tennessee crop as indicated by similar 100-110 day field Brix measurement values.

Sweet sorghum development, syrup and bagasse production Year 2

The Ceres goals for year 2 (2011) were to replicate the net plot planting data in Hawaii, Florida, Tennessee, and Alabama using at least 2 planting dates. In addition sorghum syrup would be sent to Amyris and bagasse sent to NREL from the Tennessee and Florida sites.

Following the year 1 experiences in Arcadia, a second Florida location, Clewiston (26°N, 80°W, Elevation 13 ft.), was obtained. Improved field capability at the Florida location was secured through the rental of a pilot scale mill from May through September 2011. Sufficient evaporator spare parts were obtained and improved maintenance practices implemented. In addition, Florida field practices included planting four hybrids at 3 acres per hybrid. For Florida harvesting, sugarcane harvesters were utilized to produce billets.

The first Florida planting date was March 2011 with harvesting occurring in July. Further crops were planted in April and May with harvests in August and September. The total production of Florida syrup was 1687 gallons.

During 2011 the Whiteville, Tennessee (35°N, 89°W, Elevation 492 ft.) crop was subject to some weather related issues. High winds and rain caused the plants to exhibit lodging, a process where felled stalks would regrow vertical canes. This impacted the sugar production and yield per acre. The Tennessee plantings were May and June, with harvest in September and October.

Four Hybrid varieties were evaluated at the Florida site and the sugar content crop density data showed that Hybrid 1 performed best. The genetic differences between the four hybrids were the slight variations in traits for rate of achievement of peak sugar concentrations. These included the same hybrids used in both 2010 and 2011 plantings. Analysis of sugar content for Florida hybrids showed very similar total content and distribution of sucrose, glucose and fructose at 122-125 days.

The Tennessee results showed that Hybrid 2 and 3 produced higher levels of invert sugar. Generally it appeared that Tennessee crop performed better than the Florida crop, but again was less productive than 2010, likely resulting from the lodging. There was little variation in the glucan levels for all examples except for the Florida Hybrid 1. It appeared that Hybrid 1 grown in Florida had relatively low sucrose, and slightly increased glucan content.

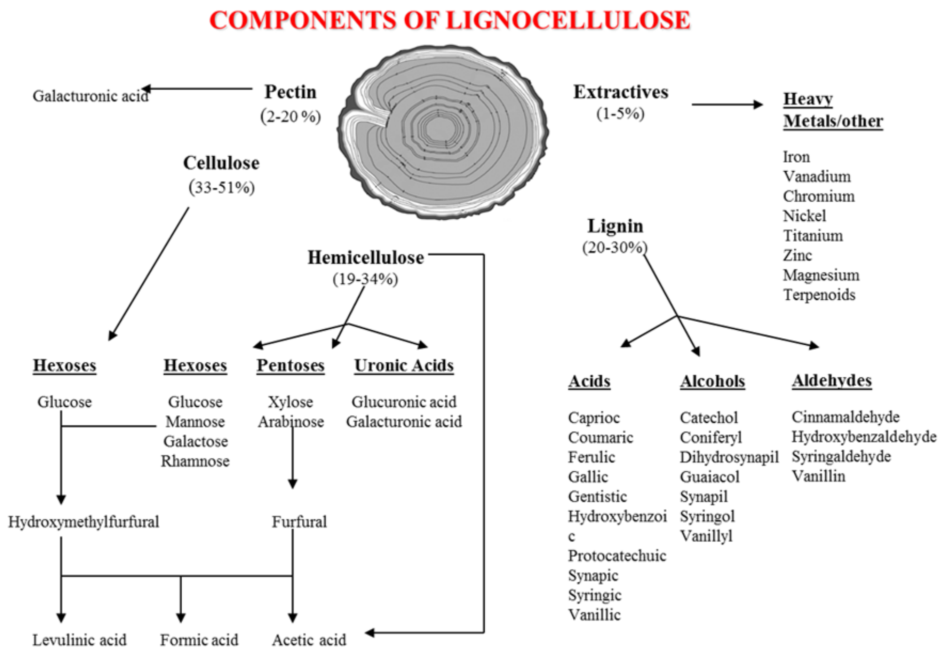
Subtask B.2 Lignocellulosic pre-treatment development and scale-up

Sorghum bagasse was provided by Ceres from the Florida and Tennessee sites. NREL was responsible for the initial analysis of sorghum bagasse. Feedstock specific analytical protocols were already established at NREL for sugars and polysaccharides. Organic acid procedures also existed at NREL and were adopted at Amyris. Measurement of salts and minerals was performed at external analytical service laboratories (McCampbell Analytical Inc. Labs, Pittsburg, CA).

Lignocellulosic biomass sugars are present in polymers in the plant cell wall and provide plant structural support. Cellulose is the main polymer constituent and hemicellulose is the second most abundant polymer of the plant cell wall. Glucose is present in cellulose (β-1, 4-glucan) which is crystalline and resistant to chemical hydrolysis. Hemicellulose contains several sugar units, the most predominant being xylose present as xylan. Other subcomponents of hemicellulose are galactan and aribinan, which yield mainly glucose and a smaller amount of arabinose. Hemicellulose is more easily hydrolyzed than cellulose, releasing a portion of the sugar during chemical pretreatment. Sorghum bagasse hemicellulose contains acetylated arabino-xylan and the hydrolysates therefore contain acetate. Pretreatment processes are designed to open up the cellulose structure to enable access for the cellulase enzymes for effective hydrolysis to glucose. Pretreatment can involve treatment with acid along with incubation at elevated temperatures for a fixed time, or be non-acid based and involve temperature and time as the pretreatment parameters. The high temperature pretreatment conditions can potentially lead to degradation of the sugars to toxic byproducts such as furfural and hydroxyl-methyl furfural (HMF). A simple depiction of lignocellulose composition and corresponding derivatives is shown in

Figure B.4 below.

Figure B.4: Illustration of Lignocellulose components



Lab Scale bagasse process development

In Year 1 of the program, NREL provided analysis of the sorghum bagasse material supplied by Ceres. The results showed that sweet sorghum bagasse had a slightly higher lignin content than corn stover, but lower than sugarcane. The bagasse glucan content was between that of corn stover and other standard sorghum material previously analyzed by NREL. The acetyl level was similar to that of corn stover.

Following the characterization of sorghum bagasse composition, NREL worked on the evaluation of the pretreatment process. Temperature, time, and the use of dilute acid were the initial variables examined. For the early work, six conditions were tested, four with acid and two without. Experiments were performed in a small scale lab procedure using a steam gun with multiple shots of each material. The target was to obtain 10 kg of pretreated material per condition.

Following pretreatment, the small scale study samples were enzymatically hydrolyzed using standard conditions (40mg/g dry weight), in bottles in a shaker incubator at 48 ^{o}C, at an initial pH of 5.1. After hydrolysis the samples were clarified in a lab floor centrifuge, then evaporated to concentrate the sugar liquor in a 3L rotary evaporator at 60 ^{o}C under vacuum. In campaign one, around 0.5-1.0L of approximately 800g/L sugar were produced from each condition. The various samples were analyzed to quantify sugar content as well as potential inhibitors such as furfural, HMF, and acetate.

Based on the analyses from the various pretreatment conditions, it appeared that the best enzymatic glucose yield occurred for Condition 2, a non-acid treatment at high temperature, followed by Condition 3 which was dilute acid at a lower relative temperature. Samples from all conditions were tested in fermentors to evaluate yield of farnesene. The results showed that the non-acid treated samples (2, 1) supported the best yields and those fermentations did not accumulate ethanol. Ethanol was produced in the cases of the lower yield fermentations and was not re-consumed during the production phase. In Campaign 2, from March through April 2011, four pretreatment conditions were evaluated in the steam gun equipment with multiple shots, this time at a larger basis to target 25kg of pretreated material per condition. This was followed by standard enzymatic hydrolysis and evaporation. The enzymatic hydrolysis was performed in a roller tube incubator (4 tubes per condition) at 48 oC and an initial pH of 5.1. Yields were calculated from a smaller sample bottle in a shaker box. The larger samples were clarified using a Western states centrifuge. The evaporation was performed in a Jaygo reactor at 55-70 ^{o}C under vacuum. Approximately 10L of hydrolysate sugar, at around 200g/L, were produced.

NREL pilot scale production of sorghum bagasse

Dried sweet sorghum was passed through a knife mill fitted with a ½" screen, then washed. A high-solids stirring reactor with a volume of 1900L was used to perform the washing step. Milled biomass was added to the reactor, which contained some water. Additional water was added and the reactor stirred for 10 min. The water was then drained. Once drained, more water was added and drained again. Then the wet biomass was either vacuum dried at 60 ^{o}C in the reactor, or collected and passed through a screw press. The water content, if dried, was below 10% and if pressed was around 50%.

The material was then pretreated in either a horizontal or vertical reactor with either dilute acid or steam at approximately 190 ^{o}C for 1-20 min. **Error! Reference source not found.** details the pretreatment conditions for each pilot run. The variations in the production runs are due mainly to operational difficulties experienced in operating the pretreatment reactors and also to some operational improvements made to the process over the course of the project.

The pretreated material was enzymatically hydrolyzed with Novozymes' Ctec 1 enzyme, loaded at 40 mg protein/g cellulose, at 50 ^{o}C for approximately 96 hours. The hydrolysates liquor was then separated from the solids in a solid bowl decanter, then evaporated at either 60 ^{o}C under vacuum or at approximately 96 ^{o}C to above 200 g/L, and then a final centrifugation step was performed to remove the additional solids. The composition of the resulting hydrolysate samples is discussed in the next section.

Operational Analysis of Pilot Scale Pretreatment Process

With its years of experience processing sugarcane bagasse, NREL approached the project with the expectation that the sorghum bagasse would perform similarly at pilot scale. Unfortunately, the field processed sorghum bagasse was not processed as efficiently as the sugarcane bagasse, and sucrose was present in the sorghum bagasse billets. During pretreatment processing, this led to crystallization of the residual sugar at the high temperatures and pressures, and to a problem with jamming the pretreatment reactor delivery screw and some valves. The bagasse, therefore, had to be washed and either dried or pressed prior to feeding it to the pretreatment reactor. This took some time to complete and delayed the supply of materials for fermentation testing.

With the sucrose issue resolved, the focus now was on selecting a pretreatment method which would enable the most desirable combination of outcomes. Given that the IBR project was evaluating a 2nd generation process, using a yeast strain that does not utilize C5 sugars, pretreatment for glucose release was the main goal. The xylose produced was to be considered a side stream which is sent to the anaerobic digester. From that perspective, the pretreatment yields were not strictly important. The objective was to use a pretreatment strategy that led to an improved digestibility of the cellulose to glucose. The horizontal reactor with steam pretreatment at 190 ^{o}C for 20 minutes allowed for good cellulose digestibility. However, in order to maintain consistently high enzymatic hydrolysis yields, the corresponding enzyme loading needed was 40 mg protein/g cellulose. New enzyme cocktails exist that may allow for similar yields at lower enzyme loadings but were not investigated for this project. Overall it is likely feasible to reduce the enzyme loading and maintain sugar yields between 85% and 90% to improve the process economics.

A further consideration at NREL was the post enzymatic hydrolysis processing. The equipment available at the NREL pilot plant was not ideal for clarification and concentration of the hydrolysate sugar solution. Therefore, some of the physical losses attributed to these steps were due to the equipment used. Separating the solids after enzymatic hydrolysis is a very difficult task to undertake but a necessary one as small, fine solids will clog filters and membranes in subsequent processing steps. NREL attempted to use their solid bowl decanter to remove the bulk of the solids, but this technique created a major loss of sugar as the solids removed contained about 70% to 80% voidage liquor. They attempted to further clarify the solution with a filter centrifuge as a final step, however, the fine solids clogged the filter and prevented continuous operation. A learning or suggestion arising from this was the potential operation of a larger centrifuge with an automatic cleaning system that would allow for a semi-continuous operation. At the time of this work, that equipment was not available at NREL. A concern, from the actual processing difficulties experienced, was that the liquor sat for extended periods of time before being concentrated or fermented. This can significantly increase the risk of feedstock contamination or formation of components that could be inhibitory to the yeast in the fermentation process. This potential loss of sugar, and increased contamination risk, could negatively affect the economics of sugar production.

In order to maximize efficiency, two methods for evaporation were explored. Attempts to vacuum dry the material at 60 °C proved to be time consuming, with the liquor spending days at temperature which leads to glucose degradation over time. Such degradation can be potentially catalyzed by other components present in the liquor. Further, from a process economic standpoint, vacuum evaporation is a costly process. The other process option was to evaporate the liquor at 96 °C and atmospheric pressure. This process took some time to fine tune due to the presence of solids in the liquor. While evidence of degradation was also observed with this process, less time was spent at the elevated temperature. The pilot 5 condition resulted in higher furfural yield per xylan. This may have resulted from extended time at high temperature during pretreatment.

The process flow diagram shown in Figure B.5 provides estimates for the material mass balances for a typical bagasse test run. Estimates are based on data provided by NREL. The post evaporation sugar concentration (Figure B.6) showed levels of glucose in the range 250g/L and xylose in the range 50g/L from the non-acid process. Based on the sugar concentrations and an average post evaporation output of 900kg (step13), the total glucose was 204kg glucose and 40kg xylose per metric ton dry bagasse.

Figure B.5: Flow Diagram of NREL pilot scale sorghum bagasse pretreatment and hydrolysis process

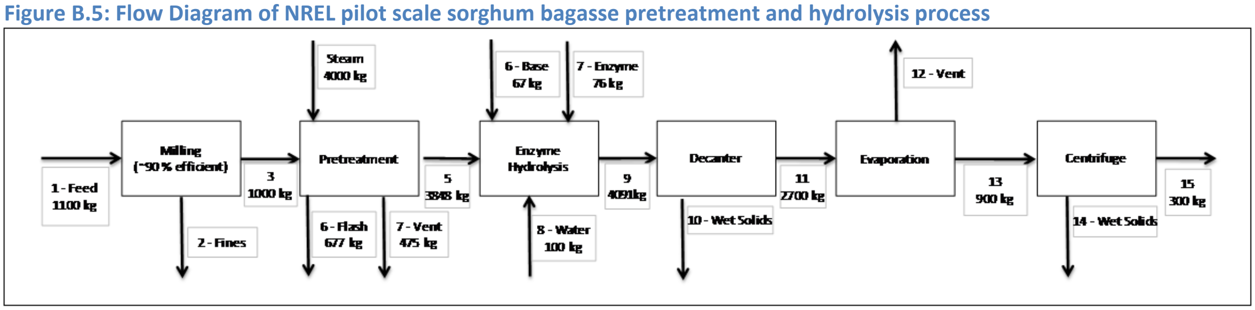
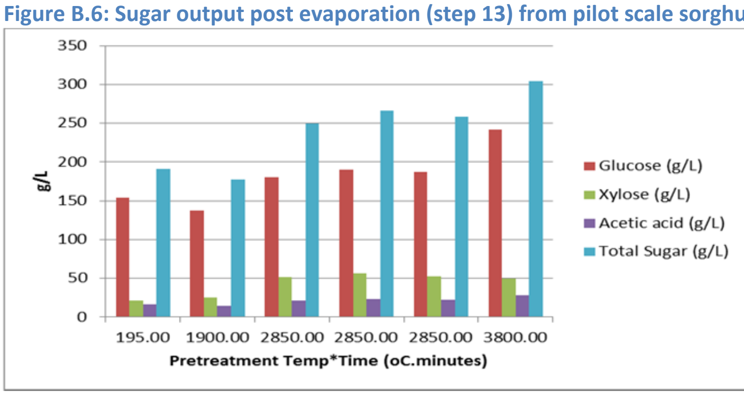


Figure B.6: Sugar output post evaporation (step 13) from pilot scale sorghum bagasse pretreatment and hydrolysis



Wood biomass sugar

Two 1000L batches of cellulose derived sugars using a proprietary process that produces “clean” cellulosic sugars from woody biomass were obtained from OTFF in Maine. OTFF operates a Kraft process for paper pulp production and has a side stream capability whereby the white liquor can be sent to enzymatic digestion for production of fermentable sugars. The cellulosic material is enzymatically treated to release glucose and xylose. Following the hydrolysis step, the sugar is purified to remove salts and organic acids that may be inhibitory in fermentation. After purification, the sugar solution undergoes evaporation to achieve a concentrated and stable sugar solution in the TRS range of 450-600 g/L which contains 80% glucose, 20% xylose by weight. The sugar did not contain any measurable inhibitors or salts.

Wheat straw sugar

Two thousand liters of wheat straw biomass derived sugar was obtained from M&G Chemtex in Rivalta, Italy. The 2000L of sugar were derived from 600kg of dry wheat straw biomass. The typical TRS sugar concentration of this product is in the range 80-100g/L, which is much more dilute than other sugar streams tested in this project.

Subtask B.3. Fermentation scale-up (leveraging analytics vendors Herguth, Medallion, or similar providing equivalent services)

Process Descriptions:

The process descriptions listed below represent the different types of fermentations strategies that are known strategies used in fermentation fed batch systems. Some were further developed or optimized for use in this project.

Single Fed-batch Fermentation: The sugar source is added using a fed-batch feeding process (see section below for various feeding strategies). Once the tank is filled to maximum capacity, the entire culture is harvested for the isolation of product.

Fill and Draw (FAD) Fermentation: The sugar source is added using a fed-batch feeding process. Once the tank if filled to maximum capacity, a percentage of the culture is removed and the fed-batch feeding process is continued. The cycle is repeated until a predetermined time is met.

Condensed Fill and Draw (cFAD) Fermentation: Similar to the FAD strategy described above, but as implied in the name, the growth phase is condensed. This is achieved by increasing the oxygen transfer rate (OTR).

Continuous (Chemostat) Fermentation: The sugar source is continuously added to the culture at a predetermined dilution rate and broth is removed to maintain constant volume. This strategy is used to maintain a constant environment and predefined level of growth. At steady state, the growth rate is equal to the media dilution rate.

Sugar Feed Delivery Strategies:

Below are the various types of sugar delivery (feeding) strategies that have been used during the course of the project. The acronyms of the feeding strategies will be used throughout the report.

Batch: The sugar source is batched into the tank. The yeast culture consumes all the sugar and converts it to biomass, byproducts, or farnesene. Once all the sugar is depleted, the fermentation is complete or moves onto the next phase of the process.

Exponential Feed Ramp: The feed rate increases exponentially in order to match the cellular growth rate of the culture, while keeping the sugar limited.

Micro aerobic Pulse Feed (MAPF): Process intended to minimize ethanol accumulation. The culture is initially grown in batch until the sugar is depleted and the dissolved oxygen (dO2) spikes. In between, doses of a low feed rate of sugar are added until the culture consumes all the sugar and another dO2 spike occurs, which triggers another dose. This is continued until the fermentation is ended.

Adaptive Micro aerobic Pulse Feed (MAPFx): The MAPFx process is the same as the MAPF process, with a difference in the pulse feed rate. The feed rate delivering the dose changes depending on the length of time the culture takes to re-consume any residual ethanol. For example, if the time between spikes is short the feed delivery rate increases. The feed delivery rate adjusts to minimize the time between a spike check. This allows the feed rate to be slightly higher than the sugar consumption rate, minimizing the formation of ethanol.

Constant Feed with Spike Checks (CFSC): This process is the same as the MAPFx process but with longer duration, lower amplitude sugar pulses. This allows the feeding strategy to get closer to a constant feed process.

Aerobic Constant Feed (ACF): The sugar is delivered at a constant rate, with no spike checks. The ACF process is used for very dilute feedstocks when the sugar concentration is very low.

Strain Nomenclature:

Several strains were in development and used in this project. These are assigned the names Strain A., Strain B, Strain C, Strain D. Increasing alphabetic order generally indicates a progression of strain performance.

Baseline Testing Using a Cane Syrup and Defined Dextrose Feed

Improved strains, through the use of synthetic biology, and improved process strategies were developed and implemented during the course of the project. These strains and process methods were initially tested in laboratory scale fermentations. This was typically at 0.5L fermentation scale for strain promotion, and 2L scale for process improvement or feedstock evaluation. The strains were initially tested in cane syrup and defined dextrose feeds in different process modes in order to get a baseline performance. The baseline performance is annotated in the figures that follow in this section. The lower green line is representative of the overall yield, titer, productivity, or steady state packed cell volume (PCV) for fermentations using defined media with dextrose as the carbon feed. The red line corresponds to the same parameters for fermentations using Florida cane syrup as the carbon feed.

Two important feedstock parameters require consideration. These are the solids content and the total reducing sugar concentration (TRS). With complex media such as cane syrup, the solids present can affect the PCV measurement. A combination of biomass and the accumulation of solids from the feedstock in the cane syrup made it difficult to differentiate between biomass and solids from feedstock, thus increasing the apparent PCV measurement.

Sorghum Syrup Fermentation Testing

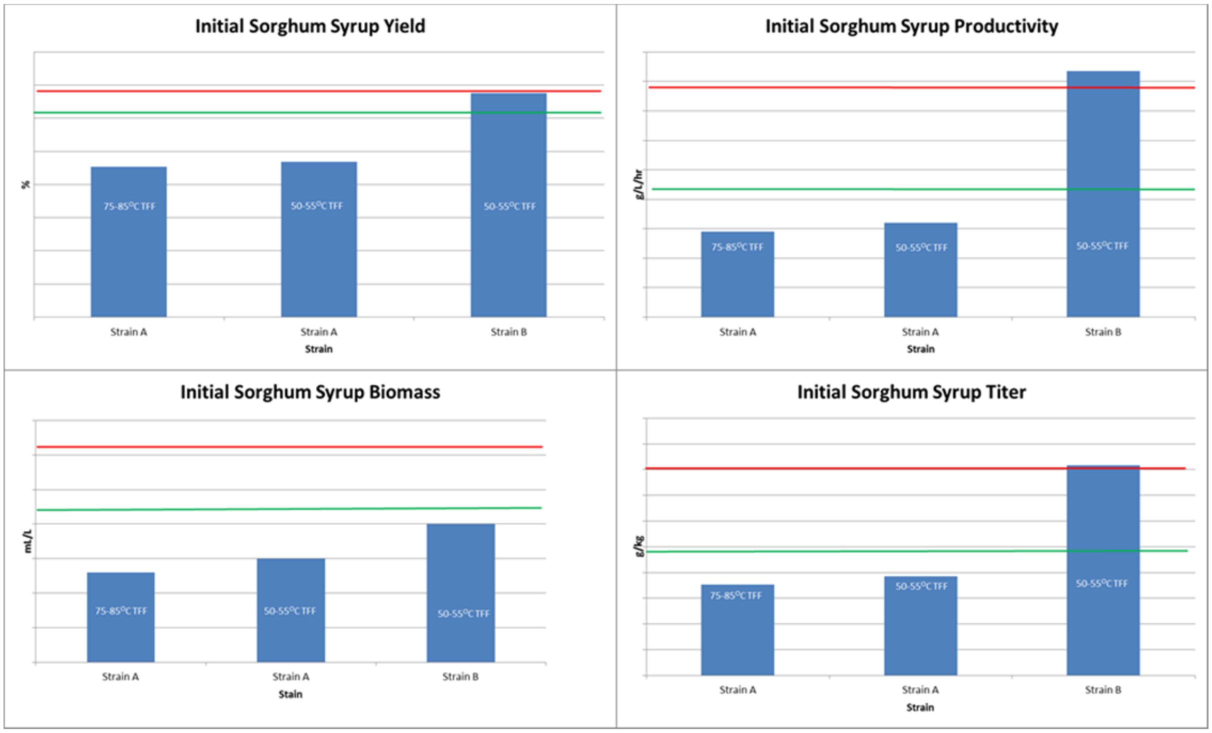
Initial 2L Sorghum Syrup Evaluations

The initial lab fermentation testing used TFF clarified –filter-sterilized Florida sorghum syrup. Using Strain A and a fed-batch MAPFx process, this feedstock did not perform as well as the baseline fermentations, which utilized dextrose (shown as green line in graphs) and Florida cane syrup (shown as red line in graphs), as indicated by lower yields, productivity, titer, and biomass formation. The organic acid analysis of the fermentation samples showed accumulation of greater than 30 g/L of glycerol. Glycerol accumulation is an indication of the culture undergoing stress, often evident for yeast in hyperosmotic conditions. In contrast, no glycerol was accumulated in any of the baseline fermentations.

Following this observation, the same Florida sorghum syrup was then tested in Strain B, also using a fed-batch MAPFx process. Although Strain B showed similar performance to the baseline results, using cane syrup, there was also an accumulation of glycerol at 11 g/L. The accumulation of glycerol in both Strain A and Strain B was indicating some effect of the sorghum syrup on the culture. The results are shown in

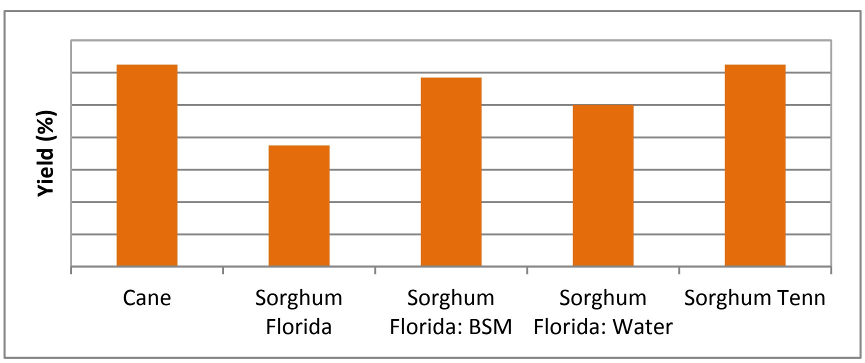
Figure B.7. The Strain A duplication was to examine hot (>75°C) TFF clarified versus 50-55°C TFF filtered material. There was no noticeable difference for the two TFF conditions using Strain A.

Figure B.7: Florida Sorghum syrup yield, productivity, titer, and biomass for Strain A and Strain B



Dilution of the Florida sweet sorghum syrup with water or addition of basal salts media (BSM) surprisingly improved fermentation yield over the untreated Florida material. It was observed that addition of BSM to the Florida sweet sorghum syrup resulted in precipitation. It was not known exactly which salts were removed via this precipitant removal step. The precipitate was removed by filtration and the clarified solution was tested in 2L scale fermentation. The results of using the clarified BSM treated syrup are shown in Figure B.8 below.

Figure B.8: Comparison of Tennessee and Florida sweet sorghum performance in fermentation versus Florida sugarcane (Strain A)



Further investigations of the Florida sorghum syrup in fermentation using Strain A showed that the Florida sweet sorghum syrup performed worse than the Florida cane syrup and Tennessee sweet sorghum syrup. Following discussions with Ceres, it was determined that the Florida sorghum crush material had been treated with lime at the Florida field site in order to aggregate solids. In the case of the Tennessee sweet sorghum, Ceres had not added lime to the crush. The data shown in Figure B.8 indicate the better performance of the Strain A in the Tennessee sorghum syrup feedstock.

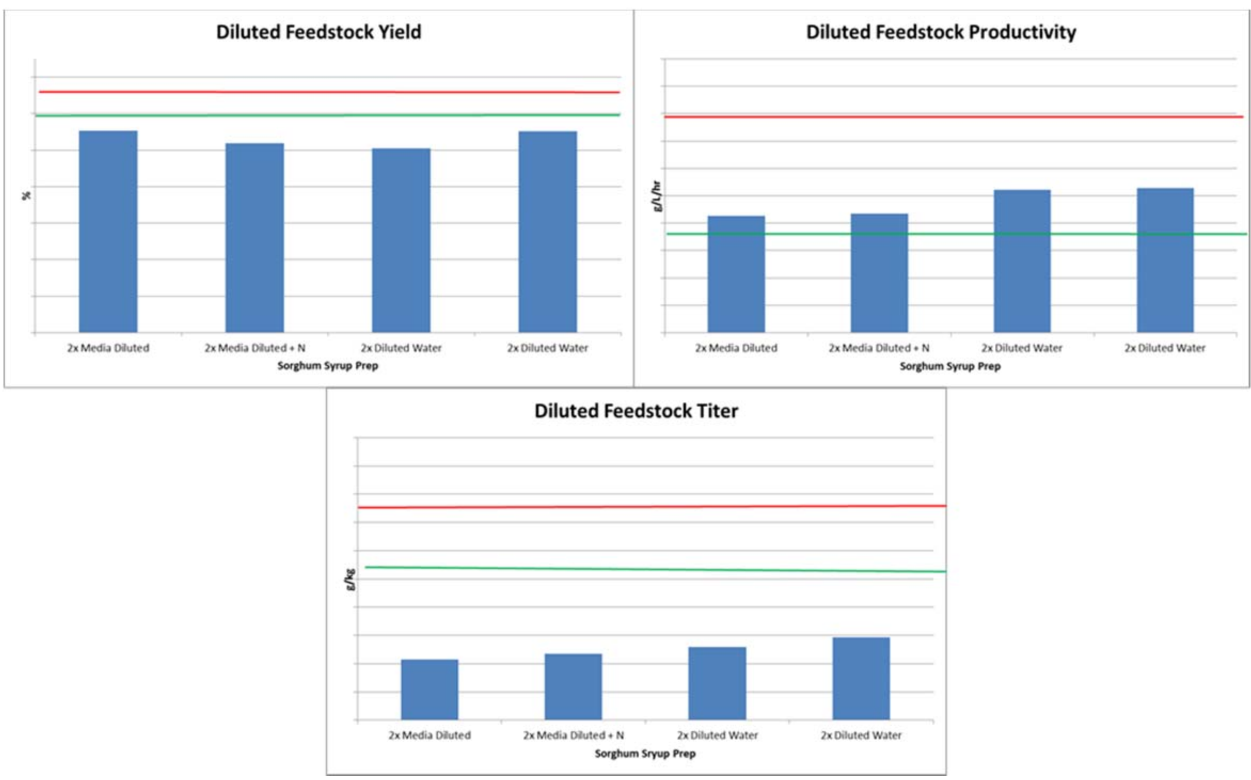
The syrup samples were analyzed to evaluate salts as well as the standard measurement of sugars and organic acids. The results (Table B.1) showed that the Florida sorghum syrup had higher calcium levels than the Tennessee sorghum or Florida cane syrups. Both Tennessee and Florida sorghum had higher levels of potassium than the Florida cane syrup. Accumulation of high calcium and potassium can lead to reduced performance of the fermentation. Reduced performance can be observed in reduction of yield or reduced cell density and productivity.

Table B.1: Elemental Analysis of Cane Syrup and Sorghum Syrup

Elements (g/kg)	Cane Florida	Sorghum Florida	Sorghum Tennessee
P	0.150	0.0668	0.472
K	4.9494	10.0393	15.195
Na	0.0035	0.1229	<0.00 44
Ca	1.1544	11.2558	1.4293
Mg	0.5615	1.2344	1.1587
Fe	0.031	0.1914	0.5059

The organic acids analyses of the three feedstock fermentations revealed similar profiles except in the case of glycerol, pyruvate and acetic acid. Both Florida and Tennessee sweet sorghum syrup fermentations produced acetate (15g/L) and pyruvate (6g/L) and Florida sorghum resulted in glycerol production in the fermentation. The results in Figure B.9 again show the improvement of performance in Florida sorghum syrup diluted with either BSM or water using Strain A. Urea was added to one fermentation condition to confirm that the culture was not nitrogen limited. The addition of a diluent lowers the concentration of the salts found in the feedstock. It was determined that the addition of urea as additional nitrogen did not provide additional benefit in the diluted cultures.

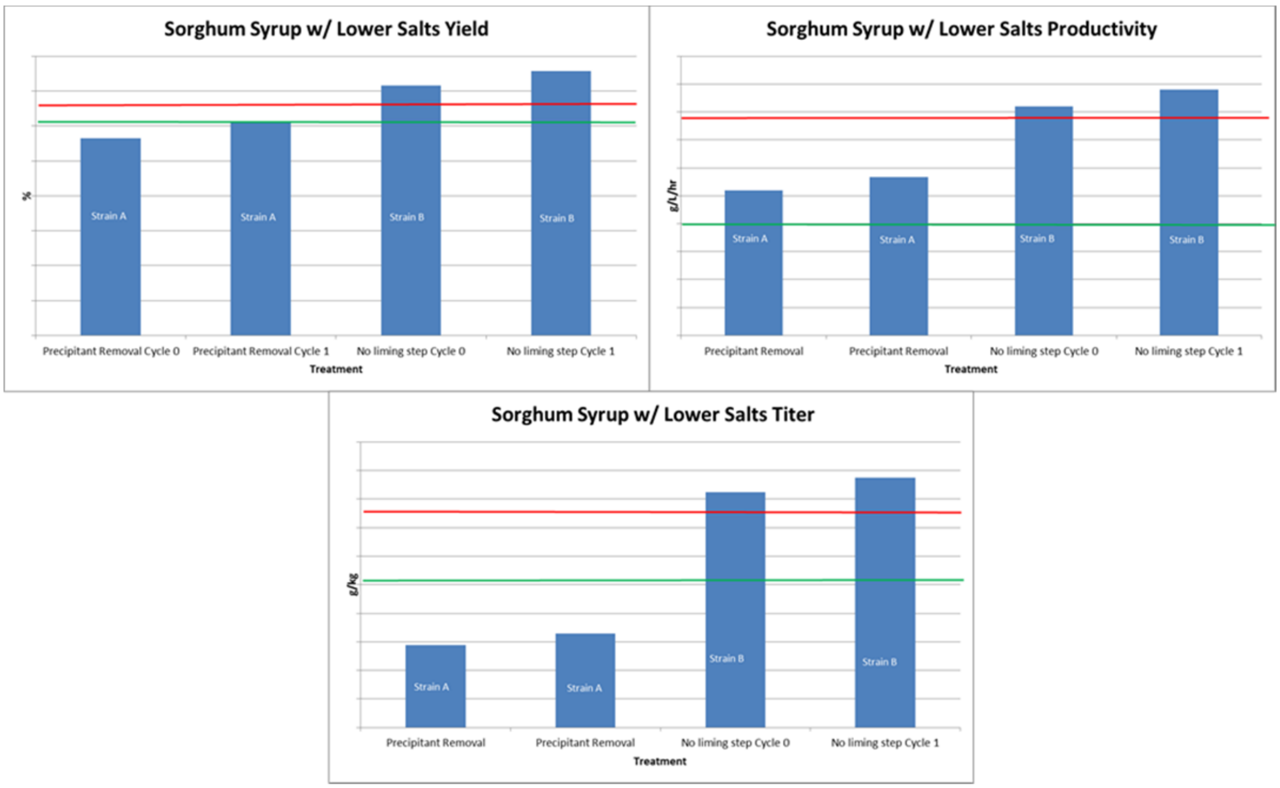
Figure B.9: Yield, productivity and titer for diluted Florida sorghum syrup



A series of fermentations were performed to evaluate the non-lime treated Tennessee feedstock using two different strains, namely Strain A and Strain B. Florida high salt Feedstock (undergoing the liming step) was prepared whereby salts were precipitated and removed and then evaluated in fermentation using Strain A. In both the Tennessee and Florida feedstocks the early FAD 0 and FAD1 draw data showed improved performance in later draw (FAD1). Later draws involve less carbon being diverted to biomass resulting in increased yield. Similarly the higher cell density present at the point of the later draw would result in higher productivity.

The yield, productivity, and titer of the Tennessee sweet sorghum syrup with no liming step using Strain B were close to the expected levels based on experience in other feedstocks tested with that strain. The Strain A yield was marginally lower than expected and had lower productivity than control in the Tennessee feedstock. In Florida precipitated feedstock the Strain A performed worse than control. This may have been due to other key components being missing after precipitation. The results are shown in Figure B.10.

Figure B.10: Improvement in fermentation yield, productivity, and titer using Tennessee sweet sorghum syrup with lowered calcium content



300L Sorghum Syrup Fermentations Prior to Performance Protocol Testing

As noted above, the sorghum syrup tested in the 300L fermentors was produced at the Tennessee site using a process without liming. Two strains were used in this testing, Strain A and Strain B. The fermentation strategy was an FAD MAPF process. The result shown below in Figure B.11 through Figure B.18 show that the Tennessee sorghum syrup based 300L FAD MAPF fermentation yield results were similar to the baseline feedstocks at the 300L pilot scale. The cane syrup fermentations had a higher productivity, titer, and biomass. This is likely due to the higher concentration of sugar seen in cane syrup, roughly 900 g/L TRS compared to the 550-650 g/L TRS seen in either the sorghum syrup or defined dextrose feed. The yield on sugar for all three feedstocks is comparable after 100 hours of fermentation. Early yield values for all feedstocks in this example are increasing in the first 100 hours as more of the carbon is directed into farnesene and less into cell mass, which reaches steady state at the 100 hour time point. Results for Strain B showed a similar trend. The yield and productivity of Strain B was similar to the values obtained with cane syrup, but the titer and biomass concentration were lower resulting from lower TRS content of the sorghum syrup. Also included in the graphs are the baseline results from other 300L fermentations.

Figure B.11: 300L Yield for sorghum and baseline feedstocks using Strain A

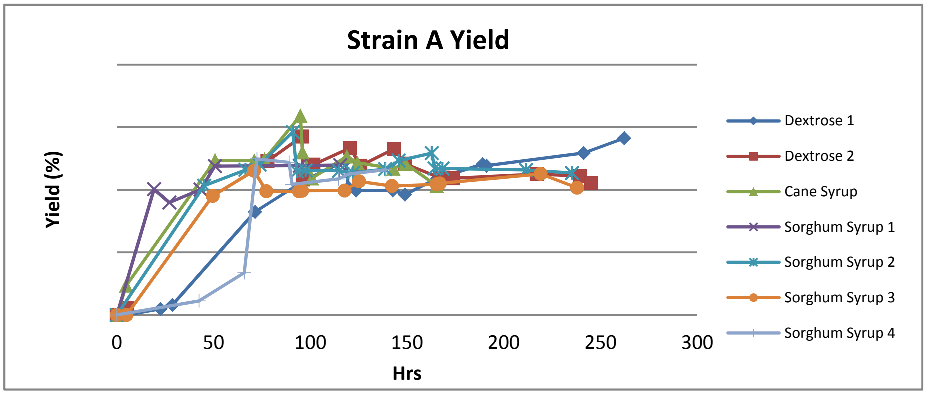


Figure B.12: 300L Productivity for sorghum and baseline feedstocks using Strain A

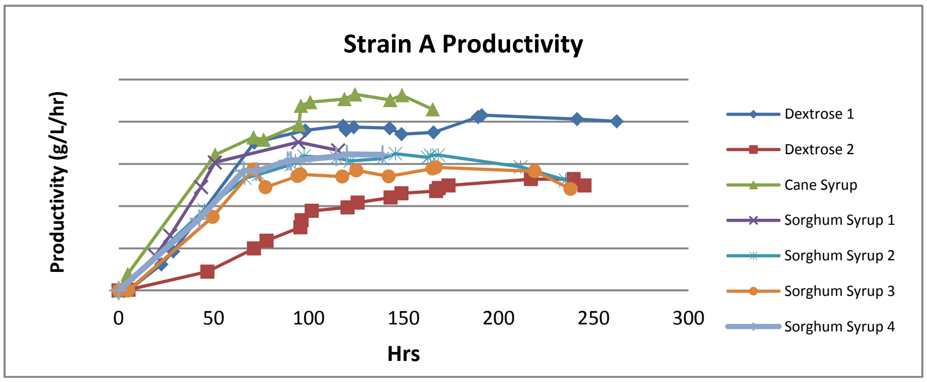


Figure B.13: 300L Titer for sorghum and baseline feedstocks using Strain A

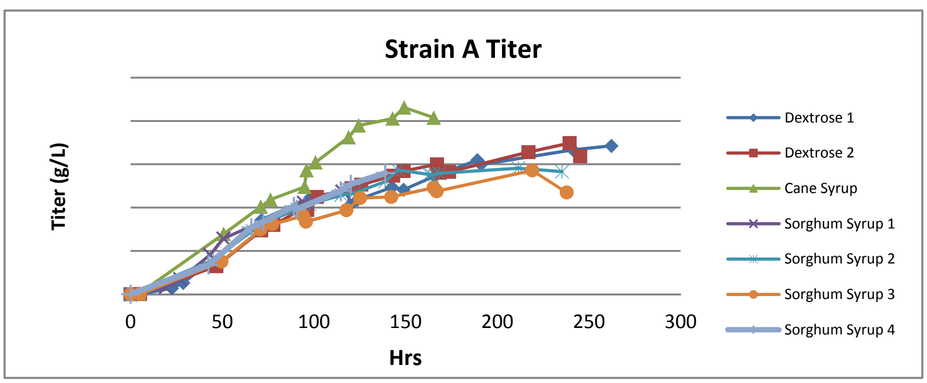


Figure B.14: 300L Cell density for sorghum and baseline feedstocks using Strain A

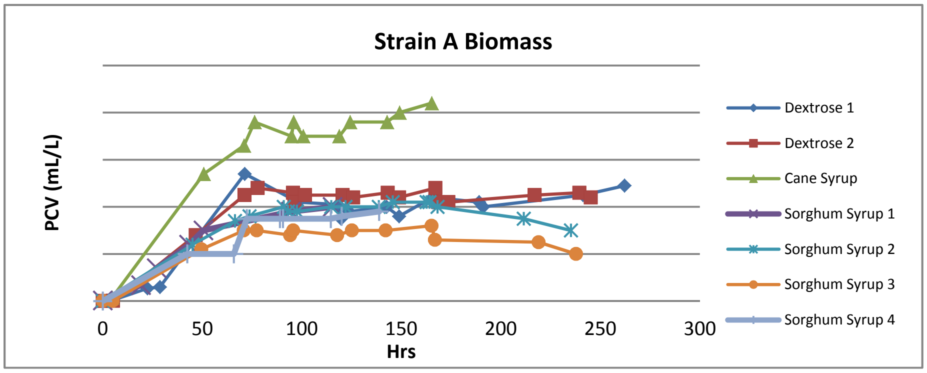


Figure B.15: 300L Yield for sorghum and baseline feedstocks using Strain B

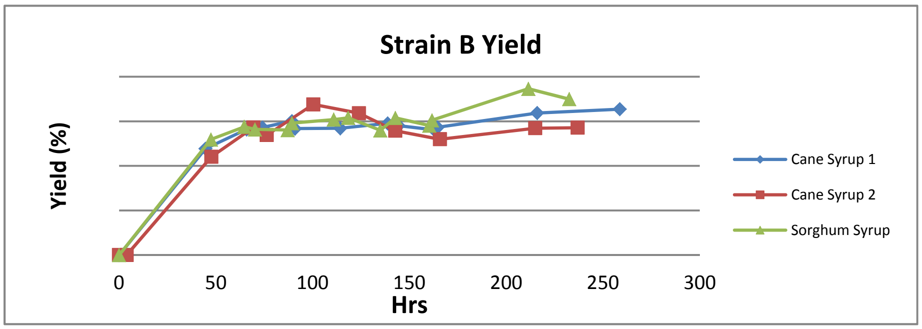


Figure B.16: 300L Productivity for sorghum and baseline feedstocks for Strain B

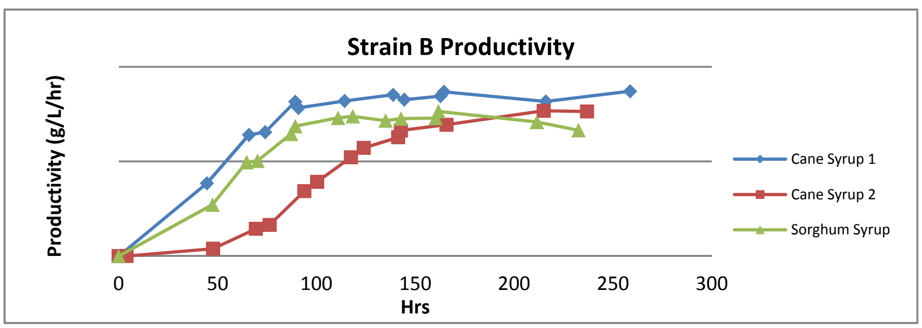


Figure B.17: 300L Titer for sorghum and baseline feedstocks for Strain B

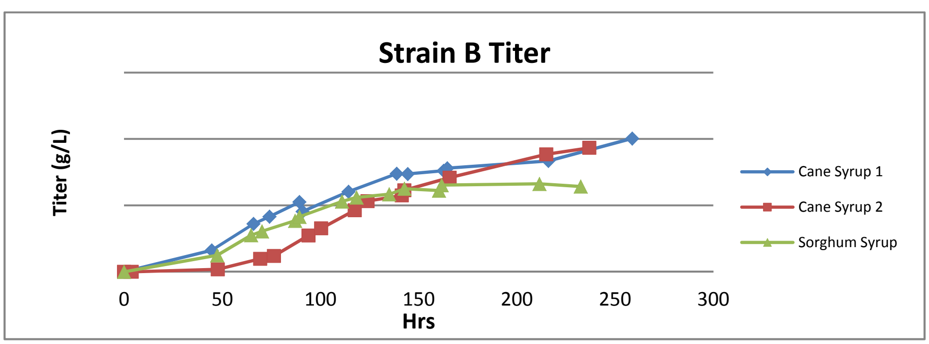
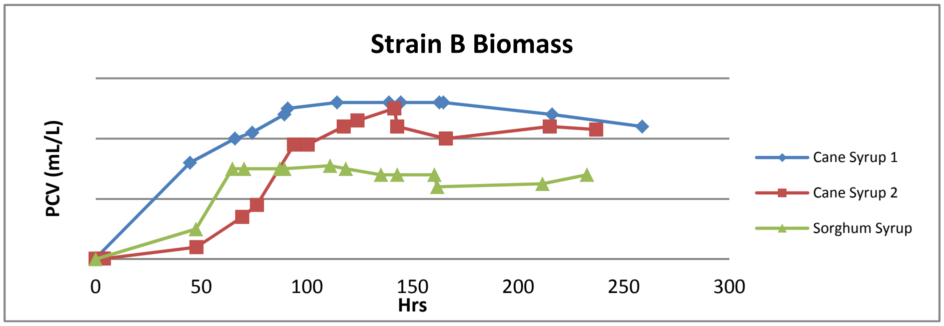


Figure B.18: 300L Cell density for sorghum and baseline feedstocks for Strain B



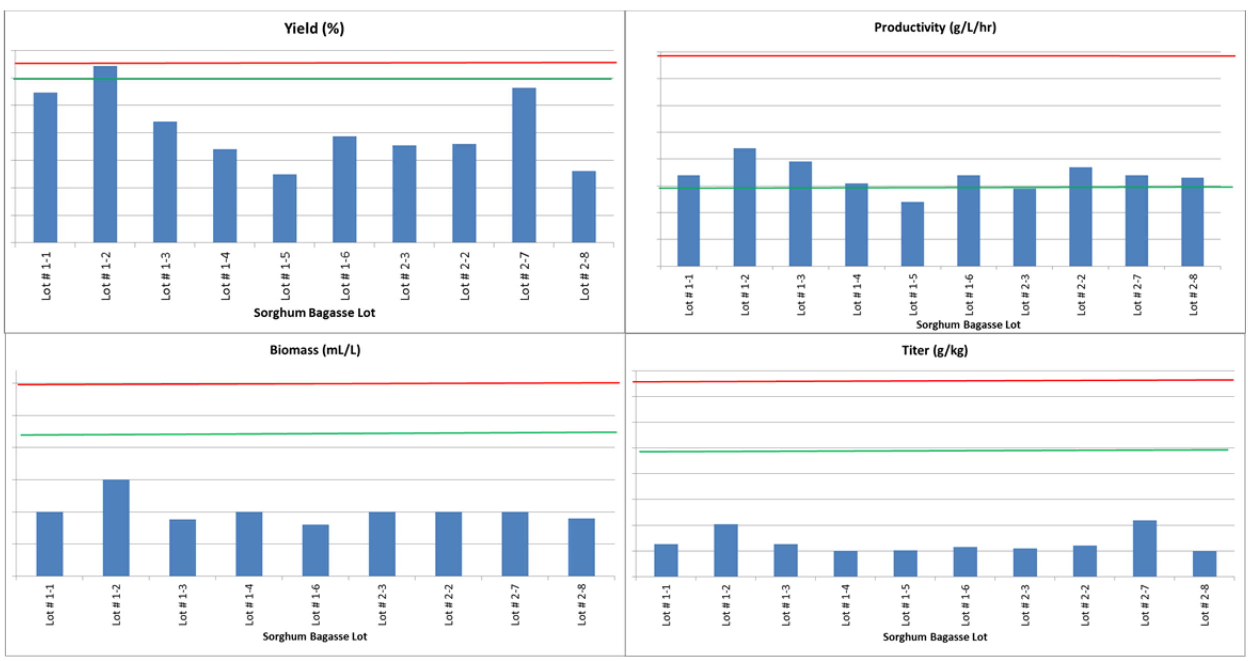
Sorghum Bagasse Fermentation Results

Sorghum bagasse hydrolysates sugars were provided by NREL using a variety of pretreatment conditions (see section B – Llgnocellulosic pre-treatment development and scale –up, and tables referenced in sections below). These hydrolysates were tested in lab fermentors to evaluate their performance in the farnesene fermentation platform. In the earliest cases the biomass build-up phase for the fermentations was accomplished using 650g/L dextrose feedstock for growth followed by the bagasse sugar for the production phase. This approach enabled a quicker biomass build-up as a result of the higher TRS concentration of the dextrose media compared to the bagasse sugar feedstock which was around 100g/L. This initial work used the early Strain A in a cFAD process strategy.

Initial Lab Scale Sorghum Bagasse Fermentation Results

The pretreatment of sorghum bagasse was discussed earlier in this report section. The initial conditions, used for pretreatment and generation of the small test samples, were shown in **Error! Reference source not found.** and **Error! Reference source not found.**. Acid pretreatment was used in the small-scale NREL pretreatment tests (shown as samples 1-3, 1-4, 1-5, 1-6 in Figure B.19). The acid treated samples showed lower yield, biomass concentration, and titer compared to the non-acid treated samples (1-1 and 1-2) shown in Figure B.19. Overall the titers and cell density were lower than the baseline feedstocks because of the lower sugar concentration in the bagasse.

Figure B.19: Fermentation Yield Results for Initial Sorghum Bagasse hydrolysate using Strain A

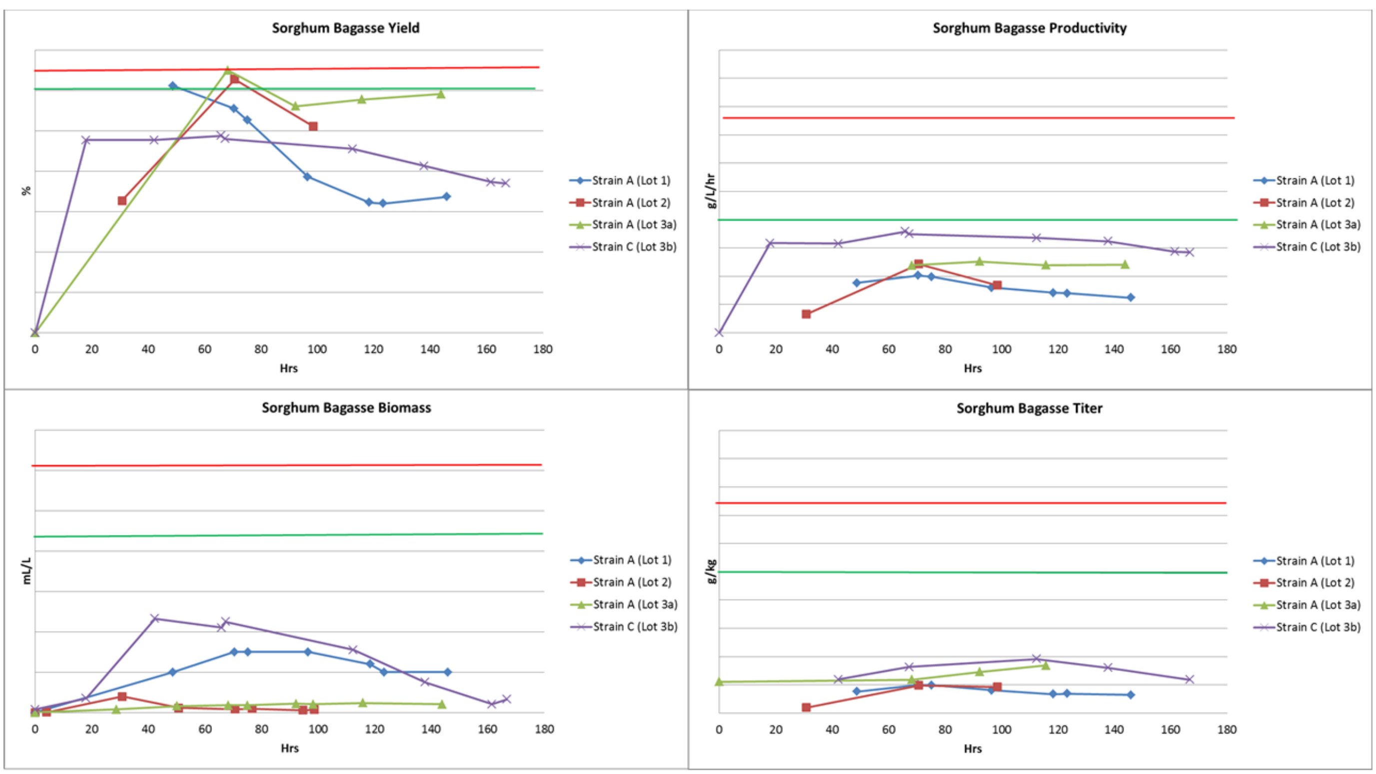


The results from the second lab pilot scale process also confirmed that the acid pretreatment condition of sample 2-3 resulted in lower yields. The second campaign showed a new result suggesting that the increased time and higher temperature condition used for samples 1-2 and 2-2, which was at 200 °C for 11 minutes, performed differently compared to the lab scale samples that were prepared at that same condition. This suggests that the scale of pretreatment may impart some features that impact the quality of the feedstock in fermentation. This may be due to differences in heating and cooling profile, sample handling or other equipment based differences. The overall results indicate that the acid treatment performed worse than non-acid conditions whether produced in a lab or pilot scale method. The optimum condition, in a non-acid pre-treatment process, is a function of the equipment and scale in addition to the variables of temperature and duration.

20L Fermentation Results of Early Large Scale Sorghum Bagasse Lots

NREL pretreated 1 mT of dry bagasse to generate larger quantities of sorghum bagasse sugar for fermentation evaluation at Amyris. In total, three large scale lots were shipped to Amyris in four shipments. The shipped materials were designated as Lot 1, 2, 3a, and 3b. The large scale lot 2 was acid treated and the other lots 1, 2, and 3 were thermal pretreatment samples The sorghum bagasse derived sugars were evaluated at the 20L scale using an initial fed-batch MAPF phase on dextrose (650 g/L) followed by a continuous fermentation and feeding process using sorghum bagasse . The specific pretreatment conditions were shown earlier in **Error! Reference source not found.**. For this testing two strains were used, Strain A and Strain C. The fermentation test results are provided in Figure B.20. The performance of the NREL lab campaign bagasse samples showed similar performance in the 20L reactors and lab fermentors. The acid treated material resulted in low cell concentration but exhibited higher yield in the 20L fermenters than was obtained in the lab test. Testing of the improved Strain C in non-acid treated bagasse sugar did not result in improved yield but did exhibit a higher productivity, titer and cell concentration compared to Strain A.

Figure B.20: 20L evaluation of NREL produced large scale sorghum bagasse samples in 20L fermentor using Strain A and Strain C



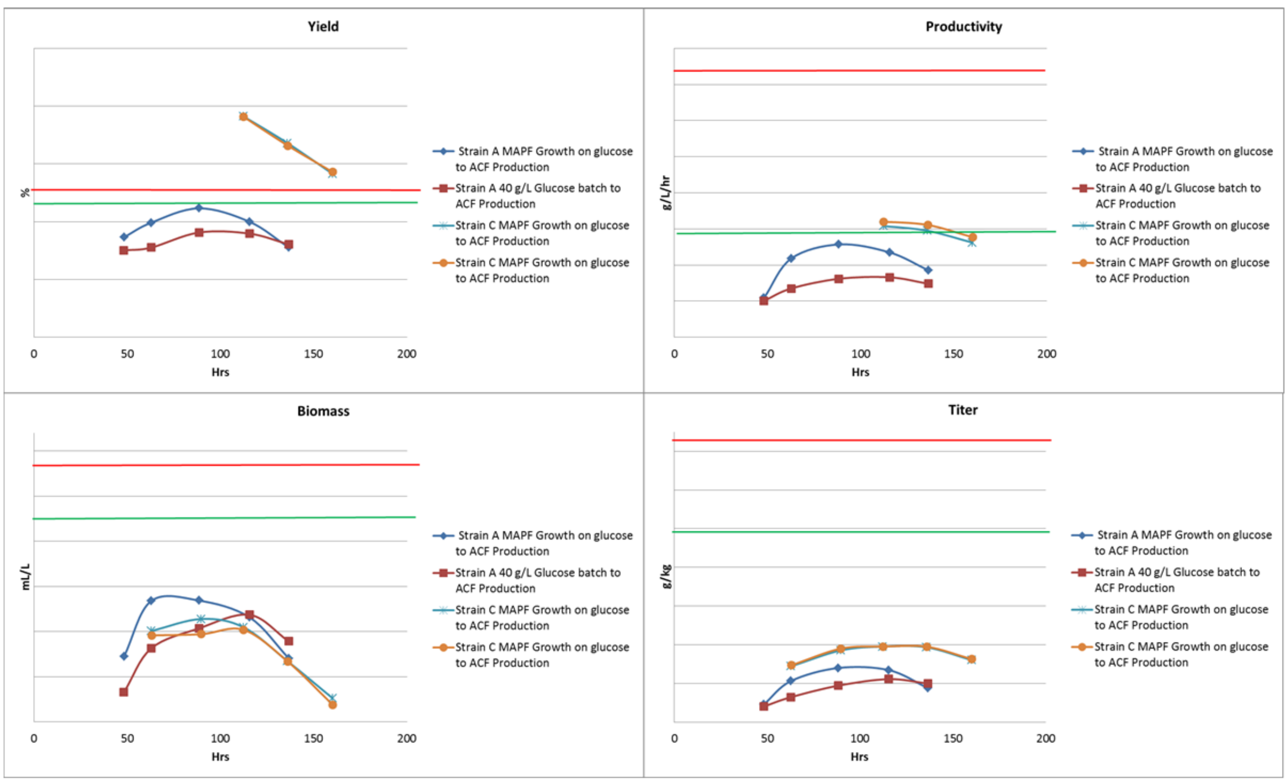
Lab Scale Results of Final Large Scale Sorghum Bagasse Lots

Two final large-scale sorghum bagasse batches were provided by NREL and labeled batches 4 and 5 (see **Error! Reference source not found.** for detail). These non-acid treated lots were tested in lab-scale fermenters, with Strain A and Strain C, using a process strategy in which biomass build occurred in defined media (dextrose) to establish higher initial cell density at the initiation of bagasse sugar feed. Once the bagasse sugar was being fed, the fermentation strategy was set to an ACF process which used a constant feed.

Overall, the performance of lots 4 and 5, using Strain A was similar to the previous 20L fermentations and large scale lots 1,3a, and 3b. Batching of the glucose for the biomass build did not boost the performance to match a fully defined dextrose 650g/L process. As shown in Figure B.22 the performance of the fermentation on the bagasse was lower than obtained for Strain A on dextrose using the MAPFx process.

In the standard process, the more advanced Strain C out-performed Strain A, in yield, productivity, and titer. Worthy of note was that the cell concentration of Strain C was lower than that for the Strain A fermentation. This showed that the Strain C had improved specific productivity (output per cell) compared to Strain A. The results are shown in Figure B.21 below.

Figure B.21: Lab scale evaluation of NREL produced large scale sorghum bagasse samples 4 and 5 in 20L fermentor using Strain A and Strain C

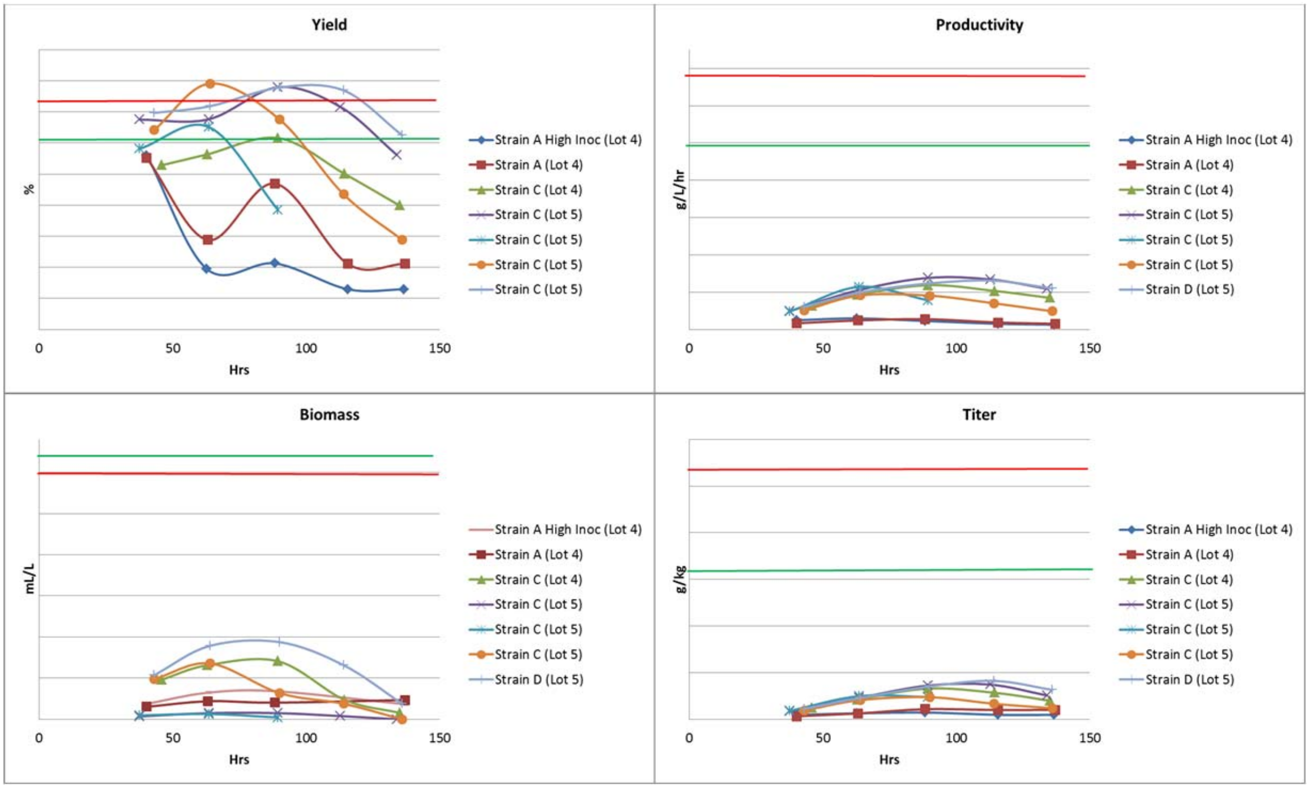


A salt analysis of the feedstock found no abnormally high concentrations of salts compared to other feedstocks. However, the all fermentations, which were run with Strain C, using sorghum bagasse sugar showed sign of stress as indicated by accumulation of >20 g/L of glycerol by the end of the fermentation. There are several potential sources of stress including a possible toxicity or nutritional effect on the cells due to the feedstock or the feed strategy used of fermentation.

Using Sorghum Bagasse for all Phases of the Fermentation Process

Biomass build and production using only sorghum bagasse sugar was evaluated. This would enable a more relevant fermentation yield assessment using only the sorghum bagasse sugar since there would be no residual dextrose present in the fermentor. The process strategy consisted of an exponential feed ramp for biomass build followed by an ACF feeding strategy. Three strains were tested: Strain A, Strain C, and Strain D. In addition a higher inoculation ratio was evaluated using Strain A. Results are shown below in Figure B.22.

Figure B.22: Lab scale fermentation evaluation of sorghum bagasse sugar used in build and production phase fermentations



The results showed that a higher cell density inoculum (40% inoculation volume) did not provide a benefit for production. The yield and titer were lower than achieved with Strain A using dextrose.

Strain C bagasse lot 5 had a better performance than lot 4, under the same process (all steps on sorghum bagasse). The yield and titer were slightly higher than lot 4 at lower biomass. Overall Strain C still outperformed Strain A in the same process.

Strain D showed the highest yield, productivity, and titer. When comparing this process to the standard Strain D, in dextrose, results, the yield performance is not much lower. It is expected that titer would be lower as a result of lower sugar concentration for the bagasse sugar versus the standard process using dextrose which had a TRS sugar contentment of 650 g/L.

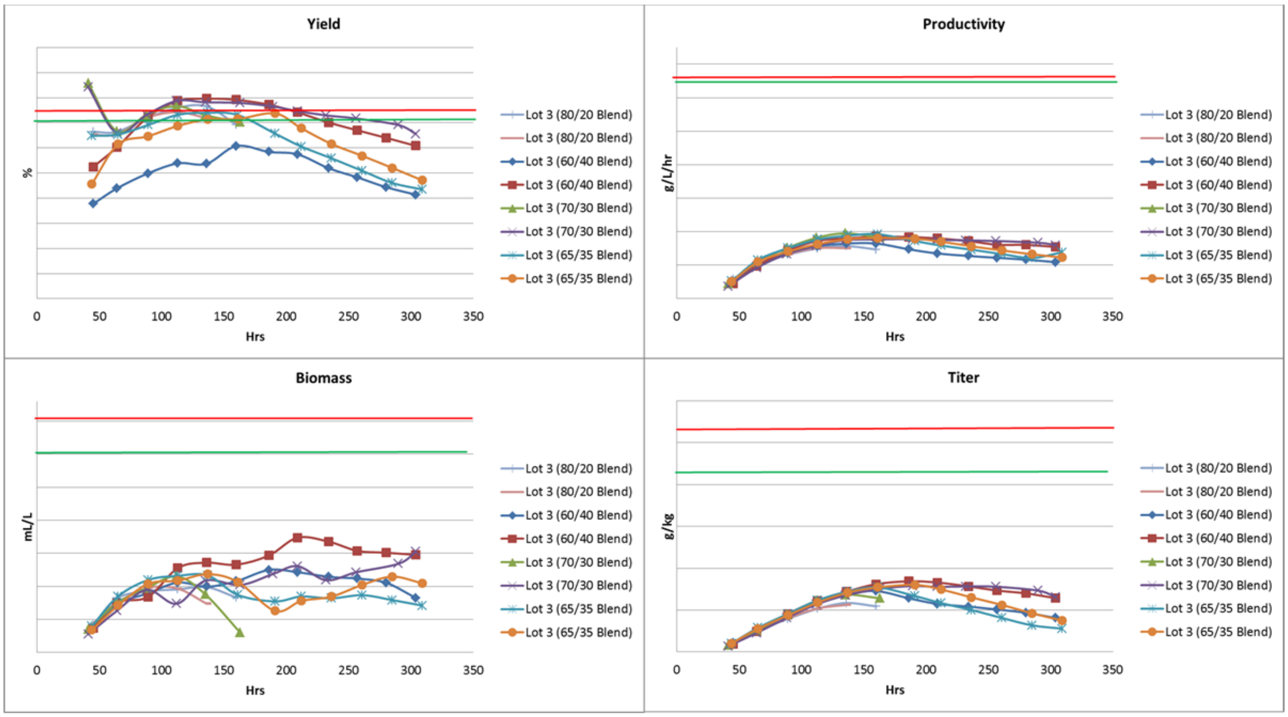
All cultures accumulated glycerol which again pointed to the presence of some type of inhibitor in sorghum bagasse. It was also evident that the oxygen uptake rate (OUR) of the cultures decreased significantly as the batches progressed. In addition, the biomass level and productivity of the Strain C and Strain D cultures dropped significantly in the later stages.

Diluting Sorghum Bagasse with Sorghum Syrup

To test the hypothesis that the sorghum bagasse may contain an inhibitor, spent media was obtained from the fermentation, at the stage that they were exhibiting growth inhibition and depressed OUR. Freshly grown shake flask cultures were placed in shake flasks containing the sterile filtered sorghum bagasse spent media and also to a 1:1 water diluted sterile filtered sorghum bagasse spent media. Glucose was added at 10g/L as a carbon source and the flasks were incubated and monitored for growth. The control shake flask, with undiluted sorghum bagasse, showed no growth while the cultures grew well in the 1:1 diluted samples. This result suggested that a toxic component was accumulating in the sorghum bagasse fed fermentations.

Following this, multiple dilutions of sorghum bagasse were prepared. Rather than diluting the sorghum bagasse with water, which would further lower the sugar concentration, sorghum syrup was used. This method enabled dilution of potential toxic components in the feed without reducing the TRS concentration. The blends tested were, 80/20 (volume bagasse/volume sorghum syrup), 60/40, 70/30, and 65/35. The strain used was Strain D. The fermentation process was an exponential feed ramp to ACF production phase. The results are as follows in Figure B.23.

Figure B.23: Lab scale bagasse/syrup feedstock blending evaluation



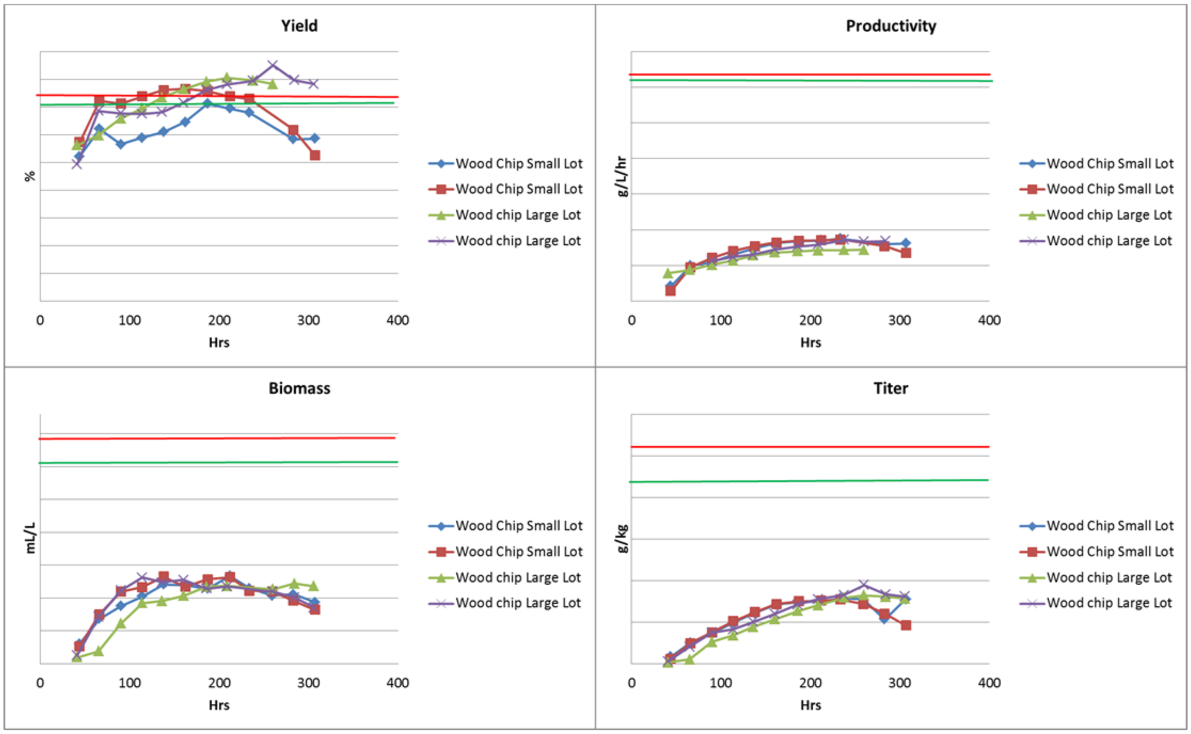
The blending results confirmed the presence of inhibitors in the sorghum bagasse. When the bagasse was diluted by only 20%, the fermentation was not able to continue past the 150 hour mark, as with previous experiments. The first of two 30% dilution fermentations ran for over 300 hours while a replicate was not able to achieve more than 150 hours. However, once the bagasse was diluted by greater than 30%, the fermentation was able to consistently extend out to 300+ hours. At 40% dilution, the fermentation succeeded to 300+ hours. Using this, data we decided to target a 35% dilution using sorghum syrup. This dilution proved to be successful, achieving a fermentation that ran over 300 hours as shown in the Figure B.23.

The cultures with more sorghum syrup added to the bagasse had a higher titer and productivity, as compared to the 35% diluted bagasse. The higher TRS of 295 g/L and lowered toxicity were contributing factors to this improvement.

Lab Scale Wood Chip Hydrolysate Fermentation Results

A small volume sample and a main full batch shipment of wood chip hydrolysate from OTFF were tested in lab fermentors. These were compared to ensure that the main lot performed similar to the small scale lot initially tested. The small lot fermentations used an exponential feed ramp to FAD ACF feeding strategy. The large lot used a FAD CSFC feeding strategy. The strain used in these fermentations was Strain D. See Figure B.24 below.

Figure B.24: Lab scale fermentation evaluation of wood chip hydrolysates feedstock



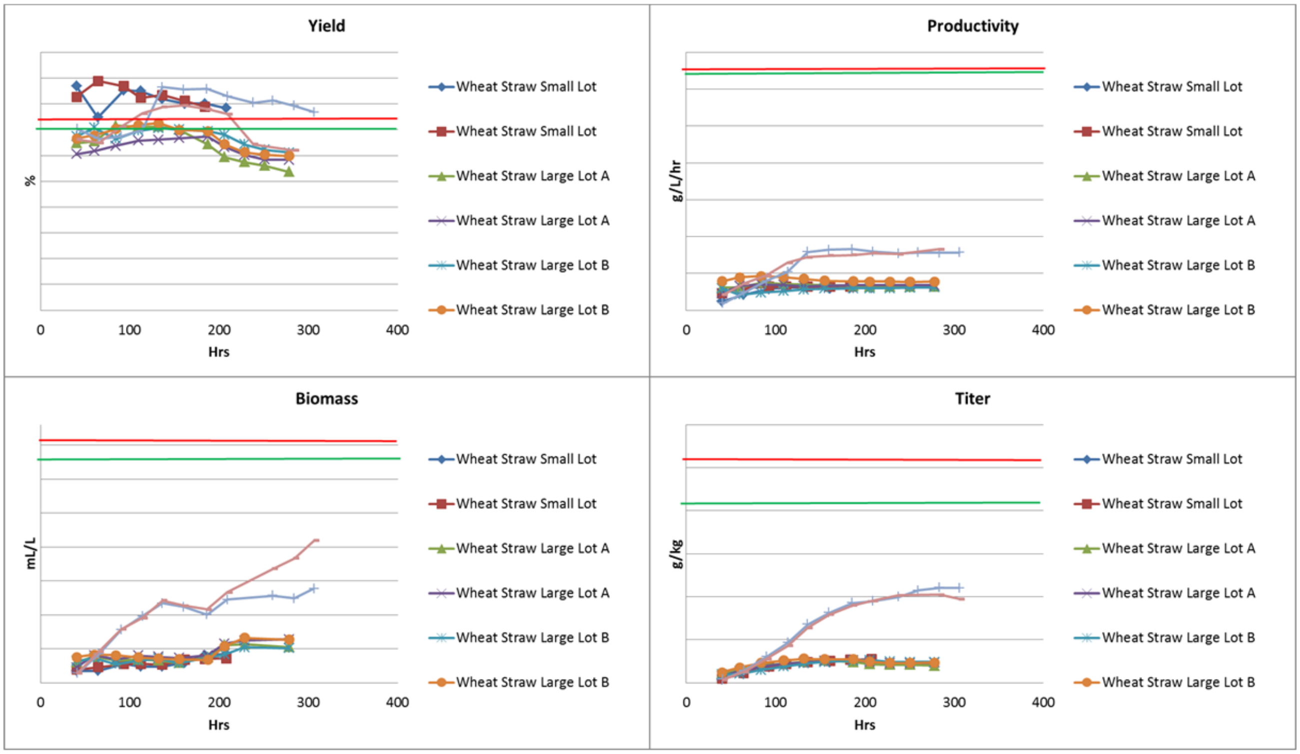
While performance for the small sample lot and main lot were similar, the yields were slightly different between the two lots which may have been due to process based effects. Most importantly, the large lot yields were comparable to the baseline fermentations. Overall, the analysis shows that there were no negative impacts from using wood chip hydrolysate as a feedstock in Amyris's fermentation process.

Lab Scale Wheat Straw Hydrolysate Fermentation Results

A small volume test lot and two large scale lots (A&B) of wheat straw hydrolysate were received from M&G Italia and tested. The small volume lot was a sub sample of large scale lot A. A wheat straw sorghum syrup (30/70) blend from lot A was also tested prior to a 300L fermentation. These fermentations used a FAD CFSC feed strategy. The strain used in these fermentations was Strain D. Results are shown below in

Figure B.25.

Figure B.25: Lab scale fermentation evaluation of wood chip hydrolysates feedstock



The small volume lot and the large lots showed similar performance in lab fermentors. The biomass, titers, and productivity were lower than baseline fermentation using Strain D and the CFSC fermentation strategy. The fermentation yields were slightly lower than the baseline process.

When blending the wheat straw with sorghum syrup (30/70), the resulting sugar concentration of the blended feedstock is increased relative to the wheat straw alone.. This translated to a much higher biomass, titer, and productivity achieved compared to undiluted wheat straw. However, the higher sugar concentration in the blend did not increase yield, as compared to the undiluted feedstock.

Subtask B.4. Anaerobic digestion development and hydrogen production

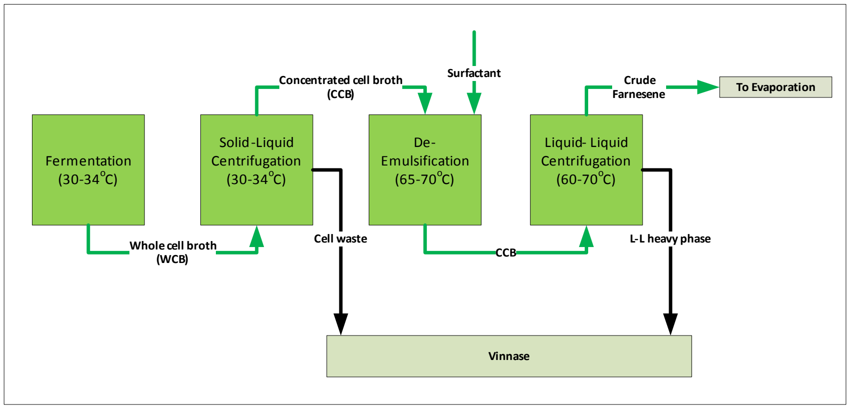
See Feasibility Analysis: In-situ Production of Hydrogen from C5 Sugars and Vinasse section

Subtask B.5. Recovery, purification and diesel finishing scale-up (leveraging vendors Intertek, Acorn Labs or similar providing equivalent services)

Recovery of farnesene from fermentation broth is achieved through a three stage process involving physical separation by centrifugation. The stages are shown in

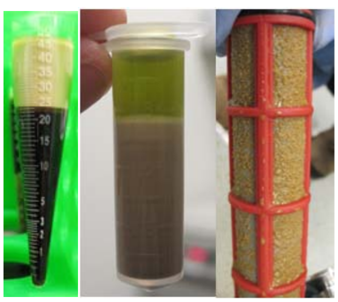
Figure B.26.

Figure B.26: Farnesene recovery process diagram



For pilot plant fermentations (20L and 300L), the fermentation broth is directed through a two stage centrifugal recovery process, using continuous flow disc stack centrifuges from Alfa Laval or GEA Westfalia. The yeast cells producing farnesene were considered to be similar to other fermentation yeasts and the equipment did not require customization for the farnesene process. The liquid-solid (L/S) centrifugation step separates the cells, along with some of the spent fermentation broth, into the heavy underflow phase. The supernatant containing the farnesene oil, farnesene emulsion, and some spent fermentation broth is collected. This supernatant is referred to as the concentrated-cell-broth (CCB). Typically the CCB contains 50% aqueous (spent media) and 50% emulsion plus oil. An example of CCB is shown in Figure B.27.

Figure B.27: Farnesene cane based CCB (left) and demulsified sorghum based CCB (middle), fouled screen post fermentation (sorghum) pre- L/S centrifuge (right)



The CCB is heated and a de-emulsification reagent is added. After stirring for a short time (minutes) to obtain a well mixed suspension and incubating the CCB at the designated de-emulsification agent concentration, the material is passed through a continuous flow liquid-liquid (L/L) disc stack centrifuge which separates the oil and aqueous phases. The de-emulsification process developed for the cane syrup based fermentation material also worked well for the sorghum syrup based material.

Early investigation of the sorghum based fermentation recovery process showed similar performance to the standard cane syrup process when TFF filtered sorghum feedstock was used in fermentation. The L/S yield was slightly lower for the higher solids containing sorghum samples. The Tennessee sorghum feedstock was clarified, prior to HTST sterilization, using centrifugation and screening. The final solids content of the feedstock was around 0.5% by volume. It is possible that these solids caused a loss of farnesene due to the product binding to the solids. This would result in product loss in the heavy phase or in the screens that were used at that time between the fermentor and centrifuge. The L/L yield data suggested that the yield was potentially the same for cane and sorghum based processes. In the first sorghum L/L sample exhibited some operational physical liquid losses which could have led to lower yield. The strategies used in these fermentations involved the FAD method where broth is removed from the fermentor (FAD0, FAD1, and FAD2) during the fed batch phase and replaced through the continued feeding of feedstock containing the sugar and nutrients. These are indicated as cycle 0, 1, and 2.

These recovery experiments were the first using sorghum syrup at the Emeryville pilot plant. The crude farnesene samples obtained from these early batches were analyzed to determine farnesene purity using Gas Chromatography with Flame Ionization Detector (GC-FID). The results showed purity was higher for the cases where low solids feedstock was used (TFF). The non-TFF sorghum fed fermentation had a purity that was slightly lower than the cane syrup. The non-TFF sorghum derived farnesene was green, as shown in Figure B.28. The data is shown in Table B.2. It is likely that this sorghum field processing does result in transfer of plant material that transmits solids containing chlorophyll and waxy components into the syrup. The TFF approach removes these components extensively whereas centrifugation is unable to remove the solids that are finer or have a density lower than the syrup thus leaving those solids in the fermentation feedstock. In a larger mill process it may be possible to obtain better separation of these solids from the syrup. Typically cane syrups are low in solids (< 0.5% volume) and we would expect the sorghum syrups to process similarly in a mill process.

Figure B.28: Appearance of crude farnesene as a function of fermentation feedstock type

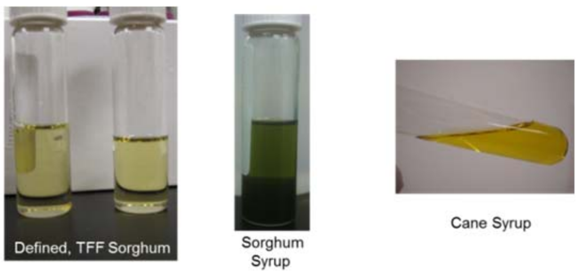


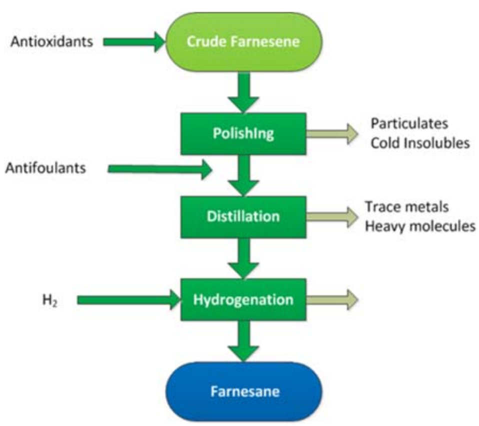
Table B.2: Crude farnesene purity as a function of feedstock in fermentation

Nutrient feed	Crude farnesene purity (% w/w)
Defined Medium	96.5
Cane syrup	95.2
Sorghum syrup (TFF)	97.3
Sorghum syrup (Non-TFF)	94.3

Production of farnesane from early sorghum syrup fermentations (Strain A)

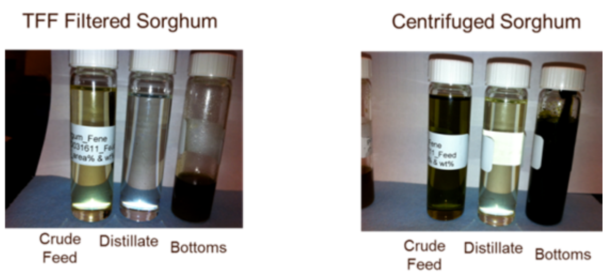
The crude farnesene is converted to farnesane through a distillation and hydrogenation process. A simple process flow scheme is shown in Figure B.29.

Figure B.29: Process scheme for converting crude farnesene into biodiesel farnesane



An antioxidant, tert-butyl catechol, is added at 100ppm to the crude farnesene after completion of the recovery process. After the addition, the crude farnesene may be stored cold prior to scheduled distillation. Stored material exhibited similar purification and hydrogenation performance for both fresh and stored starting material. Because the L/L centrifugation process is performed at > 60 ^{o}C there can be carryover of oil soluble components into the crude farnesene. Storing the crude farnesene at colder temperatures leads to some precipitation of cold insolubles. These were removed by a high capacity 1µm depth filter prior to distillation to remove solids that may affect the physical operation of the WFE, which has small gaps between the blades and wall. In addition, removal of these insolubles (lipids, sterols, hydrophobic solids) provides a slight purification of the crude farnesene. At larger scale these materials may be purified out into the bottoms fraction in a forced recirculation or falling film evaporator. The filtered crude was distilled in the Pope 1.1ft^2 wiped film short path evaporator. The conditions of this step were 150 ^{o}C jacket temperature, vacuum 5 Torr, and a flow rate of 1.2-1.6 liters per hour. The distillation step separated the contaminants into the heavy phase and the farnesene into the distillate light phase. The heavy phase contained triglycerides, monoglycerides, and salts. In the case of the non-TFF sorghum based crude farnesene; the heavy phase was green in color and had set up as a non-flowing wax. This was not observed in the cane syrup-based crude farnesene or in the TFF sorghum crude farnesene “heavy phase”. The TFF process would appear to have removed the components that resulted in green color of the crude farnesene as well as the green color and waxy nature of the distillation heavy phase. This is illustrated in the photographs in Figure B.30.

Figure B.30: Appearance of heavy and light phase materials post distillation



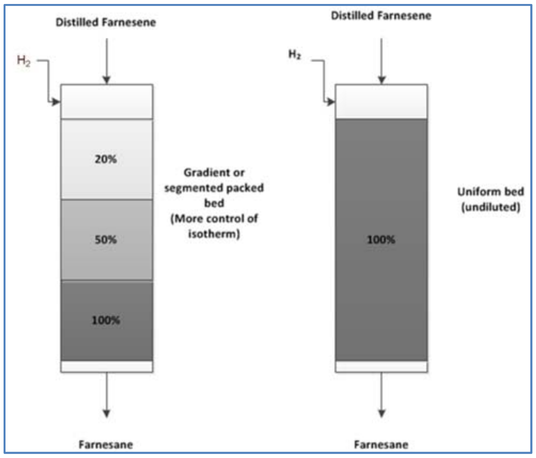
Distillation samples were analyzed by GC-FID to establish mass balance and purity as well as identification of key components. The results showed that the mass recovery (yield) of purified farnesene was higher for the filter clarified feedstock derived material.

It was decided that the TFF feedstock preparation method for sorghum syrup and cane syrup was not feasible due to the high viscosity and density of the sugar solution resulting in very low flux rates of less than 2 LMH. Such low flux limited the throughput and was not scalable. Both the TFF and non-TFF derived sorghum based crude farnesene performed adequately in distillation. Further fermentations, using the 2010 Tennessee centrifuged/screened syrup, were evaluated through the distillation and hydrogenation unit operations. The quality of distillate and farnesane produced were acceptable. In general the later FAD materials performed as well as the earlier draws suggesting that the distillation and hydrogenation steps are able to handle a broad range of crude farnesene purity input presented by the early, middle, and late fermentation output.

Hydrogenation of distilled farnesene was performed in a Parr 1L packed bed column. The catalyst is packed in a staged bed using glass beads for dilution which exposes the highest concentrations of unsaturated substrate and hydrogen to a diluted bed, thus decreasing the potential for overheating. The conditions were jacket temperature of 150 ^{o}C, bed temperature <250 ^{o}C, H_2 pressure 500 psig, and a farnesene flow rate of 0.3-1.2LPM. A depiction of the hydrogenation reactor is provided in

Figure B.31. The purity of material obtained after hydrogenation was within specification and the yields were around 95%.

Figure B.31: Graded hydrogenation tubular reactor



Process integration using sorghum syrup

Three separate batches of the Sorghum media were prepared and used as feedstock for three similar fermentation batches. The fermentations were operated in MAPF mode using a FAD approach. The fermentation broth was harvested and the particulate cellular material separated from the farnesene containing broth using a disc stack continuous flow nozzle centrifuge followed by separation of the lighter density farnesene product using a L/L centrifugal separator. The crude farnesene obtained from centrifugation was purified in a short path single effect evaporator. The distilled farnesene was then hydrogenated in a fixed bed hydrogenation reactor. The farnesane purity obtained met the specification of 96 wt% or greater.

Overall Process Flow of Farnesene

There is scope for process development and strain improvement that would improve the LS and LL step yields. Such improvements could result in an overall process yield of >85% from raw sugar to fuel.

Performance of lignocellulosic sugar based process

Production of farnesane from wheat straw and wood chip lignocellulosic sugar feedstock-fed 300L fermentors was evaluated in the pilot plant. Two batches of wheat straw, two batches of wood chip and one wood chip (30%)/sorghum syrup (70%) blend batch were completed. The fermentations were operated in CFSC mode using a FAD approach. The fermentation broth was harvested and the particulate cellular material separated from the farnesene containing broth using a disc stack continuous flow nozzle centrifuge followed by separation of the lighter density farnesene product using a L/L centrifugal separator. The crude farnesene obtained from centrifugation was purified in a short path single effect evaporator. The distilled farnesene was then hydrogenated in a fixed bed hydrogenation reactor.

Overall Process Flow

Results from the lignocellulosic sugar feedstock based processes clearly indicate that fermentation performance was best in the wood chip based feedstock and the sorghum syrup supplemented wood chip blend. This is due to the ability of the higher sugar concentration to support higher density fermentation and enable the fermentation to operate in a production phase with low growth rate, resulting in better product yield from the fermentable sugar.

The recovery of product was poor in the wheat straw based process. The lower titer and higher percentage losses in the L/S and L/L centrifugations contributed to the low product recovery. Lower purity of crude farnesene post recovery was evident in the wheat straw based process. The purity of the distilled farnesene was only slightly lower for the wheat straw case versus the wood chip and wood chip-sorghum syrup blend. The farnesane purity was similar for all feedstock examples although the losses throughout the process are highest for the lower sugar content wheat straw case. This indicates the process is capable of generating acceptable biodiesel end-product quality from a variety of starting feedstocks but yield and economics are a function of the feedstock type. It was evident that feedstocks with TRS > 250g/L are desirable for achievement of acceptable fermentation yield, productivity, and titer as well as higher crude farnesene recovery yield.

General Comments

Strain improvements led to increased yield, productivity, and net titer for the sorghum syrup fed fermentations. The yield, productivity, and titer of the strains in the wood chip and wood chip-sorghum blend were higher than the wheat straw. The dilute sugar concentration of the wheat straw feedstock led to low volumetric productivities and reduced yield.

Subtask B.6. TEA and LCA for Amyris Diesel production

See Technoeconomic Analysis section.

Task C: Development and scale-up of value-added products from diesel fermentation intermediate

Task Objective: The third task in this project focuses on the use of the C15 isoprenoid fermentation product used for diesel production as a raw material for the synthesis of a number of large-market, high-value chemicals.

Subtask C.1. Laboratory-scale development and chemical product synthesis

- *Completed March, 2011*

Subtask C.2. Mid-scale synthesis and characterization

- *Completed July, 2011*

Subtask C.3. Pilot-scale production and chemical process technoeconomics

- *Completed June, 2013*

Accomplishments Against Goals

Subtask C.1. Laboratory-scale development and chemical product synthesis

The goal of providing laboratory scale process development for use of the C15 isoprenoid fermentation product to produce a higher value product was accomplished with the synthesis and testing of the Plasticizer product and developing a process that could be scaled.

Subtask C.2. Mid-scale synthesis and characterization

The goal of mid-scale synthesis and characterization was met with the multi-kilo scale reactions performed in the skid mounted chemistry unit as a scalable process. Material from this production was characterized, and provided the basis for determining product specifications.

Subtask C.3. Pilot-scale production and chemical process technoeconomics

The goal of piloting was performed at a CMO at a scale of nearly 2 mT. The technoeconomics were derived from the optimized reaction to meet the piloting goals.

Project Activities

Flexasene 1010 was originally developed as a precursor to a plasticizer candidate. Plasticizers are additives that can affect the properties of a material such as fluidity, or plasticity. The compound was used in a polyvinyl chloride (PVC) plasticization study at Batelle Labs to determine which compounds performed well under testing for ease of process, Shore D hardness, elongation to break and tensile strength. Flexasene 1010 was one of six candidates that performed reasonably well plasticizing PVC. The hydrogenated version of Flexasene 1010 is Flexasene 1011. This saturated compound performed well in a second round plasticization study, and Flexasene 1011 was chosen to for initial scale up at 15 gallon scale. The process that was first scaled up included a Diels-Alder reaction with trans-β-farnesene and DMM diluted with solvent, a distillation step to remove the solvent, the hydrogenation of the residual Flexasene 1010, and a distillation to purify the saturated Flexasene 1011. Process development work targeted the optimization of the Diels-Alder reaction, which is the reaction of the conjugated diene present in β-farnesene with the dienophile DMM. This resulted in next generation processes that eventually eliminated the need to dilute with solvent, which eliminated the distillation step necessary for removal of the solvent. Side reactions were also minimized after optimization of the reaction temperature and the reactant addition rates which further benefited the overall process by eliminating the need for the final purification distillation step. After polymerization testing of the Flexasene 1010 and Flexasene 1011, it was determined that Flexasene 1010 also performed reasonably well as a plasticizer. Based on these results, the product that was successfully scaled to over a metric ton basis was the unsaturated compound Flexasene 1010. The elimination of the hydrogenation operation and the purification step significantly lowered production cost.

Subtask C.1. Laboratory-scale development and chemical product synthesis

Flexasene 1010 synthesis at laboratory scale

Summary of results

Through various experiments conducted using a Diels-Alder reaction at varying temperatures, it was determined that higher reaction temperatures led to higher conversion of farnesene to product. Both toluene and xylenes were tested as the diluting solvent for this reaction. The boiling points of these solvents limited the range of the reaction temperature to 110 °C and 140 °C. In a side by side comparison of toluene and xylenes, the conversion rate for the xylenes reaction was significantly higher and further development was done with xylenes. Using xylenes as the solvent and a reaction temperature of 140°C, Flexasene 1010 was synthesized at bench scale. The high boiling point of xylenes allowed for a higher reaction temperature reducing the cycle time of the batch reaction for more reasonable throughput.

Reaction Temperature Comparison

Farnesene (1.0 milliequivalents or mEq) and DMM (1.0 mEq) were mixed in batch mode and heated to 95 °C, 105 °C, and 120 °C. Results indicated that the higher temperature had a higher conversion of farnesene to product. The temperature of 120 °C was selected for further experiments. Results indicated that higher temperatures would increase the reaction rate with little impact to impurity levels, this led to the evaluation of xylenes in subsequent experiments.

Solvent Selection

Due to the exothermic nature of the Diels-Alder reaction, as well as some equipment constraints, dilution of the reaction was chosen to provide a heat sink to absorb excess heat produced by the reaction. Both toluene and xylenes were used as candidates to carry out the synthesis of Flexasene 1010. Using xylenes as the solvent allows for carrying out the reaction at higher temperature, therefore achieving a higher conversion in a shorter amount of time (reduction of cycle time). Xylene was thus chosen for further process development, including distillation removal. This enabled operation of the reaction at 120°C or greater.

Purification at Laboratory scale

The initial candidate for the added-value product plasticizer was Flexasene 1011. Flexasene 1011 is the hydrogenated (saturated) product of Flexasene 1010, referred to as the precursor. It was necessary to remove the solvent used to dilute the Flexasene 1010 reaction prior to hydrogenation since xylenes is a solvent that contains double bonds and would be hydrogenated along with the precursor. The hydrogenation of xylenes produces a strong exotherm beyond the capacity of the equipment for heat removal, thus for safe, efficient processing, the solvent needed to be removed prior to hydrogenation. Simulating the equipment capacity of the 15 gallon reactor skid with regard the temperature and pressure, an experiment was performed to determine if the solvent could be removed *in-situ*. It was determined to be not possible to remove the solvent from the reaction mixture in the reactor.

Therefore, it was concluded that the removal of the solvent would need to be performed in a separate operation in a distillation unit. Bench scale removal of xylenes by distillation was performed to establish conditions for scale up to the WFE. The results from procedure performed on aliquots from two lots can be seen in the table below.

Table C.1: Xylenes and Lights Removal by Distillation

Temperature (°C)	Pressure (torr)	Fraction removed	GC-FID area%*
130	50	Xylenes	
130-160	2.4	Lights	
160-196	2.4	Product	97.8% Molecule C; < 1% thermal dimers

Key Variables and Process Controls

Once the process to be scaled was determined, key variables were studied and optimized. Since oxygen ingress was found to impact side reactions such as polymerization producing heavier impurities, the reaction mixture was sparged with nitrogen prior to heating. This operation was included in the SOP. The sparging minimized the formation of oligomers and polymers. Based on calorimetry testing results, it was determined that the Diels-Alder reaction is exothermic, the heat of reaction is -143/kJ/mol of farnesene and the adiabatic temperature rise is 158.4 °C. As a safety control, the farnesene was dose controlled to minimize the pooling of farnesene in the dimethyl maleate during the reaction, which could lead to an exotherm if not controlled. The in process controls (IPCs) used for dosing included sampling of the reaction mixture during processing and analyzing the sample by Gas Chromotography with a GC-FID. The resulting analysis provided the levels of farnesene available in the reaction mixture by area%. If the farnesene area% was too high, the addition rate could be decreased. The dose controlled addition of farnesene also created a reagent ratio that favored the Diels-Alder reaction over the thermal dimerization reaction with the excess availability of dimethyl maleate. The reaction temperature was also studied to decrease cycle time. An increase in reaction temperature increased the Diels-Alder reaction rate, leading to decreased cycle times. Since the key variable controlling the thermal dimerization side reaction was excess dimethyl maleate through dose control, the dosing of farnesene was maintained at a low addition rate even at higher reaction temperatures.

Heats of Reaction

Prior to scale up, the calorimetry testing was performed at an external laboratory that was familiar with such testing. The results are captured in the table below.

Table C.2: Results of Calorimetry Testing

Thermal Inertia, [-]	Onset Temperature, T_0 [°C]	Maximum Temperature, T_m [°C]	Adiabatic Temperature Rise of System, ΔT [K]	Specific Heat Capacity of Sample, C_p,s [J/g-K]	Heat of Reaction, ΔH_R [kJ/mol farnesene]
1.093	92.3	250.7	158.4	2.278	-143.4

Subtask C.2. Mid-scale synthesis and characterization

Process Development and Scale Up

Summary of Results

Flexasene 1010 was scaled in the Skid Mounted Reactor Vessel, a 15 gallon stainless steel reactor, producing a total of 74 kg in three batches. The solvent was removed by wiped film evaporation and the product carried forward for hydrogenation in a fixed bed tubular reactor to produce the plasticizer product. The hydrogenated product, Flexasene 1011 was further purified through distillation in the WFE.

Process Development at 1 Liter Scale

The reaction in xylenes was repeated at the 1-L scale tracking the key process variables. The maximum farnesene accumulation was about 25%. As one of the key process variables, accumulation of farnesene in the reaction mixture needed to be minimized to favor the Diels-Alder reaction over the dimerization of farnesene as well as oligomerization. The coupling of farnesene to farnesene is referred to as dimerization, the addition of farnesene to farnesene as well as DMM is referred to as oligomerization. The dimer and oligomer compounds impact the polymerization process using the plasticizer product as an additive for PVC products.

Onset Temperature for Safety Parameters

It was observed that a small amount of product starts forming at 60°C, and significant conversion is observed at 80-90°C. A study was performed to determine the impact of temperature on reactivity. This was necessary for determination of safety parameters to define the maximum temperature at which the reaction mixture can be considered safe, or safely unreactive, which in this case was 50 °C.

Solvent Dilution and Temperature

In order to increase productivity as the process moved to larger scales, volume efficiency (more product using less volume) needed to be optimized. It was determined that the best method in which to achieve this was to reduce the dilution of the reactants by the solvent reduction. To evaluate the impact of this proposed change on safety, a bench study on the effect of dilution on the exotherm observed during reaction was conducted at 1-L scale. Table C.10, has the reaction conditions that were used, and Table C.11 shows the results of changes in composition as the reaction progressed which indicated that the higher temperature of 140 °C would be safe.

Table C.3: Reaction conditions for effect of dilution on exotherm study

Process condition	Value
Reaction temperature (°C)	120, 140
Dilution with xylenes (v/v)	14
Farnesene (Meq)	1.0
DMM (Meq)	1.1
Farnesene addition rate (mL/min)	0.43
Addition time (h)	22.5
Post addition temperature (°C)	140
Post addition time (h)	9.5

DMM was diluted with xylenes 14 times by volume, and the temperature of the mixture was raised to 120 °C. The maximum farnesene accumulation was 20% by GC-FID area and no exotherm was observed, so the reaction temperature was increased to 140 °C. At this temperature, farnesene was consumed faster and no exotherm was observed. Based on these results, it was determined that the reaction temperature could be increased to 140 °C without increasing safety concerns.

Scale Up in 15 Gallon Skid Reactor

Flexasene 1010 was synthesized in three batches using the 15 gallon skid mounted reactor vessel. For Batch 1, no exotherm was observed and the consumption of farnesene was consistent, varying 4.4 area% by GC-FID from maximum farnesene level to minimum farnesene level during the addition of the Diels Alder reagent.

For batch 2 and batch 3, the dilution was reduced to increase volume efficiency and cycle time after noting that no exotherm was observed in the first batch reaction. The dilution change still met the minimum stir volume, while remaining below the maximum vessel volume with reagents and solvent charged. The reaction rate increased as expected, minimizing the area% of farnesene in the reactor contents during the addition, which is favorable to the Diels-Alder reaction.

Each batch reaction was monitored by GC-FID analysis. The reactants, farnesene and DMM were tracked as well as the product. The profiles of each reaction can be seen in the graphs below.

Figure C.1: Reaction profile Batch 1

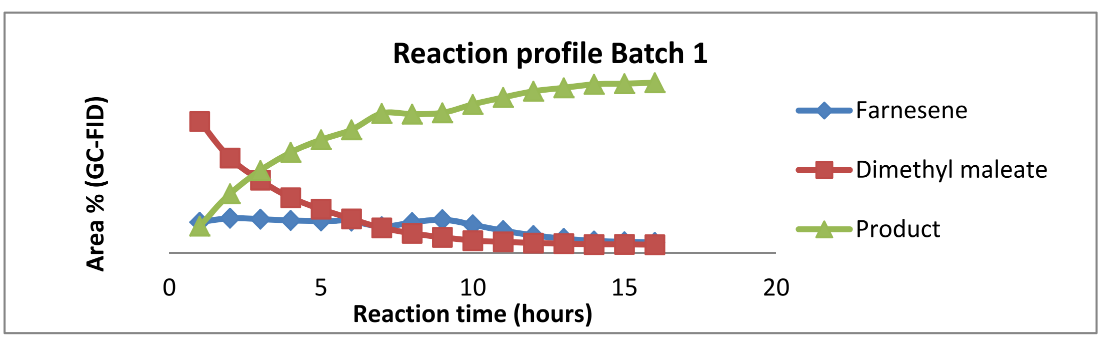


Figure C.2: Reaction profile Batch 2

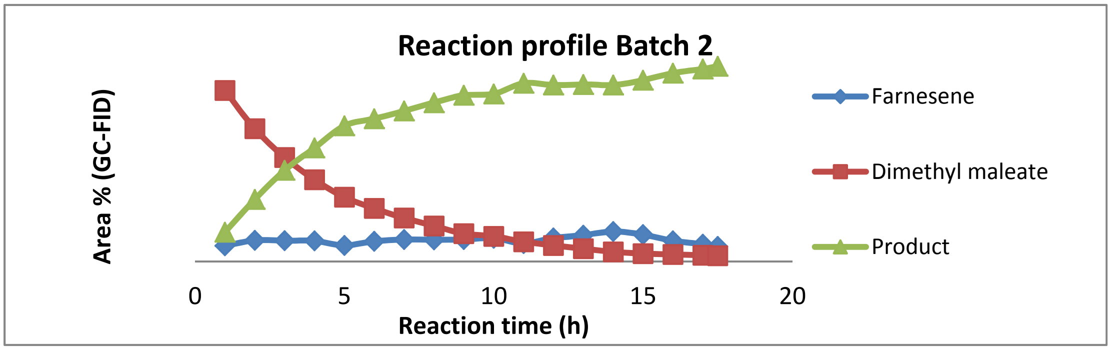
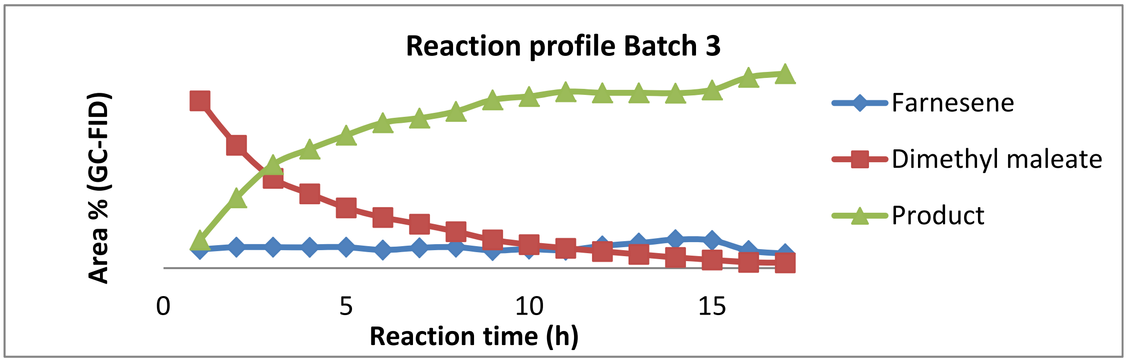


Figure C.3: Reaction profile Batch 3

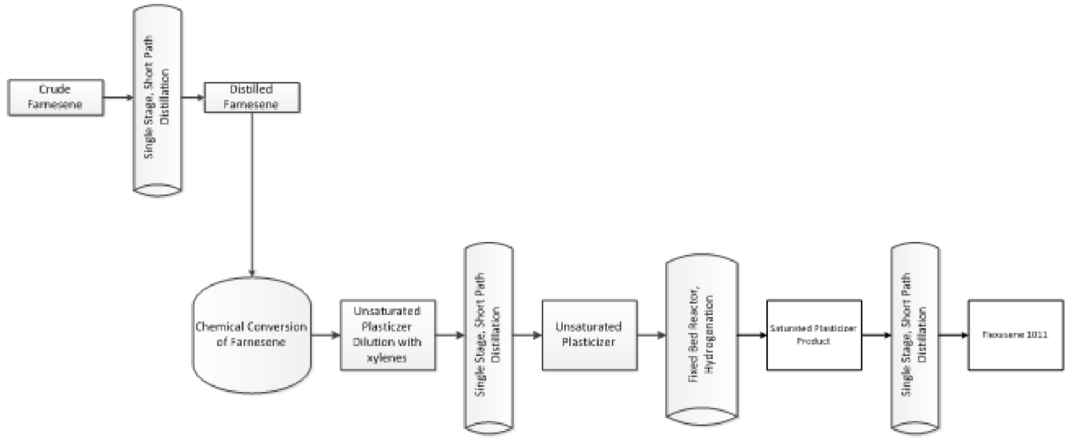


Purification: Distillation Removal of Xylenes from Flexasene 1010

The removal of xylenes from the Flexasene 1010 product was achieved using a WFE. The mass balances and GC-FID area% analysis of the distillate (xylenes) and bottoms (product) are presented below for the three batches distilled for solvent removal.

Once the xylenes were removed from Flexasene 1010, the unsaturated precursor was hydrogenated in the tubular fixed bed reactor to produce the saturated plasticizer product, Flexasene 1011. The catalyst loading was varied in the tube, 25%, 50% and 100% for a total of 477g of catalyst. This loading helped to prevent the exotherm from the initial hydrogenation of completely unsaturated material. The saturated Flexasene 1011 was then purified through distillation to remove the heavier impurities formed during processing.

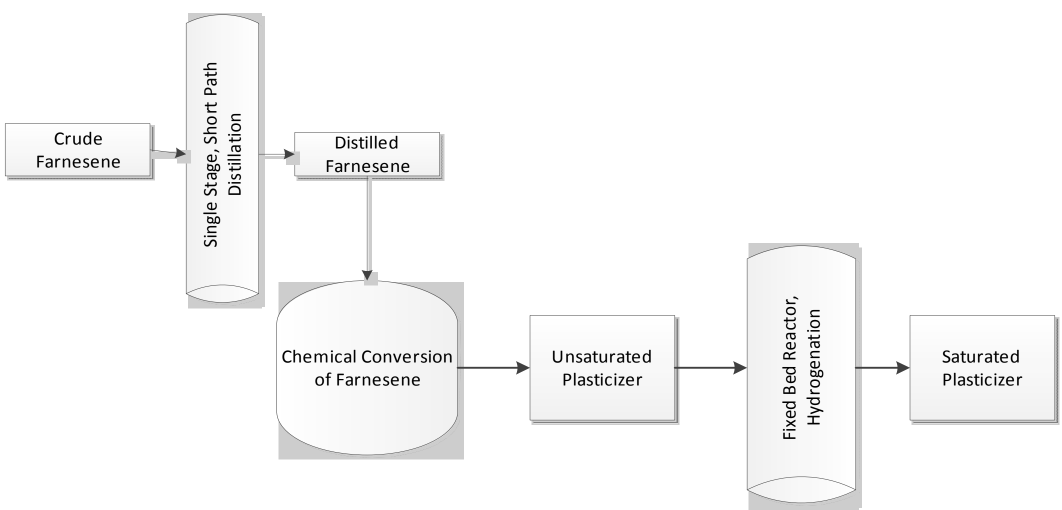
Figure C.4: 15 Gallon Reactor Scale Up Process Flow Diagram (PFD)



Scale Up of Undiluted Plasticizer Process with Equipment Modifications

Amyris Farnesene derived from fermentation utilizing sorghum syrup based media was used in the second scale up of the process after modifying the 15 gallon reactor. The overhead mounting of the stirrer was changed to a side mount. The minimum stir volume was reduced from ~20 L to ~7 L by changing from a side mounted to an overhead mounted set up for the stirrer. In order to maintain similar agitation, a second impeller was added to the stir shaft and a baffle was also added. The thermocouple provided the second baffle. These changes to the reactor allowed for an undiluted reaction and removed the need for a distillation to remove any solvent diluent needed to meet the previous minimum stir volume. The temperature of the reaction was also increased along with a reduction in the addition rate resulting in an undiluted reaction that produced Flexasene 1010 without distillation to remove solvent.

Figure C.5: Process Flow Diagram for the Undiluted Process in 15 Gallon Reactor Skid



The following process description is for the undiluted reaction. The reactor was purged with nitrogen to displace air and prevent oxygen ingress. The DMM was charged. The liquid was stirred under a nitrogen blanket and heated to 160 ± 5 °C. The trans-β-farnesene (farnesene) was charged using a diaphragm pump and feed line. The GC purity for the starting material was 97.4 wt%, the water content was 96 mg/L by Karl Fischer method, and color in Saybolt units +28.

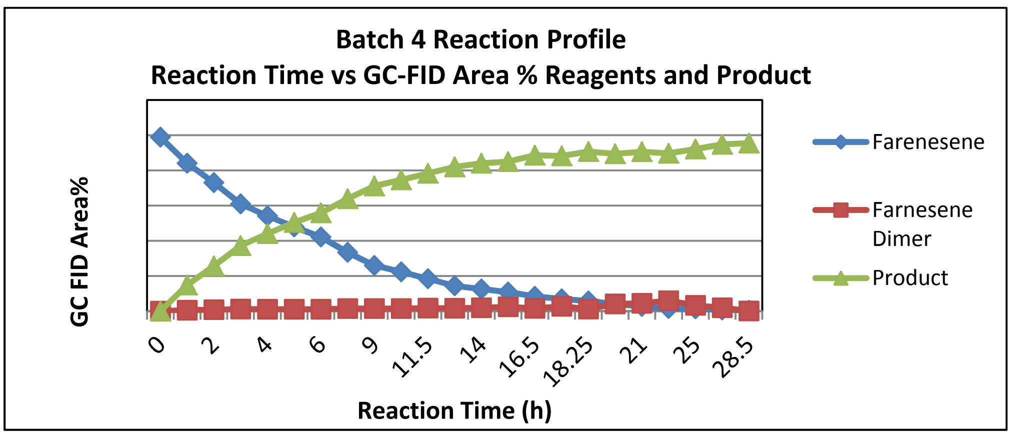
At reaction temperature, the progress and feed rate was monitored by GC_FID Area%. By Gas Chromatograph (GC) Area%, the farnesene content in the reaction mixture was maintained at ≤ 5% of the reaction mixture. This measurement ensured the amount of DMM was much higher than the amount of farnesene in the reaction mixture, ensuring the Diels-Alder reaction would be favored over the side reaction of fene dimerization. It also prevented any pooling of farnesene which could lead to an exotherm for safety. The reaction was considered complete when product by GC Area% ≥ 90%. Reaction product was 95.5% by GC Area%. The reaction was suspended by cooling to 25 °C overnight, since 24 hour coverage was not available. On completion the reactor contents were cooled and a final sample was taken prior to transfer from the vessel.

The reaction product was hydrogenated on a fixed bed reactor. The catalyst loading was varied in the tube at, 25%, 50% and 100% vol catalyst/vol bed for a total of 534 g packed adjusted catalyst per bed. This loading helped to prevent an excessive exotherm during the initial hydrogenation of completely unsaturated material. After hydrogenation, no further purification was needed, resulting in the final plasticizer product.

The data summarized in the table below indicates that the overall yield from the theoretical reaction yield to the saturated plasticizer was 87.67% (RSD 1.7%) through to the hydrogenated product. The Diels Alder reaction was able to obtain conversion yields of close to 96% over the reaction duration of 28.5 hours as indicated in the reaction profile (figure below)

The reactants, farnesene and DMM as well as the product for the Diels-Alder reaction were tracked by GC-FID. The resulting profile can be seen in the following graph.

Figure C.6: Reaction Profile



The optimized process without solvent dilution increased volume efficiency and eliminated the distillation step to remove the solvent. It also eliminates the solvent waste stream.

Alternative Feedstock Performance with Optimized Process for Flexasene 1010 as the Plasticizer Candidate

An evaluation of the optimized process to produce the Plasticizer candidate from Amyris Farnesene derived from fermentation utilizing wheat straw, wheat straw and sorghum syrup, woodchip, and woodchip and sorghum syrup-based feedstock was performed. These reactions were not performed at mid-scale due to the constraints of farnesene volumes produced from fermentation. The performance for the alternative feedstocks was tested on laboratory scale using a further optimized process. This process was run at a higher temperature, 180 °C, and a slower addition rate. The higher temperature increased the rate of the Diels-Alder reaction and the slower addition rate minimized competing dimerization and oligomerization reactions. The cycle time was reduced from 28.5 hours to 16 hours.

Based on plasticizer testing results, it was determined that Flexasene 1010 performed similar to the Flexasene 1011 material, and the process was optimized to meet specifications developed for Flexasene 1010. The use of Flexasene 1010 as the plasticizer candidate eliminated the hydrogenation step from the process. Based on polymer information, specifications for Flexasene 1010 were determined and can be seen in the table below.

Flexasene 1010 Specifications

International Union of Pure and Applied Chemistry (IUPAC) name: (*E*)-dimethyl 4-(4,8-dimethylnona-3,7-dien-1-yl)cyclohex-4-ene-1,2-dicarboxylate

Table C.4: Flexasene 1010 Specifications

Test Description	Specification Limits Minimum	Maximum	Unit of Measure	Test Method
Flexasene Content and Identity (by HPLC)	90 0.95	 1.05	% (w/w) RRT	SOP00270A
Impurities Oligomers + Polymers Dimer DMM Farnesene Total GC Impurities		 2.0 2.0 1.5 2.0 10	 Area % % (w/w) % (w/w) % (w/w) Area %	 TM-0013 TM-0013 TM-0013 TM-0013 TM-0013
Water (Karl Fisher)		500	ppm	SOP00202A
% hydrolysis products of Mol. C (total acid and anhydride)		0.1	mg KOH/g	TM-0012

The component concentrations were measured using quantitative gas chromatography and the total content was determined in critical streams (Process Flow Diagram Figure). The critical streams are indicated in the table below.

Table C.5: Process Flow Stream data for Plasticizer Process Flow Diagram

Stream Description	Data required
Farnesene Reactor Feed	• Farnesene g/L (GC) • Volume (wt)
Dienophile Reactor Feed	• DMM g/L (GC) • Volume (wt)
Post Reaction Harvest	• Component GC-area % • Volume (wt)

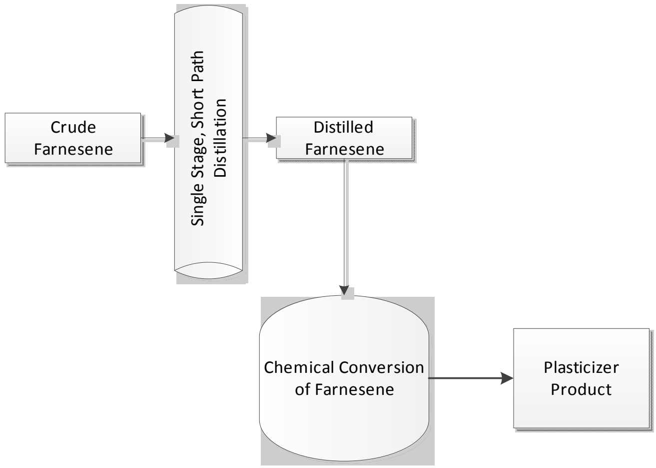
Process Method and Testing Specifications

Due to material volume constraints, the test reactions were performed at a scale of 286 g farnesene. The reactor setup included a 1000 mL, 3-neck round bottom flask with a condenser, overhead stirrer, inlet adapter, nitrogen line, and a thermal couple. The original reactor setup including a metal heating mantel was switched to a soft heating mantel when one of the reactor flasks cracked. Based on the line of the crack, which matched the rim of the heating mantel, it was determined that the crack was due to glass expansion during heating. No further incidents occurred once the heating mantel was changed.

The reactor was purged with nitrogen to displace air, and the DMM was charged. The liquid was stirred and sparged for 20 minutes with nitrogen and a nitrogen blanket was maintained in the vessel. The DMM was heated to 180 °C ± 3 °C and the trans-β-farnesene (farnesene) was transferred into a 500 mL flask with an outlet adapter and nitrogen line. The farnesene was sparged with nitrogen for 20 minutes and maintained under a nitrogen blanket at ambient temperature. Once the DMM in the reactor vessel reached 180 °C the farnesene was transferred to the reactor vessel using a diaphragm pump At reaction temperature, the progress and feed rate was monitored using GC-FID Area% analysis of the reaction mixture. The feed rate was kept constant and set for a 12.5 hour addition time. At this rate the Diels-Alder reaction was favored over the side reaction of fene dimerization, and also prevented any pooling of farnesene in the reaction mixture. An increase in the amount of farnesene relative to the amount of DMM in the reaction mixture could lead to an exotherm due to the amount of farnesene available to react. The reaction was considered complete when product by GC Area% ≥ 90%.

The process flow schematic is provided in Figure C.7 below.

Figure C.7: Process Flow Diagram for Unsaturated Plasticizer Flexasene 1010, Optmized Process



Results

The data showed that the overall yield from the theoretical reaction yields to the plasticizer ranged from 98.99% to 99.18%. The Diels Alder reaction was able to obtain conversion yields of close to ≥ 95% for a cycle time of 16 hours as indicated in the Reaction Profiles (see Figure C.8-11 below).

The alternative feedstock reactions were tracked by GC-FID. The reaction profiles can be seen in the following graphs.

Reaction Profiles

Figure C.8: Reaction Profile for Wheat straw Feedstock

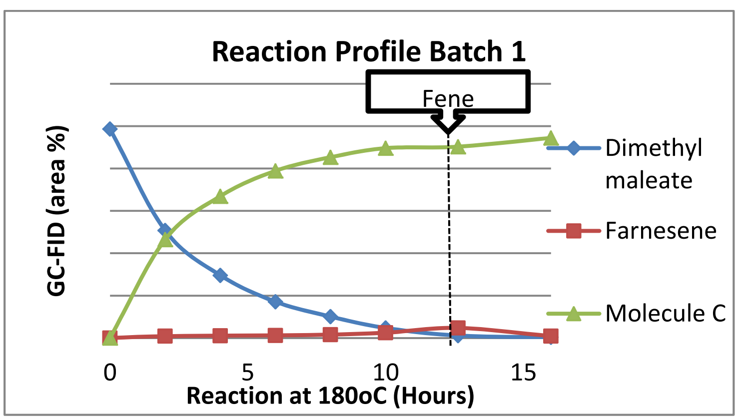


Figure C.9: Reaction Profile for Wheatstraw/Sorghum syrup Feedstock

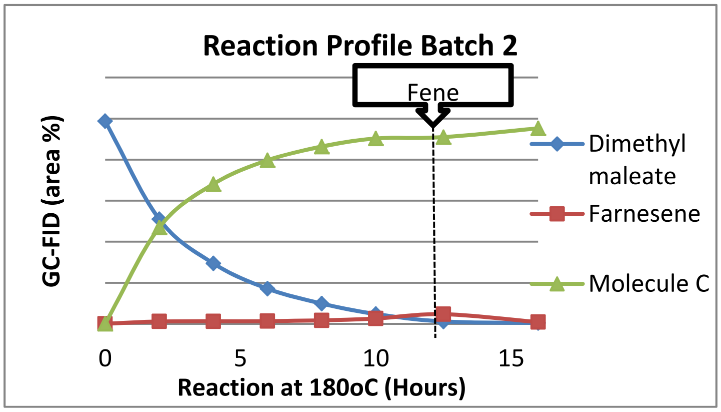


Figure C.10: Reaction Profile for Woodchip Feedstock

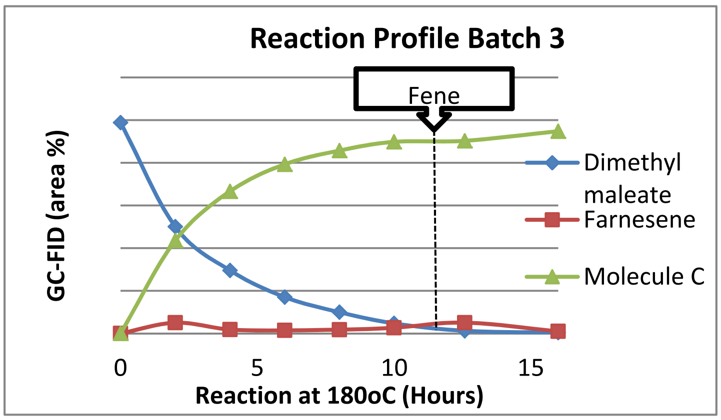
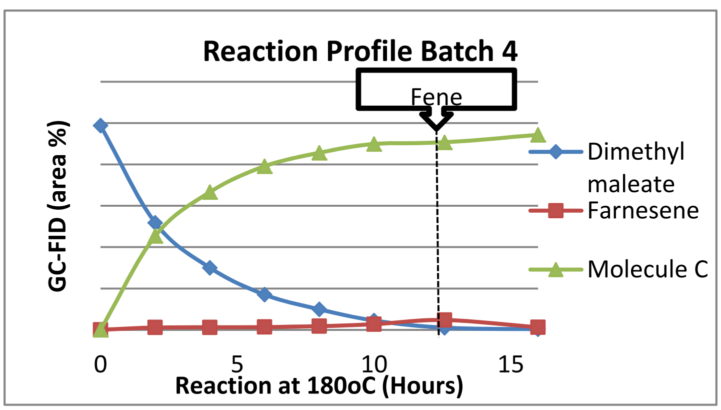


Figure C.11: Reaction Profile for Woodchip/Sorghum syrup Feedstock



Subtask C.3. Pilot-scale production and chemical process technoeconomics

Pilot-Scale production

The modifications made to the skid mounted reactor, changing the mounting of the stirrer from side to top mounting, and adding a baffle to maintain mixing reduced the minimum stir volume of the reactor vessel. This allowed mid-scale reactions to be run undiluted. This process change and scale up resulted in the removal of the distillation operation to remove the solvent diluent. The removal of a diluent also minimized side reactions as the reaction kinetics were improved with the undiluted process. The material produced at mid-scale met specifications without further processing, or purification steps. These process conditions were used for the sorghum syrup performance protocol on the 15 gallon reactor skid. Having proven this process at mid-scale, the same process was scaled in the CMO piloting campaign. Two batches of 1.5 mT each were produced during a Flexasene 1010 campaign at a CMO. The campaign was successful producing product that met specifications without further processing.

Estimation of rate of Heat Generation

At a farnesene addition rate of 33 kg/h and using the experimentally determined heat of reaction, the rate of heat generation was estimated at 5,532 kcal/h. This calculation is used to determine the heat removal necessary during production and if the equipment can safely maintain temperature parameters.

Reaction Profiles

The batch reactions were monitored by GC-FID and the profiles can be seen in Figure C.12 and

Figure C.13. Note: Flexasene 1010 is also referred to as Molecule C (Mol C) during this campaign.

Figure C.12: Reaction profile for Batch 1

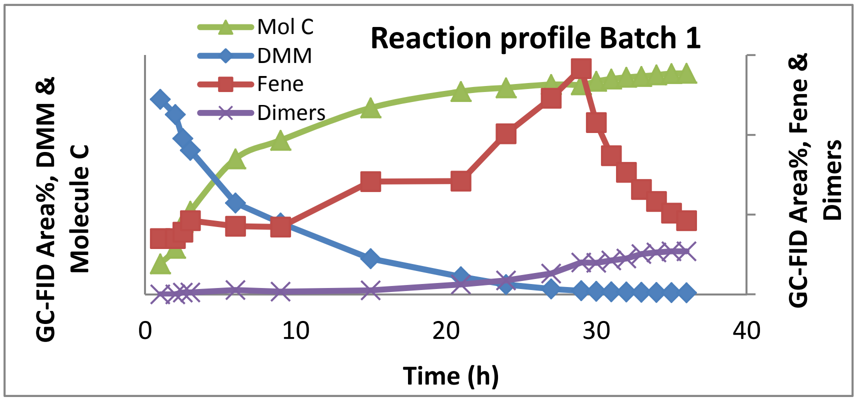
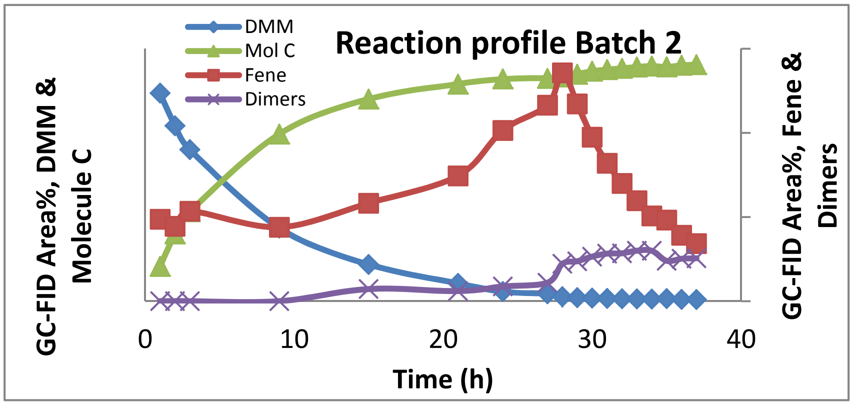


Figure C.13: Reaction profile for Batch 2



Mass Balances

Bench scale experiments using a solvent diluent had several distillation unit operations to remove the solvent after the Diels-Alder reaction followed by distillation for purification due to the side reaction impurities. It was at this scale that it was noted that the dilution was impacting the reaction rate and that with a controlled addition of the farnesene, the reaction could be run without dilution. However, the initial scale up of the reaction was performed using solvent and resulted in <87% yield (by area%) and required distillation to remove the solvent prior to hydrogenation and distillation after to remove heavier impurities from side reactions. The Piloting scale was performed without dilution and at a higher temperature than the mid-scale reactions. This production run produced >94.9% wt Flexasene 1010 product that did not require further purification.

Task D: Regulatory and end-user process and product acceptance

Task Objective: The establishment of market readiness for Amyris Diesel and chemical products is an essential component of the IBR project.

Subtask D.1. Fuel product approval and acceptance

- *Completed June, 2013*

Subtask D.2. Chemical product approval and acceptance

- *Completed March, 2013*

Subtask D.3. Use approvals for genetically modified microbes (GMM)

- *Completed May, 2013*

EPA regulates the commercial use of GMMs. The Amyris microbe S. cerevisiae is eligible for exemption from EPA review as it is recognized as posing only low risk. The Amyris manufacturing facilities complied with specified EPA criteria including the Tier 1 filing to maintain this exemption, as well as the use of compliant containment structures and safety procedures. No further GMM approvals were pursued or required. Additional details were provided in NEPA discussion in Task A.

Subtask D.4. Commercial production process approvals and acceptance

- *Closed out May, 2013*

Target completion is beyond this project scope. Additional details discussed in the Summary section up front.

Accomplishments Against Goals

Subtask D.1: Fuel product approval and acceptance

- Renewable diesel (Farnesane) multi-media review and acceptance by CARB
- OEM validation by CUMMINS ENGINE COMPANY, CATERPILLAR, MWM-NAVISTAR, MERCEDES BENZ (Truck & Bus Division), VOLSWAGEN AG, SCANIA AB and MAN
- Establishment of renewable diesel product specifications by Colonial Pipeline
- Robust on-highway demonstrations with FedEx, Sierra-Nevada Brewery and Volkswagen of America
- Bunker diesel fuel demonstration by the DOT Maritime Administration (blended fuel)
- Renewable diesel specifications by US Navy – Direct Sugar to Hydrocarbon (DSH-76)
- Combustion kinetic and chemical modeling by Cummins and Princeton University
- Combustion and spray visualization at high pressure and pressure validating CFD modeling – Cummins and Caterpillar
- EPA TSCA review and Chemical Abstract Service (CAS) registration of Farnesane - pending EPA PMN registration
- EC Registration, Evaluation, Authorization and Restriction of Chemicals (REACh) registration of Farnesane as diesel and aviation turbine fuel
- Proposed ASTM standard specification for aviation turbine fuel containing synthesized iso-paraffinic hydrocarbon (Farnesane)
- Gas turbine fuel analysis by National Aeronautical and Space Administration (NASA) Dryden Flight Research Center – presentation at CRC 2013 Conference
- Princeton University analysis and publication, Comparative Evaluation of Global Combustion Properties of Alternative Jet Fuels (Farnesane as a surrogate jet fuel)

Additional data collection and filings to support follow-on registration:

The project collected significant data in terms of quality and quantity required to support further fuel registrations, new chemical registrations, end-user acceptance, and OEM validation (diesel and jet fuel). Operational data was also collected supporting efforts to obtain engine OEM commercial warranty coverage. Data collection was on neat (100%) Amyris Diesel as required by the OEMs. The project acquired extensive data verifying real-world fuel storage, transport, distribution, and application.

Life cycle analysis and certification:

The project achieved a primary LCA analysis completed for diesel fuel under the EPA RFS II and CARB protocols. The file is ready for submission to EPA for certification. Assessment activities are ongoing with the international RSB, a recognized global sustainability auditing and certification body. The purpose of this activity is to certify by independent body the Amyris sugar pathway. The activity will be completed post IBR project timeline.

OEM engagement, testing and on-highway validation:

The projected acquired extensive data from OEM engagement including CFD modeling, combustion and kinetic modeling, spray visualization, engine bench and vehicle chassis testing, and on-highway validation.

The project completed three (3) comprehensive US on-highway demonstrations involving major OEMs, namely Cummins, Volkswagen and Navistar. The demonstrations included heavy-duty Kenworth trucks operated by FedEx, a medium-duty diesel-hybrid commercial truck operated by Sierra Nevada Brewery, a medium-duty regional transit bus operated by ETMA, and light-duty passenger vehicles provided by Volkswagen operated by Amyris. The programs confirmed drop-in compatibility in terms of engine performance, vehicle reliability, fuel economy, fueling, fuel storage, and emissions (exhaust and evaporative) levels.

Filing and approvals (TSCA and REACH):

TSCA: The project acquired data justifying a substantially similar finding for farnesene as a listed TSCA chemical and data supporting a farnesane PMN application. The farnesane PMN application is progressing through the final phase of agency review with approval expected near-term.

REACh: Under the EC REACh directive, the two chemical substances (farnesene and farnesane) were successfully registered.

CARB multi-media fuel approval:

CARB has concluded, based on submitted farnesane data, that no further multi-media testing is required. The farnesane data is now supporting evidence justifying an ARB proposed regulation classifying renewable diesels (those compliant with ASTM D975 specifications) as ‘diesel’. The farnesane data supplements the CARB diesel ‘no significant adverse impacts finding’ regarding multimedia exposure. Note: Biodiesel will continue to be subject to multi-media evaluation.

CARB fuel registration:

CARB has approved Amyris renewable diesel fuel registration up to 35% blends.

Additional Pipeline spec testing:

Through data sharing and manufacturing process explanation, the project pursued the first US pipeline renewable diesel product code and corresponding product specifications listing. The product code and material specifications are necessary for renewable diesel material allowance into the US fuel distribution pipeline network. In 2012, Colonial Pipeline established their renewable diesel specifications under Section 3 of the Shippers Manual as Product Code 3.2.7 Renewable Diesel, namely;

Renewable diesel is a liquid fuel derived from 100% hydrotreated bio-mass feedstocks that meets the registration requirements for fuels and fuel additives established by the EPA under section 211 of the Clean Air Act and the requirements of ASTM D975.

Ultra Low Sulfur Diesel (ULSD) product containing greater than 5% renewable diesel may be entered into the Colonial pipeline distribution network as Grade 65 - 15 ppm Sulfur Distillate or Grade 69 – 15 ppm Sulfur Distillate Blendstocks. The Colonial Pipeline specifications permit the distribution of renewable diesel as conventional ULSD throughout their US pipeline network.

Subsequent adoption by other major US pipeline operators is expected.

Fuel storage:

Farnesane as both a diesel fuel and as an aviation turbine fuel blending component was assessed for long-term thermal and oxidation stability and the effectiveness of commercial oxidation stabilizing additives on farnesane was investigated. The assessment was performed by subjecting farnesane to accelerated methods (e.g. ASTM D381) and physical properties analysis following an extended 16 month storage period. The product was found to maintain storage stability equal to or better than fossil-derived fuels.

The US Air Force Research Laboratory (AFRL) assessed storage stability through measurement of peroxides and potential gums (e.g. ASTM D5304) under accelerated storage conditions using high temperatures. The AFRL facility measured peroxide formation in 10% and 20% farnesane-containing jet fuels over the course of 6 weeks at 65°C. Results were consistent with fossil-derived jet fuels. The Air Force analysis also investigated compatibility with storage stability additives. Stability additives were found to be compatible and effective when additized to farnesane as a diesel fuel.

REACh/TSCA data acquisition:

New chemical substances are required to be registered in the US under the TSCA and in Europe under the Registration, Evaluation, Authorization and Restriction of Chemical substances (REACh) Directive. These regulations require assessing chemical substances by its physicochemical, toxicological, ecotoxicological and biodegradation properties. The project pursued data acquisition for properties which were not available. The resultant data is used to determine the chemical substance classification and labeling requirements.

The project specifically focused on acquiring enhanced biodegradation data, eco-toxicity studies, and assessment of physical-chemical properties within production variation. EPA and ECHA guidance for low soluble test materials like farnesane required development of alternate procedures while maintaining the test protocol limits. For example, the farnesane and farnesene enhanced biodegradation testing was extended to 60 days to demonstrate degradation >50% (measured biodegradation was 60%). As a result, both farnesene and farnesane can be classified favorably as non-persistence.

EPA PMN approval for farnesane:

The farnesane PMN application was submitted under eTSCA submittal system during December 2011 and subsequently was subject to further EPA mandated studies. The testing phase was subject to numerous delays by third-party laboratory issues or extensive lead-times seeking EPA guidance and data interpretation. The PMN application has recently been supplemented by new eco-toxicity data and is awaiting EPA approval.

Subtask D.2: Chemical product approval and acceptance

- EC REACh registration of 3 new chemical substances
    - Farnesene (Farnesane precursor)
    - Farnesane as specialty chemical
    - Renewable terpene synthase product

Registration package preparation/Filing and approvals (TSCA and REACh):

The project achieved eight (8) new chemical substance registrations under EPA TSCA and CAS with production applications as base lubricants and specialty chemicals. Under the EC REACh directive, two (2) new chemical substance registrations were achieved in addition to the registration of farnesene. Of the eight new EPA chemical substances, four have progressed to commercial products while the remaining substances continue through the product validation process.

Chemical properties characterization:

The achieved TSCA and REACh registrations required the acquisition of significant physical and chemical properties, certain toxicity studies, and ecotoxicity evaluations under EPA and ECHA guidelines. All acquired data was reviewed and accepted by the respective regulatory bodies.

End-user validation testing:

The eight new chemical substances have either been validated (four substances) or continue to undergo the validation process by commercial downstream users through fit for purpose testing. Testing protocols are inclusive of industry standards and proprietary methods.

Project Activities

Subtask D.1: Fuel product approval and acceptance

Cummins:

Cummins validated Amyris Diesel utilizing computational analysis, dynamometer performance assessment and on-highway analysis. Figure D.1, Figure D.2 and Figure D.3 illustrate engine dynamometer data from neat (100%) Amyris Diesel operation. The data shows a significant reduction in particulate matter (PM) emissions while engine performance (power and torque output) is maintained.

Figure D.1: Relative Particulate Emission Comparison (engine dynamometer) – S50 Diesel vs. Amyris Diesel

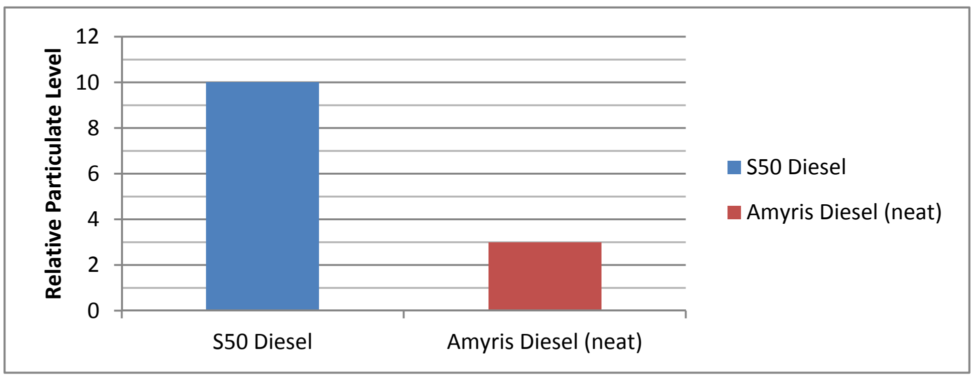
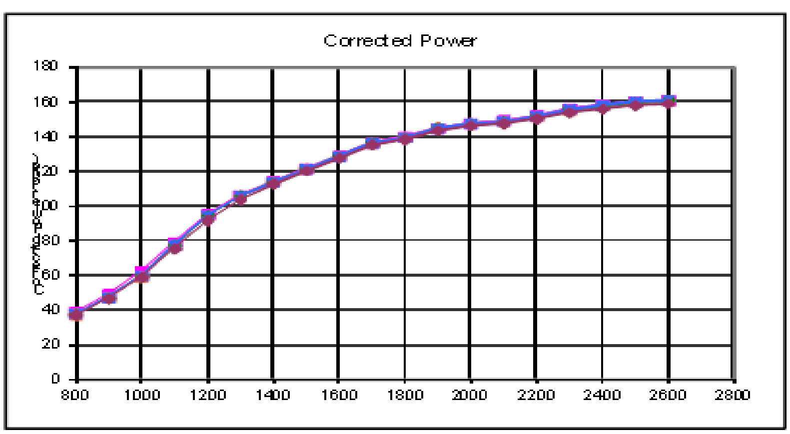
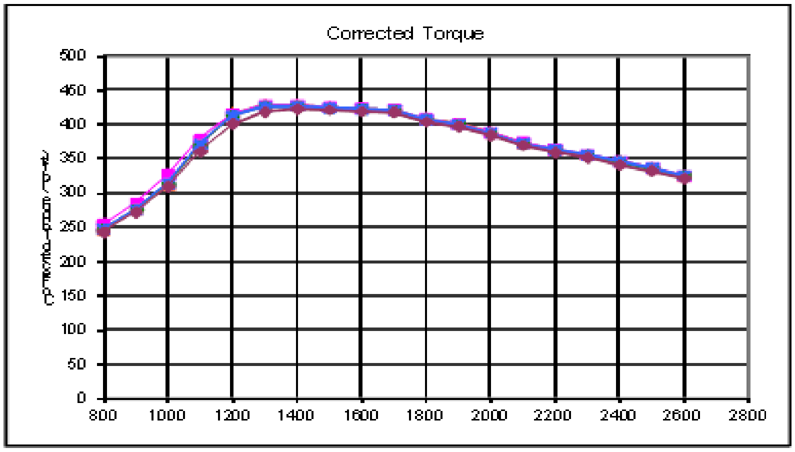


Figure D.2: Engine Power Kilowatts (kW) Output Comparison (engine dynamometer) – S50 Diesel vs. Amyris Diesel



Diesel S50
Amyris Diesel (neat) 100

Figure D.3: Engine Torque Output Comparison (engine dynamometer) – S50 Diesel vs. Amyris Diesel



Diesel S50

Amyris Diesel (neat) 100

Caterpillar:

Caterpillar validated Amyris Diesel utilizing combustion and spray visualization at high pressure and temperature CFD modeling at a 25% blend ratio - 25% Amyris Diesel/75% ULSD. The relatively low 25% blended ULSD achieved shorter (improved) ignition delay, shorter (improved) flame lift-off length and similar peak luminosity from soot. The observed earlier timing of peak soot luminosity is partly due to shorter ignition delay. Overall the combustion results at tested conditions are similar to ULSD.

MWM-Navistar: Maxxforce 7.2H

MWM-Navistar validated Amyris Diesel on an engine dynamometer basis at 20% blend ratio (20% Amyris Diesel/80% ULSD) through neat Amyris Diesel (100%). The dynamometer results for neat Amyris Diesel in comparison to S50 diesel Showed that operation with neat Amyris Diesel maintained similar power output, while lowering oxides of nitrogen (NOx) and PM exhaust emissions, and improving fuel consumption.

Subtask D.2. Chemical product approval and acceptance

Multi-media data: Toxicity data explanation and summary

Summary of chromosome aberration test results:

Chromosome aberration testing is one of many genotoxicity tests used to screen for possible mammalian mutagens and carcinogens. This *in vitro* test evaluates the potential of a test article to induce structural chromosomal aberrations in human peripheral blood lymphocytes (HPBL), in the presence and absence of an exogenous metabolic activation system (S9). The assay evaluated the clastogenic potential of the test article. Many compounds that are positive in this test are mammalian carcinogens; however, the contracted laboratory reported that there is not a perfect correlation between this test and carcinogenicity.

Farnesane testing was conducted at Bioreliance of Rockville, Maryland from September through November, 2012. Following test method OECD 423, Cyclophosphamide and mitomycin C were evaluated as the concurrent positive controls for treatments with and without the required exogenous metabolic activation system.

In the chromosome aberration assay, the cells were treated for 4 and 20 hours in the non-activated test system and for 4 hours in the S9-activated test system. All cells were harvested 20 hours after treatment initiation. The highest dose analyzed under each treatment condition exceeded the limit of solubility in treatment medium at the conclusion of the treatment period, which met the dose limit as recommended by testing guidelines for this assay.

There is no meaningful correlation between the dose administered and the effect of chromosomal aberration.

Eco-tox data explanation and summary

Summary of water solubility testing:

As a precursor to aquatic toxicity testing, a 96-hour functional solubility in well water study was conducted at Wildlife International in Easton, Maryland. The study included analytical method development for analysis of farnesane.

The solubility of farnesane was determined in freshwater at 0, 1, 2, 3 and 4 days at 24°C ± 1°C. A stock solution of farnesane was prepared in acetone, which was used to prepare the solutions for the solubility trials. A solvent concentration of 0.1 mL/L was used as per OECD guideline. Saturated aqueous solutions of the test substance were prepared at a nominal concentration of 100 µg a.i./L in 14-L clear water accommodated fraction (WAF) bottles. Duplicate bottles were placed onto stir plates with a magnetic stir bar and maintained at 24 ± 1° C. At each interval, triplicate subsamples of the aqueous phases from each bottle were collected from mid-depth using a volumetric pipette. Prior to collecting the samples, the stir bar was turned off for approximately 10 minutes. Each sample was extracted as per the method outline and analyzed for farnesane. The pH of the freshwater prior to preparation of the samples was 8.1.

Analytical method for the solubility trials:

The method used for the analysis of farnesane in freshwater was developed by Wildlife International, Ltd. The solubility of farnesane was determined as follows: glass centrifuge tubes were rinsed with toluene. Recovery samples, used for quality control (QCs), were prepared by directly fortifying the freshwater with the appropriate Farnesane stock solutions. Toluene was added to each sample and samples were vortexed for approximately one minute. Aliquots of the organic extracts were transferred to autosampler vials and samples were submitted for analysis by GC/MS.

Concentrations of farnesane in the samples were determined using a Hewlett-Packard Model 5890 GC equipped with a Hewlett-Packard Model 5971A Mass Selective Detector operated in the selected ion monitoring (SIM) mode. Calibration standards of farnesane, ranging in concentration from 10.0 to 100 µg a.i./L, were prepared in toluene using a stock solution of farnesane in acetone. Calibration standards were prepared and analyzed with each sample set. Linear regression equations were generated using the peak area responses versus the respective concentrations of the calibration standards. The concentration of farnesane in the samples was determined by substituting the peak area responses of the samples into the applicable linear regression equation.

The method limit of quantitation (LoQ) for these analyses was defined as 10.0 µg a.i./L, calculated as the product of the concentration of the lowest calibration standard (10.0 µg a.i./L) and the dilution factor of the matrix blank samples (1.00). Four matrix blank samples were analyzed to determine possible interferences. No interferences were observed at or above the LoQ during the sample analyses.

Freshwater QC samples were fortified at 50.0 µg a.i./L using a stock solution of farnesane in acetone and were analyzed concurrently with the test samples. The measured concentrations for the matrix fortification samples ranged from 48.1 to 95.8% of the nominal concentration.

Summary of 96 hour algae test:

A 96-hour toxicity test with freshwater alga (*Pseudokirchneriella subcapitata*) was conducted at Wildlife International in Easton, Maryland. Testing was conducted using the following methods/guidelines: Organisation for Economic Co-operation and Development (OECD) Guideline 201, EU Directive 92/69/EEC, Method C.3, and USEPA Office of Chemical Safety and Pollution Prevention (OCSPP) Number 850.4500. The purpose of the study was to determine the toxicity of farnesane to the freshwater green alga, *Pseudokirchneriella subcapitata*, during a 96-hour exposure period.

The green algae, *Pseudokirchneriella subcapitata*, was exposed to six test concentrations, a solvent control (0.1 mL N,N-dimethylformamide per liter of culture medium), and a negative control(culture medium) under static conditions for 96 hours. Six replicate test chambers in the negative control group and four replicate test chambers in each treatment group and in the solvent control group were maintained.

Concentrations of farnesane in the samples were determined using an Agilent Model 5890 GC equipped with a Hewlett-Packard Model 5971A Mass Selective Detector operated in the SIM mode. The method LoQ was defined as 2.00 µg a.i./L, calculated as the product of the concentration of the lowest calibration standard (2.00 µg a.i./L) and the dilution factor of the matrix blank samples (1.00).

Nominal test concentrations were selected in consultation with the Sponsor and were based upon the results of an exploratory range finding toxicity test and the solubility of the test substance.

Nominal test concentrations selected were 3.1, 6.3, 13, 25, 50 and 100 µg a.i./L. Measured concentrations were determined from samples of test medium collected from each treatment and control group at test initiation and at test termination (96 hours).

Summary of chronic fish early life stage test:

Testing was conducted at Wildlife, International in Easton, Maryland. The objective of the early life stage study was to determine the effects of farnesane on the time to hatch, hatching success, survival, and growth of fathead minnows (*Pimephales promelas*) during early life stage development. The following guidelines were followed: OECD GUIDELINE 210 and USEPA OPPTS (Office of Prevention, Pesticides & Toxic Substances) NUMBER 850.1400 (draft).

The testing exposed fathead minnow embryos to a geometric series of five test concentrations, a negative control (dilution water), and a solvent control (0.1 mL/L HPLC-grade dimethylformamide) all under flow-through conditions. The exposure period included a 4-day embryo hatching period, and a 28-day post-hatch juvenile growth period. The results of the study were based on the mean measured concentrations.

Deliveries of the test solutions to the test chambers were initiated seven days prior to test initiation in order to achieve equilibrium of the test substance. Four replicate test chambers were maintained in each treatment and control group, with one incubation cup in each test chamber. Each incubation cup contained 20 embryos, resulting in a total of 80 embryos per treatment. At test initiation, embryos <24 hours old were impartially distributed to incubation cups and exposed to test solution in the test chambers. After a 4 day embryo hatching period, the larvae were released into the test chambers, where exposure continued during a 28-day post-hatch juvenile growth period. Observations of the effects of farnesane on time to hatch, hatching success, growth, and survival were used to calculate the NOEC, the lowest-observed-effect-concentration (LOEC), and the maximum acceptable toxicant concentration (MATC).

Summary of chronic daphnia test:

Testing was conducted at Wildlife, International in Easton, Maryland. The objective of this study was to determine the effects of farnesane on the survival, growth and reproduction of the cladoceran (*Daphnia magna*) during a 21-day exposure period under flow-through test conditions. The following guidelines were followed: USEPA OPPTS NUMBER 850.1300 and OECD GUIDELINE 211.

The cladoceran, or daphnids (as referenced from this point on),were exposed to a geometric series of five test concentrations, a negative control (dilution water) and a solvent control (0.1 mL/L HPLC-grade dimethylformamide). Two replicate test chambers were tested for each treatment and control group. Each replicate contained two compartments with five daphnids, resulting in a total of 20 daphnids in each treatment and control group. Nominal test concentrations were selected based on exploratory range finding toxicity data. Mean measured test concentrations were determined from samples of test water collected from each treatment and control group at test initiation, at approximately weekly intervals during the test, and at test termination. The analysis of farnesane in freshwater samples was based upon methodology developed by Wildlife International.

Delivery of the test substance to the test chambers was initiated approximately five days prior to the introduction of the daphnids to the test water in order to achieve equilibrium of the test substance in the test chambers. At test initiation, neonate daphnids were impartially assigned to exposure chambers. First-generation daphnids were observed daily during the test for mortality, the onset of reproduction, and clinical signs of toxicity. Following the onset of reproduction, the number of second-generation daphnids were counted three times per week (i.e., Monday, Wednesday and Friday), and at test termination (Day 21). Body lengths and dry weights of the surviving first-generation daphnids were measured at the end of the exposure period. Observations of the effects of farnesane on 1) survival, 2) reproduction, and 3) growth were used to determine the NOEC, the LOEC, and the MATC. EC50 values were determined based on reproduction and on first-generation immobility at test termination.

Table D.1: Farnesane chronic daphnia test results

Summary of Survival, Reproduction and Growth of *Daphnia magna* Exposed to Farnesane for 21 Days

Mean Measured Concentration (µg a.i./L)	Percent Adult Survival[1,2]	Mean No. Neonates Per Reproductive Day ± Std. Dev.[3]	Mean Length ± Std. Dev. (mm)	Mean Dry Weight ± Std. Dev. (mg)[1]
Negative Control	95	14.7 ± 1.5	5.0 ± 0.00	1.13 ± 0.19
Solvent Control	90	15.1 ± 1.5	4.9 ± 0.10	1.19 ± 0.17
Pooled Control	93	14.9 ± 1.4	5.0 ± 0.07	1.16 ± 0.17
12	95	15.3 ± 1.1	4.9 ± 0.05	1.26 ± 0.20
20	100	15.3 ± 0.39	4.9 ± 0.08	1.13 ± 0.12
36	90	14.8 ± 0.10	4.8 ± 0.00*,[4]	1.14 ± 0.10
54	100	13.6 ± 1.1	4.7 ± 0.10*,[4]	1.25 ± 0.11
77	95	10.1 ± 1.3*	4.5 ± 0.15*	1.27 ± 0.12

* Indicates a statistically significant decrease in mean total length (Bonferroni's t-test, $p \leq 0.05$) or in reproduction (Dunnett's one-tailed test, $p \leq 0.05$) in comparison to the pooled control.

[1] There were no statistically significant decreases in survival (Fisher's Exact test, $p > 0.05$) or in mean dry weight (Dunnett's one-tailed test, $p > 0.05$) in comparison to the pooled controls.

[2] 21-day EC50 for survival: >77 µg a.i./L

[3] 21-day EC50 for reproduction: >77 µg a.i./L

[4] While statistically significant differences were noted in lean total length at the 36 and 54 µg a.i./L treatment groups in comparison to the pooled controls, the slight differences (4-6% from the pooled control mean) were not considered to be biologically meaningful or treatment related.

Subtask D.3. Use approvals for genetically modified microbes (GMM)

See Section A, Subtask A.1.

Subtask D.4. Commercial production process approvals and acceptance

See Executive Summary section.

Product Produced or Technology Transferred: Intellectual Property and Data Sharing

Publications, conference papers, or other public releases of results

Nothing to report.

Website or other internet sites that reflect the results of this project

Nothing to report.

Networks or collaborations fostered

Nothing to report.

Technologies/techniques

Nothing to report.

Inventions/patent applications, licensing agreements

Ten invention reports and five families of patent applications have been filed under contract DE-EE0002869, which are listed in the table below:

Primary DOE Case No.	Secondary DOE Case Nos. (if any)	Title	Patent Application No. (Amyris Ref. No.)
S-127,288	N/A	Partially Hydrogenated b-Farnesene	PCT/US2012/024922 (AM-4400 PCT)
S-127,413	S-127,800 S-127,801	Plasticizers Derived from b-Farnesene	PCT/US2012/028956 (AM-5000 PCT)
S,127,492	N/A	Process Improvement to Method for Making Squalane	PCT/US2011/037341 (AM-3500 PCT); US Application No. 13/112,991 (AM-3500); Brazilian Application No. 112012028932-2 (AM-3500 BR); European Application No. 11724846.8 (AM-3500 EP); Japanese Application No. 2013-511388 (AM-3500 JP)
S-127,573	S-127,289 S-127,290 S-130,043	Methods for Making Base Oil	PCT/US2012/024926 (AM-5200 PCT)
S-127,856	N/A	Diels-Alder Ester-Based Lubricants	PCT/US2012/048203 (AM-5300 PCT)

Other products, such as data or databases, physical collections, audio or video, software or netware, models, educational aid or curricula, instruments or equipment

Nothing to report.

Technoeconomic Analysis

Foreword

The purpose of this TEA is to analyze Amyris's biofuel conversion technology for the production of renewable diesel and chemicals.

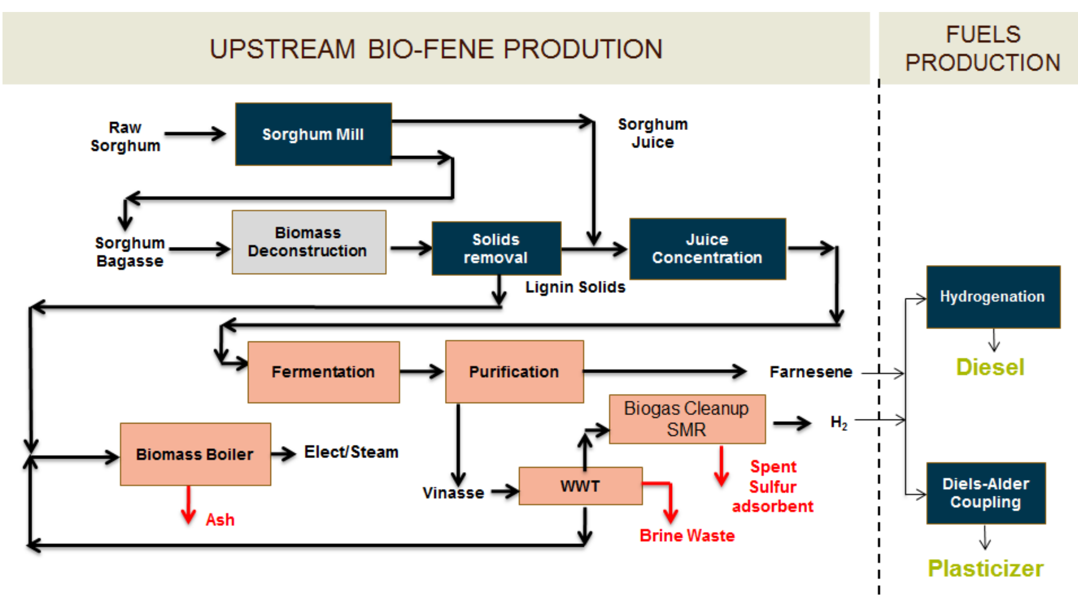
The numbers used in this study are derived upon certain assumptions that are, to the best of our knowledge, representative of the likely costs to build and operate a SSIBR. All numbers represented are approximate, even if not explicitly stated, and are not to be interpreted as a guarantee, or promise, to deliver or execute on these costs as they are subject to market forces and other forces unknown or beyond our control. For these reasons, the actual economics presented in this report may not be indicative of actual performance, but are meant to be a representation of the potential performance given our current state of knowledge.

Background

Amyris's objective is to provide renewable molecules that satisfy demand growth for non-petroleum fuels and chemicals. Proving a capability to innovate as well as execute on a commercial scale, Amyris's technology platform is focused on the production of No Compromise® products (*e.g.* products that perform comparably to, or better than incumbents at comparable prices). Amyris recognizes the potential economic opportunity alternative sugar-feedstocks enable (*e.g.* cost-advantaged sugar, geographical diversity, environmental advantage, *etc.*), and it is driving a research and development program to access these feedstocks. As part of this comprehensive commercialization pathway, Amyris developed a rigorous process economic model to forecast technological milestones and production costs associated with biomass-derived fuels and chemicals.

This section provides a description of the hypothetical production economics associated with an integrated domestic sweet sorghum biofuels refinery. The comprehensive design converts sweet sorghum into Amyris renewable diesel and chemicals. Material flow through the plant is envisioned, as detailed in the block flow diagram below, Figure TEA.1.

Figure TEA.1: Process description of the SSIBR



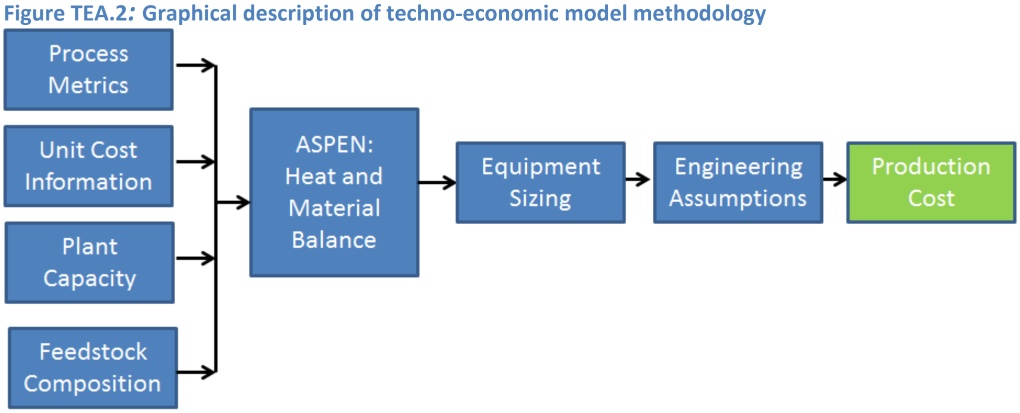
The techno-economic model illustrates the production cost associated with Amyris's renewable products manufactured from sweet sorghum feedstock. The model provides utility in the assessment of the products' competiveness in the broader market, as well as the overall viability of the bio-refinery.

In summary, a comprehensive TEA was undertaken utilizing model input parameters, such as feedstock composition, process yields, raw material unit costs, and purchased capital cost information collated from various partners to illustrate the production costs associated with the proposed SSIBR. The combined information was synthesized in a thermodynamic process simulation tool (i.e. ASPEN Plus) and production costs and sensitivity effects were calculated. Nth plant production costs reveal the potential for profitable and cost competitive renewable diesel pricing. In addition to the baseline process purposed for the SSIBR (i.e. C5 sugars to 'green' hydrogen production), the sensitivity analysis below illuminates the cost advantaged nature of a yeast strain capable of fermenting both C6 and C5 sugars to farnesene.

Methodology

A techno-economic analysis explores the interplay of technical, process, and economic factors and their effect on production economics. In addition, a TEA model provides a means to analyze specific unit operation performance targets and prioritize resource allocation to areas based on their impact on production cost. A simple diagram describing the methodology of the TEA employed in this study is presented in Figure TEA.2.

Figure TEA.2*:* Graphical description of techno-economic model methodology



The specific inputs and outputs of the TEA are described.

- Inputs:
  - Plant Capacity (e.g. tonnes/d of raw feedstock)
  - Process description, yields, productivities
  - Raw material unit cost information
  - Purchased capital cost
  - Engineering assumptions (e.g. contingency, installation factors, etc.)
- Outputs
  - Production cost
  - Comprehensive Heat and Material Balance

Design Basis

Amyris's core biotechnology platform is driven by a technology engine that modifies yeast metabolism for the production of renewable fuels and chemicals. As yeast organisms do not typically consume C5 sugars, the integrated process proposed to maximize carbon and energy efficiency of a SSIBR requires the flow of un-fermented C5 sugars and additional carbon to the waste water treatment section of the SSIBR. Utilizing waste water nomenclature, the carbon entering the water treatment section is referred to as chemical oxygen demand (COD). As the wastewater COD flows to the anaerobic digestion unit, the COD and waste nutrient are degraded into biogas, and metabolically transformed into a mixture of CH_4, CO_2, N_2, & H_2S. In the proposed process scheme, the produced biogas is subsequently upgraded into a purified CH_4 and CO_2 gas stream and chemically reformed to renewable 'green' hydrogen (CH_4 reformed to H_2). The *in-situ* production of hydrogen from COD derived biogas offsets external carbon inputs that would be required during hydrogenation of the intermediate fermentation product to renewable diesel. For example, a process without *in-situ* 'green' hydrogen production would require the input of an additional carbon stream, such as natural gas, in order to supply a feedstock for hydrogen production. In addition to minimizing external carbon inputs, the reformed biogas can provide an additional revenue source to the SSIBR in the form of a surplus hydrogen co-product.

The design basis for the SSIBR is benchmarked to the NREL cellulosic ethanol plant at 2000 dry tonnes/d of feedstock. The design case described in the following document represents an advanced Nth plant, where process and engineering challenges have been addressed and solved (e.g. a best case option). The feedstock composition and water content assumed for the Nth plant SSIBR design case is presented in Table TEA.1.

Table TEA.1*: Sweet Sorghum composition and sugar content assumptions*

Nth Field Sorghum Juice Assumptions								
kg Sugar/wet tonne	73							
kg bagasse/wet tonne	365							
kg juice/wet tonne	635							
Sugar concentration g/L in Juice	132							
Sorghum Bagasse Composition % Dry Weight								
Extract	**Glucan**	**Xylan**	**Galactan**	**Arabanan**	**Surcose**	**Lignan**	**Ash**	**Acetate**
14%	37%	20%	1%	2%	3%	19%	1%	3%

As the design basis is linked to a 'best case option', it is instructive to understand the distance between the present state-of-technology and the Nth plant design basis.

Table TEA.2*: Unit Operation Conversion assumption for the state-of-technology and Nth plant cases*

Process Metrics	**Nth Plant Base Case**
Juice Extraction yield % g/g	95%
Solids Loading to Pretreatment Reactor %g/g	30%
C5 sugar yield through Pretreatment %g/g	80%
Solids Loading to Hydrolysis Reactor	25%
C6 sugar yield through Enzyme Hydrolysis	90%
Clarification yield % g/g	98%
Vacuum Distillation yield %g/g	98%
C6 fermentation yield % of theoretical	90%
C5 fermentation yield % of theoretical	N.A.
Purification from Whole Cell Broth %	90%
Hydrogenation Conversion	99%
Enzyme Loading mg/g	20

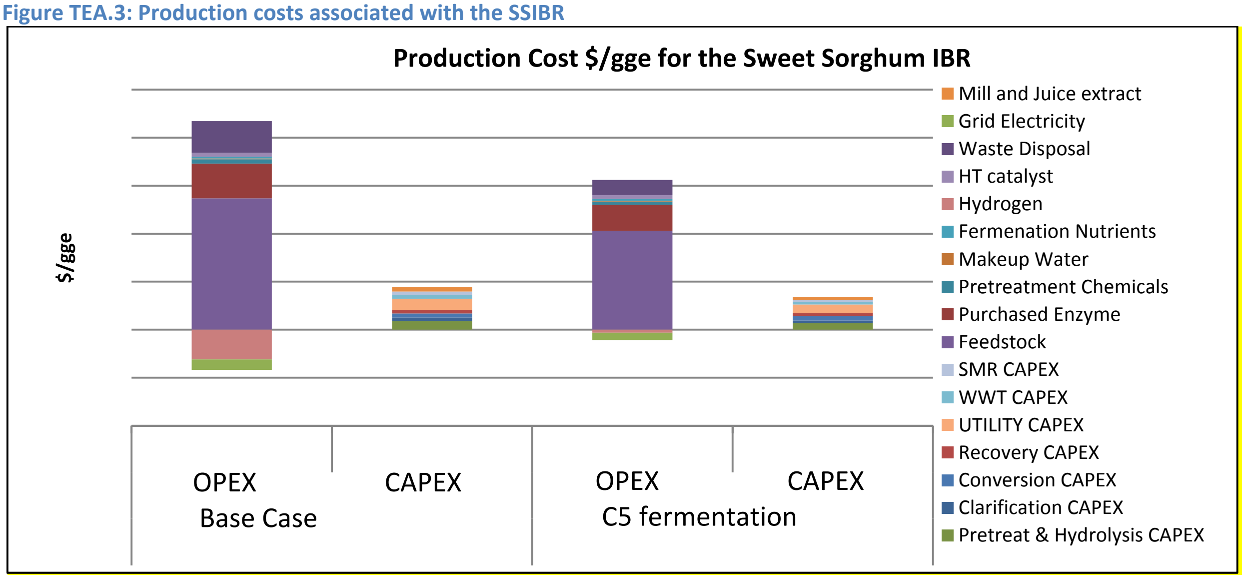
Beginning with an Nth plant design capacity of 2000 dry tonnes/d, feedstock and process inputs were defined by the various SSIBR partners. Ceres defined crop yield and sorghum bagasse composition data, NREL the process conditions and corresponding monomeric sugar yields associated with the bagasse pre-treatment, Amyris the fermentation and downstream processes and yields associated with finished fuels and chemicals, ICM the process conditions and waste degradation metrics associated with sorghum derived vinasse, and Praxair the process conditions and hydrogen yields associated with biogas upgrading and reforming.

Information and process metrics combined from all the partners provided the engineering basis to design a comprehensive heat and material balance for the SSIBR, which was constructed in ASPEN, a simulation program used to predict process performance based on pre-defined assumptions that is widely used throughout the industry. Utilizing the thermodynamic ASPEN simulation, raw material consumption was defined, capital equipment was sized, and capital costs were estimated. These feedstock and process assumptions are detailed in Table TEA.1 and Table TEA.2.

Based on the feedstock design basis described in Table TEA.1, Table TEA.2, and raw material unit costs, an analysis of the production economics for the SSIBR was conducted.

The production costs associated with renewable diesel derived from sweet sorghum details two Nth plant design cases, the base case C5 sugar to hydrogen process described previously and a C5 sugar co-fermentation to the intermediate fermentation product, farnesene, Figure TEA.3. The model's output production cost is presented in dollars per gallon of gasoline equivalent (gge), and it is broken into variable costs associated with raw material utilization (OPEX) as well as depreciable capital charges associated with the construction of various sections of the SSIBR (CAPEX).

Figure TEA.3: Production costs associated with the SSIBR



Sensitivity Analysis:

In order to assess the impact of specific improvements or challenges on the overall production economics, single point sensitivities from the base case Nth plant design were explored. The output of these sensitivities as well as a description of the individual process metrics perturbed is presented in Figure TEA.4.

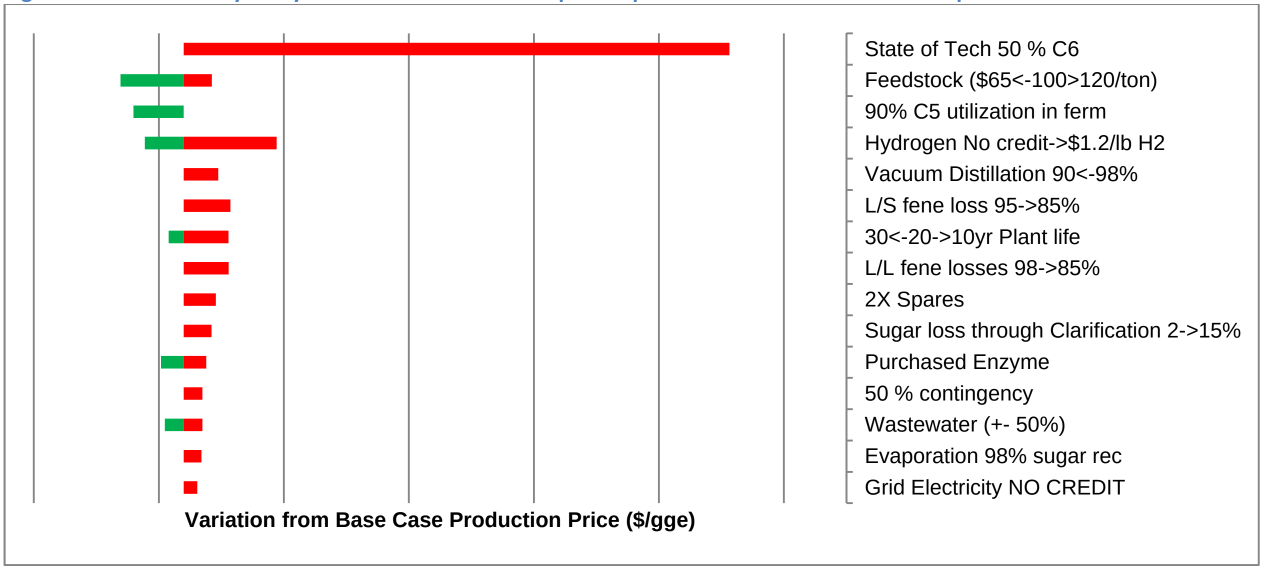
The specific sensitivities explored are described. Bold descriptions refer to Figure TEA.4.

- **State-of-technology**: A snapshot of present technology status relative to the Nth plant design case
- **Feedstock Costs**: Impact of cheaper or more expansive sweet sorghum feedstock
- **90% C5 Utilization**: Impact of achieving 90% of theoretical C5 co-fermentation yield on production cost
- **Hydrogen Credit**: Impact of inability to utilize 'green H_2' in production
- **Vacuum distillation**: Impact of product yield through vacuum distillation
- **L/S fene loss**: Impact of product loss in liquid solid separation post-fermentation
- **Plant life**: Impact of depreciable plant life on production cost
- **L/L fene loss**: Impact of product loss in L/L separation post-fermentation
- **2x spares**: Impact of up front purchase of spare equipment on production cost
- **Sugar loss through clarification**: Impact of solids clarification sugar yield pre-fermentation on production cost
- **Purchased Enzyme**: Impact on production cost of enzyme cost (+/- 50%)
- **Wastewater**: Impact on production cost of wastewater cost (+/- 50%)

- **Evaporation:** Impact of sugar loss on production cost during sugar concentration pre-fermentation

As is detailed in the sensitivity plot below, yield is a key cost driver for a commercially successful sweet sorghum based farnesane process. The sensitivity analysis below for an integrated bio-refinery was generated from internal Amyris documents and NREL's dilute acid deconstruction process assumptions (Humbird et al 2011).

Figure TEA.4: Sensitivity analysis associated with the impact of process metric deviation on Nth plant SSIBR economics



Detailed assumptions on specific unit operation process metrics, raw material unit costs, and associated purchased capital costs are detailed in Table TEA.2.

The cost delta between state-of-technology and Nth plant economics illustrated in the sensitivity chart (Figure TEA.4), includes process assumptions for all bio-refinery unit operations (e.g. pretreatment, deconstruction, clarification, sugar concentration, etc.) and is not limited to Amyris's fermentation and separation technology. The state-of-technology represents a snapshot of the present moment, where individual unit operations may still require continued research and development before commercial deployment. Similar to the Nth plant design cases, production price estimates for the state-of-technology were generated by entering the process metrics detailed into the ASPEN simulation to generate production costs. The successful implementation of an integrated bio-refinery will require innovation and expertise in multiple process operations.

In addition to process development efforts related to strain engineering, the effect of the three subsequent downstream Amyris separation unit ops, L/S centrifuge, L/L centrifuge, and vacuum distillation, are detailed in the sensitivity chart.

There is a significant incentive to drive technology development toward the utilization of C5 sugars in the fermentation process with the aim of maximizing carbon utilization and overall process yield.

The total installed capital costs for the hypothetical SSIBR are based on Amyris experience and NREL's cellulosic ethanol dilute acid deconstruction process economics (Humbird et al 2011). Capital costs associated with the sweet sorghum mill were substituted from NREL's energy cane assessment (Bain et al 2007). Given the high level of uncertainty for many of these capital cost estimations, the analysis leaned toward a conservative assumption of a four-fold installation factor for all purchased capital, as well as 35% contingency factor.

Carbon, Energy, and Water Efficiency:

Carbon, energy, and water efficiency are key factors in defining the overall economic and sustainability metrics of an integrated bio-refinery. The mass and energy balances used to define conversion of sweet sorghum to renewable fuels and chemicals allows for the quantification of carbon, energy, and water utilization.

Not surprisingly, the state-of-technology scenario is significantly dis-advantaged from both a carbon and water efficiency basis when compared to the Nth plant scenario. This is largely accounted for by the differences in fermentation yield, where in the state-of-technology case, a majority of the carbon is funneled into the production of biomass and CO_2. An advantage of the integrated system is the ability of the plant to recoup a portion of this 'biomass carbon' in the waste water treatment and boiler unit operations and transform it into hydrogen and electricity.

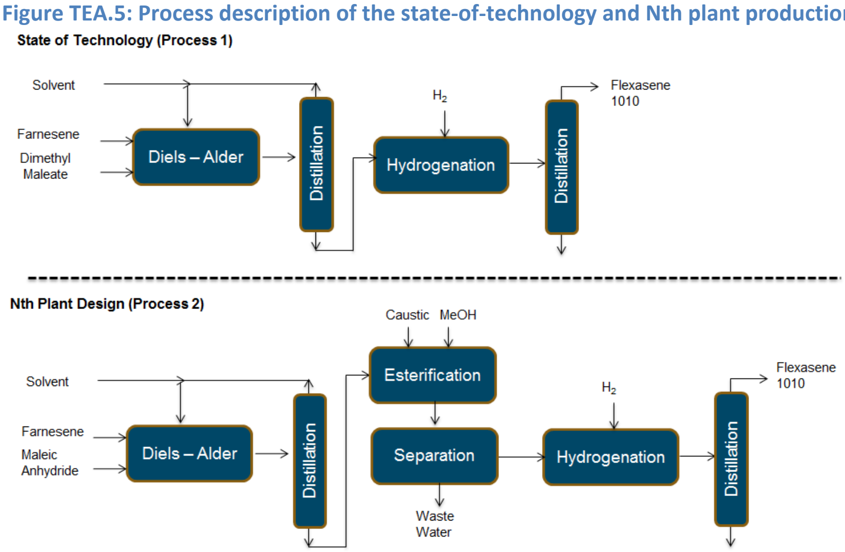
In the state-of-technology case, the presence of a larger heat load (i.e. biomass carbon) applied to the boiler system creates a cascade effect on the water economy in the plant; whereby, greater percentages of water are lost in the combustion stack and cooling tower evaporators in the state-of-technology design verses the Nth plant.

It is informative to view the water sustainability metrics relative to the most advanced IBR design case, NREL's lignocellulosic ethanol model (Humbird et al 2011). In comparison with the with the lignocellulosic ethanol design case published by NREL, the SSIBR is slightly disadvantaged exhibiting a total water make up of 10.1 gal H_2O/gge and the 2011 NREL ethanol design case 8.3 gal H_2O/gge. The difference can largely be attributed to the greater heat load generated in the aerobic process, where a larger volume of cooling water is required for heat management in the fermentation section. Water management and utilization will continue to be a key environmental and regulatory metric that commercially successful IBRs must work to reduce and manage.

Farnesene-Based Plasticizer production cost

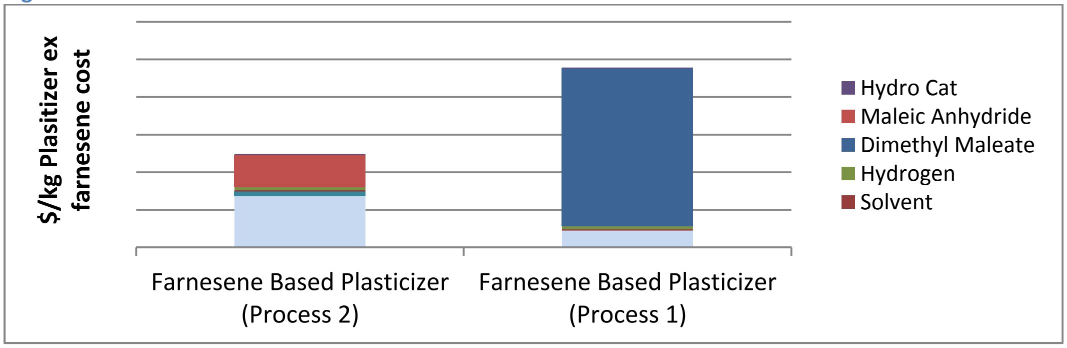
Similar to a petroleum-based refinery the sweet sorghum bio-refinery is designed with the ability to maximize profitability through the production of multiple products. The alternative product focused on in this analysis is Amyris's renewable plasticizer, flexasene 1010. The Nth plant and state-of-technology processes are depicted in Figure TEA.5. As is described in the Task C section above, the state-of-technology yields for flexasene 1010 are quite high, approximately 95%. The alteration of the production process for the Nth plant design is driven by the high raw material cost associated with the reactant, DMM. The Nth plant design envisions a process capable of utilizing maleic anhydride, a far more cost-effective starting raw material.

Figure TEA.5: Process description of the state-of-technology and Nth plant production processes for flexasene 1010



A comparison between the state-of-technology and Nth plant production economics is detailed in Figure TEA.6 below.

Figure TEA.6: Production costs associated with the SSIBR



Findings

Toward proving out a commercially viable SSIBR process, a comprehensive TEA was performed utilizing input parameters from consortia partners. In summary, Nth plant production costs calculated in the TEA analysis suggest the ability to produce renewable diesel at a cost competitive price.

The key conclusions of the TEA indicate that the commercial success of an SSIBR will rely on the continued development of several key unit operations utilized in the SSIBR.

- Clarified and Concentrated Sugar Feeds
- Continued Work Towards Enhanced Strain
- Improvement of Downstream Product Recovery from Whole Cell Broth
- Quantification, Characterization, and Management Strategies for SSIBR Waste Streams
- Systems Integration of the Envisioned Integrated SSIBR Unit Operations

In addition, it is clear that the ability to co-ferment C5 sugars into the intermediate product farnesene is significantly cost-advantaged over the proposed design case (i.e. C5 sugars to biogas to hydrogen). Continued progress in both the biological and engineering aspects of the SSIBR will be key factors in realizing the commercial potential of the envisioned process.

Feasibility Analysis: *In-situ* Production of Hydrogen from C5 Sugars and Vinasse

A key feature of the proposed SSIBR is the ability to efficiently utilize C5 sugars to offset external carbon inputs. The process purposed in the SSIBR aims to metabolize C5 sugars and vinasse into biogas and then chemically reform them into hydrogen. The *in-situ* produced hydrogen is envisioned to provide the necessary raw material to chemically process the fermentation intermediate farnesene into the finished renewable diesel. In order to assess the technical challenges associated with the described process option, an exploratory study was pursued to assess the following.

- Prove the efficacy of a commercially available upflow anaerobic sludge blanket (UASB) digester operation to degrade a vinasse waste stream heavy in C5 sugars, and metabolize this influent carbon (i.e. COD) waste stream into biogas.
- Assess the economic and engineering challenges of chemically upgrading and transforming a model biogas stream into pressurized, pure hydrogen for utilization in the SSIBR hydrogenation units.

Feasibility Analysis: Anaerobic Digestion of Sweet Sorghum Derived Vinasse

Objective

The objective of this study was to assess the feasibility of anaerobic digestion as a means to process Amyris derived waste vinasse to produce methane, the key biogas species that will be chemically reformed into hydrogen. In addition to enabling significant COD reduction in the waste water, the study focused on identifying the composition of the biogas stream. Particular focus was placed on the generation of hydrogen sulfide (H_2S) in the biogas stream, as this is a catalyst poison known to effect methane reforming to hydrogen.

Feedstock

Two feedstocks were processed over a two month period in two anaerobic digester reactor configurations.

- Single stage mesophilic (90 – 100°F) anaerobic digestion
- Two stage mesophilic (90 – 100°F) anaerobic digestion

The technology of choice, single-phase digestion, is the simpler and less expensive process to employ commercially. Two-phase digestion is more robust and can handle more complicated waste streams. These two reactor configurations were tested for the determination of the most appropriate commercial technology. Available compositional analysis of the two feedstocks is shown in Table TEA.3.

Table TEA.3: Comparison of model feedstock derived vinasse compositions tested

Amyris Derived Viansse Feed stock	COD ppm	TKN ppm	pH	TS %	DSS %	TSS %
Sugarcane	262,000	5,900	5.5	14	8.1	5.9
Wood Chip based cellulosic sugar from OTFF	150,000	n.a.	n.a.	n.a.	n.a.	n.a.

Data Analysis:

Pilot-plant-scale UASB reactors were used to treat fermentation vinasse and waste material from Amyris fermentations. The reactor, made out of opaque PVC and clear plastic pipe, contains biological pellets formed by the agglomeration of microbes. The pellets contact the vinasse during up-flow to produce biogas of which methane is a substantial component. As the biogas is directly metabolized from the influent COD, the ability of the pellets to reduce the COD and thereafter metabolize the degraded feed into biogas is measured and reported as percent reduction at a particular concentration or loading rate. The biogas was captured and analyzed by mass spectroscopy. The UASB reactor was operated with an initial organic loading rate (OLR) of 3 grams COD/Liter/Day that was increased to 12–15 grams COD/Liter/Day. During this period, we obtained COD removal of 70 to 80% between feed and effluent with corresponding biogas methane content of 90+ mole %. In order to assess digester stability during the processing of high COD feedstock, two reactor configurations were tested.

Both the single stage and two stage systems were in the operational mode of an UASB, and the two-phase system included a column of pellets ahead of the standard single-phase. This pre-column of stationary pellets was operated at 4.0 pH while the main column was operated at 7.2 pH. In the two-phase arrangement, the pre-column acted as a proton donor to its single-phase system. Although more complex to set up, the two-phase system exhibited more stable performance and displayed fewer peaks and valleys in the data compared with the single stage setup (Figure TEA.7 and Figure TEA.8 vs.

Figure TEA.9 and Figure TEA.10).

Figure TEA.7: Mesophilic digestion performance data for single-phase digestion of Amyris sugarcane derived vinasse

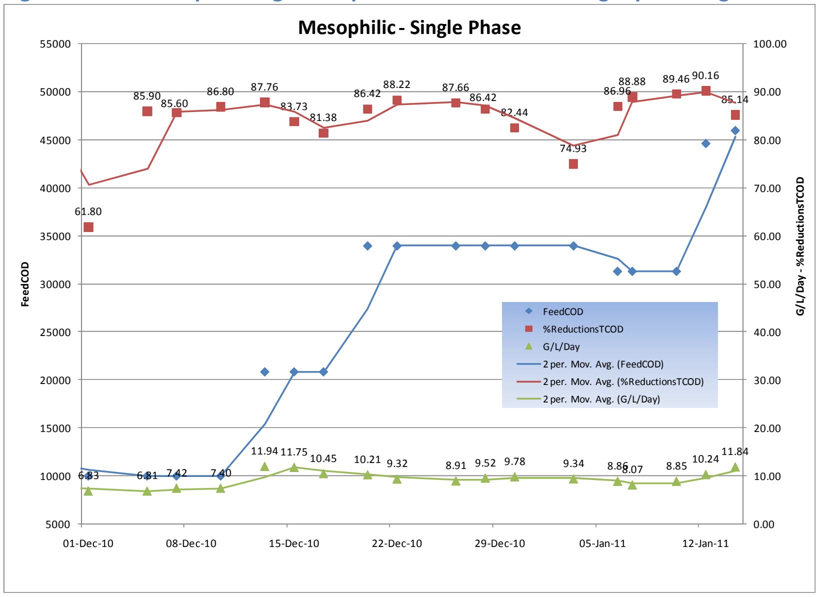


Figure TEA.8: Mesophilic digestion performance data for single-phase digestion of Amyris lignocellulosic derived vinasse

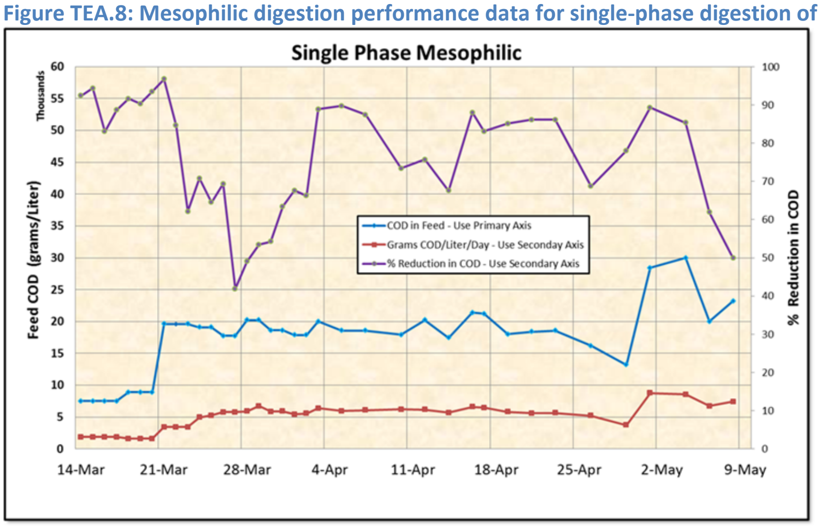


Figure TEA.9: Mesophilic digestion performance data for two-phase digestion of Amyris sugarcane derived vinasse

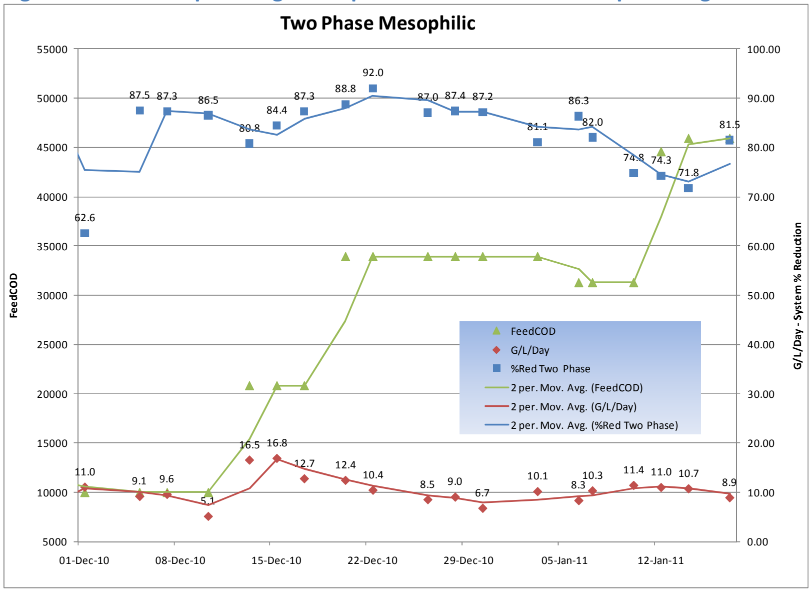
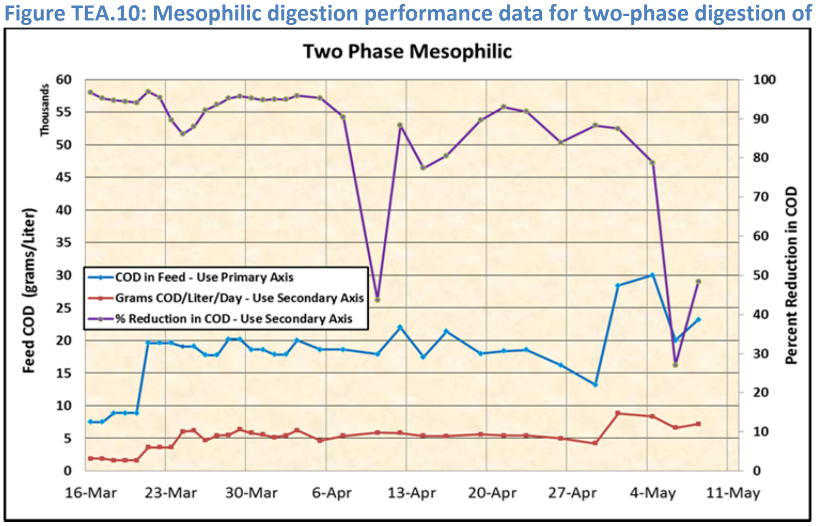


Figure TEA.10: Mesophilic digestion performance data for two-phase digestion of Amyris lignocellulosic derived vinasse.



As provided, the COD concentration of both feedstocks was too high for effective digestion and dilution of the waste stream was required prior to use. Digestion systems acclimate slowly to new feed types, and the concentration of the feed was aggressively diluted for enhanced acclimation of the system. The concentration was increased over time until steady state was observed.

Interestingly macro nutrients appear sufficient to support a robust anaerobic biomass culture, which may provide production cost savings for the integrated bio-refinery. For example, anaerobic bacteria require higher concentrations of many trace minerals relative to other microorganisms and augmentation of these nutrients is performed as standard testing protocol. Elements added to the system were: Co, Zn, Mn, Mo, Se, W, B, Ni, Fe, Ca, Mg, P and S. As a rule, commercial installations generally do not add this full complement of elements, and optimize the externally added minerals to a minimal set over time. Adjustments were made with $Mg(OH)_2$ for increasing pH and H_2SO_4 for lowering pH as needed for the different reactor conditions.

Single phase mesophilic performance results are shown in Figure TEA.7 and two-phase mesophilic performance is shown in Figure TEA.8. As can be seen from the Figures, the COD feed concentration was raised several times during the testing period. Preliminary work (not shown in graph) began biomass acclimation at 5,000 ppm COD concentration. Post acclimation, the feed concentration was raised to approximately 10,000 ppm COD and held for a significant amount of time to achieve acceptable and stable performance. Starting middle of December the concentration was increased aggressively to a feed concentration target of 45,000 ppm COD. The performance of the system exhibited stable and consistent performance at a loading rate of approximately 10 g/L/d.

During the laboratory tests, biogas production (not shown) proceeded well with a high BTU gas production (e.g. 60% (v/v) > CH_4). There is little observable H_2S in the gas (around 1000 ppm). Hydrogen sulfide is a metabolite of sulfuric acid used for pH control rather than an inherent quality of the feed. Carbon dioxide concentration of the gas is low, but it is important to highlight that this may not be indicative of a full-scale system, as CO_2 control is performed differently in commercial units than in lab testing. However, it is very likely that the biogas from a commercial application will have 75% to 85% methane by volume with the balance CO_2 and trace impurities such as H_2S.

In conclusion, the model waste streams tested in the laboratory (e.g. sugarcane and lignocellulosic derived vinasse) exhibit significant COD reduction, as well as acceptable biogas composition and BTU value. However, it is important to note that residual impurities arising from lignocellulosic biomass fermentations will assuredly produce new challenges to the digestion process. Expected challenges include: potential for high H_2S in biogas stream, especially for dilute acid pretreatment feedstocks, poorly digestible soluble and insoluble components particularly from lignin, the production of volatile silicates, which can cause accelerated erosion of capital equipment, as well as feedstock toxicity issues leading to degranulation and system instability. Poorly digestible components will reduce theoretical gas yield as well as produce effluent that may require more complex treatment trains. The additional treatment would possibly be performed in the following manner:

- Building a larger primary unit with lower load rate and longer retention time
- Aerobic polishing of effluent water, which may be necessary to meet regulatory nitrogen plant emissions
- Combination of anaerobic and aerobic polishing of effluent water

In addition, contaminants in the biogas stream pose a different problem as the biogas is envisioned to be purified, upgraded, and reformed into hydrogen. There are many alternatives to gas cleanup and the exploration of those alternatives is explored in the bio-gas purification and reforming feasibility section immediately following this section.

Feasibility Analysis: Bio-gas purification and reforming

Objective

The primary objective of this study was to develop a preliminary process design for the production of hydrogen from digester gas and to provide a budgetary cost estimate. In order to assess, the distance from conventional hydrogen production a comparison with natural gas (NG) based hydrogen production is included.

Design Basis

The variability in natural gas reforming feedstock is known to affect hydrogen production costs (e.g. pressure, capacity, composition, etc.). At the time this study was undertaken, specific data on Amyris vinasse derived biogas did not exist. In lieu of this information, a model biogas stream was assumed for engineering process design and production price estimation.

Major Design Assumptions

- Capacity - 2.2 MMSCFD of digester gas feed
- Digester feed gas operating conditions – Pressure of 5 psig and Temperature of 80 ºF.
- Digester Gas Composition - CH_4 62.9%, CO_2, 35.9%, N_2 0.8%, H_2O 0.2%, H_2S 1000 ppm, NH_3 100 ppm, O2 100 ppm.

Product Slate

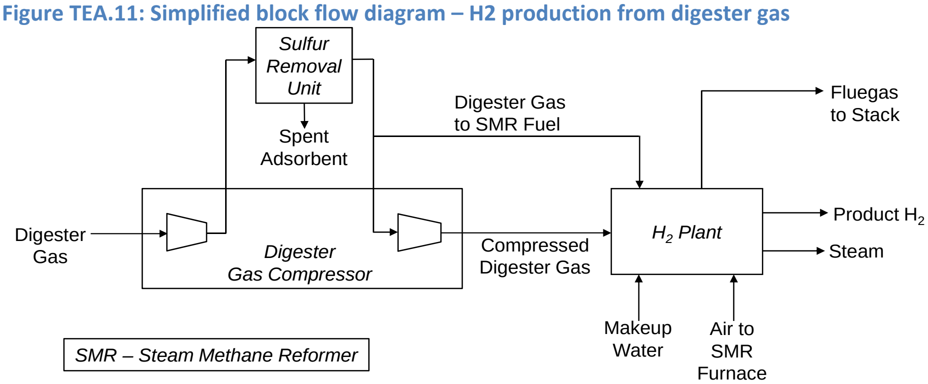
A conventional natural gas (NG) reforming unit will typically deliver two consumable products to a refinery. In order, to benchmark the biogas reformer to the incumbent NG reforming unit, the engineering analysis employed in the feasibility study focused on a biogas reforming design that would deliver equivalent products. Desirable target outputs are as follows:

- Pressurized hydrogen at 250 psig at nominal purity of >99.99% (v/v)
- Superheated steam (375 psig, 700 ºF) by-product, available for use for efficient heat management schemes throughout the SSIBR project.

Process Description

Digester gas feed, available at 5 psig, is first compressed to 30 psig and fed to the sulfur removal unit, which reduces H_2S concentration from 1000 ppm to about 5 ppm. The sulfur removal unit efficiently and economically scrubs the H_2S formed during anaerobic digestion from the biogas stream. As H_2S is a known reforming catalyst poison, sulfur removal is a necessary process step for viable biogas reforming. The amount of sulfur removed is equivalent to about 186 lbs/day. A simplified block flow diagram for the production of hydrogen from digester gas is shown in Figure TEA.11.

Figure TEA.11: Simplified block flow diagram – H2 production from digester gas



Based on the scale and engineering heuristics detailed in Table TEA.4, a solid scavenger system was selected for the current sulfur removal application. The system selected system utilizes an iron-based scavenger that will need to be periodically discharged. Two identical beds in lead-lag configuration are used. Each of the beds is designed to be able to achieve the desired level of sulfur removal for a period of six months. Use of the lead-lag configuration allows the beds to be re-loaded with fresh scavenger material without disrupting the operation of the overall process.

Table TEA.4: Commercially available sulfur removal technologies

Parameter	Technology			
	Liquid Scavengers	Solid Scavengers	Liquid Redox	Amine + Claus
Levels of Sulfur Removal	100 lbs/day	300 lbs/day	< 20 ton/day	> 15 ton/day

A major fraction, 98.5%, of the digester gas from the sulfur removal unit is compressed to 355 psig and used as feedstock in the hydrogen plant. The specific pressure value was selected to enable production of H2 at the desired pressure of 250 psig while accounting for pressure drops within the hydrogen plant. The remainder of the digester gas is used as fuel within the reforming section of the hydrogen plant.

The compressed digester gas is initially fed to the sulfur polishing unit, which typically consists of a hydrotreater to reduce most sulfur species to H_2S as well as a ZnO-based bed to reduce the H_2S concentration to ppb levels. Higher levels of H_2S will poison the catalyst used in the reforming section of the steam methane reformer. The sulfur free digester gas is then mixed with superheated steam and sent to the steam methane reformer, where the digester gas is reformed to produce a syngas containing mainly H_2, CO, CO_2 and some unconverted CH_4. Key reactions in the reforming step include:

$$CH_4 + H_2O \rightarrow CO + 3\ H_2 \qquad (1)$$

$$CH_4 + CO_2 \rightarrow 2\ CO + 2\ H_2 \qquad (2)$$

Syngas exits the reforming section at temperatures in the range of 1400 – 1600 °F. Heat is recovered by steam generation. Syngas is then sent to a water gas shift (WGS) reactor to convert additional CO to H2, through the following reaction:

$$CO + H_2O \rightarrow H_2 + CO_2 \qquad (3)$$

Shifted syngas is sent to a pressure swing adsorption (PSA) unit to produce >99.99% hydrogen. The tailgas from the Pressure Swing Adsorption (PSA) unit is recycled for use as fuel within the steam methane reformer. Heat recovery trains on the syngas and flue gas as well as the water management system are not discussed in this report.

Performance Summary

A performance summary of the proposed biogas reforming unit developed for the SSIBR is detailed in Table TEA.5. The biogas reforming unit maintains a hydrogen production capacity of 2.8 MMSCFD produced from 2.2 MMSCFD of digester gas. Interestingly, roughly 80% of the total power requirement for hydrogen production is utilized during digester gas compression. The makeup water requirement flow rate is 30 gal/min. Approximately 1700 ft^3 of iron-based scavenger will be required every six months for use within the sulfur removal unit. The process exports 8,500 lb/hr of superheated steam (700 ºF, 375 psig). The solid sulfur scavenging unit produces roughly 90 tons of solid waste in the form of spent scavenger, which will require landfill disposal.

Table TEA.5: Performance summary for digester gas to hydrogen process

Biogas Reforming Unit Inputs/Outputs	Digester Gas Compressor	Sulfur Removal Unit	Hydrogen Plant
Inputs			
Digester Gas, MMSCFD	2.2		
Power, kW	400		100
Makeup Water (gal/min)			30
Iron-based adsorbent per Change out*, ft^3		1700	
Outputs			
H_2, MMSCFD			2.8
Superheated steam, lb/hr			8500
Solids to Landfill per Change out*, tons		90	

*Change out every 6 months

Impact of CO_2 Removal

The digester gas feed contains a significant amount of CO_2. Hence, an additional case which removed 95% of the CO_2 from the digester gas leaving the sulfur removal was evaluated. Hydrogen production went up from 2.8 MMSCFD to 3.2 MMSCFD in CO_2 removal case. Given the relatively small scale of hydrogen production, it is unlikely that the incremental H_2 could justify the additional capital.

Comparison with Conventional NG Based Hydrogen Production

The typical capacity of a natural gas based hydrogen plant is around 100 MMSCFD. The digester gas based process is about 2 orders of magnitude smaller. For these types of plants, capital costs generally scale favorably for larger sizes. Natural gas is usually available at pressures in the range of 300 – 600 psig as compared to digester which is available at about 5 psig. Digester gas compression adds both capital and operating costs. Table TEA.6 summarizes some of the key differences when producing hydrogen from digester gas versus the more conventional natural gas.

Table TEA.6: Natural gas versus digester gas based hydrogen production

Feedstock and Operating Parameters for Hydrogen Production	Conventional NG based Steam Methane Reformer Plant	Digester Gas based Steam Methane Reformer Plant
H2 Capacity, MMSCFD	100	2.8
Feedstock	NG	Digester gas
Feedstock Pressure (psig)	300-600	5
Reformer Operating Pressure (psig)	500	330
Sulfur removed, lbs/day	15-25	186

The lower operating pressure for the reformer in the case of digester gas will reduce the amount of unconverted methane in the produced syngas. The natural gas and the digester gas based process differ in the amount of sulfur present in the feed. Natural gas typically contains about 5 – 10 ppm H_2S. For a 100 MMSCFD hydrogen plant, amount of sulfur removed is about 15 – 25 lbs/day. By contrast, digester gas contains 1000 ppm H_2S, the amount of sulfur that needs to be removed is about 186 lbs/day. Sulfur management adds cost and operational complexity.

Biogas Reforming Feasibility Summary

A preliminary design to produce 2.8 MMSCFD hydrogen from 2.2 MMSCFD digester gas was developed. The amount of hydrogen produced could be increased by about 15% with the inclusion of a CO_2 removal unit.

The relatively small scale and additional equipment requirements for feed compression and sulfur removal will adversely impact capital cost relative to conventional hydrogen production from natural gas. As such, a process for hydrogen production from digester gas will be favored in geographies where natural gas is either not available or very expensive. Given the likely hood that an IBR will be geographically isolated from major refining operations, the biogas reforming unit may prove a superior option when compared against shipment of the fermentation intermediate to hydrogenation sites proximal to NG sources. Furthermore, up-stream pretreatment of the biogas to lower sulfur levels would favorably impact cost and complexity of the hydrogen production process. Increasing the amount of hydrogen produced by combining biogas streams from multiple digesters could provide additional economy of scale benefits.

Feasibility Analysis Findings

A key feature of the SSIBR process, C5 and vinasse degradation to biogas for chemical reforming was experimentally tested and engineering analyzes performed. Results from the feasibility study prove the ability of a commercially available UASB digester to efficiently degrade vinasse laden with C5 sugars into biogas, 80-90% COD reduction. In addition, the digester study revealed the biogas to be heavy in CH_4 and light in H_2S content, a beneficial composition for subsequent biogas reforming.

The engineering analysis performed by Praxair illuminated the process challenges and production economics of in-situ hydrogen production from biogas reforming. Specifically highlighting potential process issues associated with biogas streams heavy in H_2S content, and process configurations associated with CO2 removal from the upgraded gas stream.

Concluding Remarks

Expansion of the Amyris pilot plant was completed successfully to plan and within budget. The success of the project is largely attributed to Amyris's ability to leverage previous experience in pilot plant design, construction and operation, and to execute with minimal incremental permitting and little or no downtime of ongoing pilot operations during the upgrade design and installation phases.

The project evaluated sorghum hybrids, from planting through harvesting, across four geographies. Overall, the south east plantings (Florida, Tennessee, and Alabama) provided consistent yields across the planting windows while the Hawaii plantings showed the yield decreasing with later plantings.

Various feedstocks were evaluated for production of Amyris's advanced biofuel farnesane including sorghum syrup, sorghum bagasse, cane syrup, wheat straw hydrolysate, and woodchip hyrdrolysate. Success at pilot scale was a key metric for demonstration of commercial readiness. Amyris was able to meet specifications for production at pilot scale of farnesane from sorghum syrup and lignocellulosic based sugars per the project requirements.

In addition, we demonstrated production of an alternative chemical product which could be utilized as a plasticizer. The mid-scale synthesis goals were met with the multi-kilo scale reactions performed in the skid mounted chemistry unit as well as at a CMO at 2mT scale.

Regulatory and end-user acceptance of Amyris diesel and chemical products was addressed and, with the exception of LCA certification and commercial approvals, all diesel fuel and chemical products objectives were achieved. During the course of the project, Amyris acquired extensive physical-chemical properties and toxicological data with production representative materials, secured significant OEM data contributions, engaged US military testing, and achieved both commercial and military end-user validations. The project also achieved significant progress towards ASTM validation for farnesane as an aviation turbine fuel blending component. DOE funding and collaboration provided the necessary confidence for acquiring OEM, military and end-user resource commitments. Such commitments resulted in new chemical substance registrations (US and Europe), further federal and California regulatory approvals, global OEM validation and diesel engine warranty coverage, US military renewable (and high purity) fuel opportunities, and pipeline distribution product code and specifications. These achievements are essential tools for 1) ensuring successful commercial introduction, 2) contributing to US energy independence (diesel and aviation turbine fuels) and 3) development of new market opportunities (e.g. US military fuels, aviation turbine fuel and specialty chemicals).

Amyris developed a rigorous process economic model to forecast technological milestones and production costs associated with biomass-derived fuels and chemicals. The comprehensive model considered the conversion of sweet sorghum into Amyris renewable diesel and chemicals. The model input parameters, such as feedstock composition, process yields, raw material unit costs, and purchased capital cost information were collated from various partners to illustrate the production costs associated with the proposed SSIBR. The combined information was synthesized in a thermodynamic process simulation tool (ASPEN Plus) and production costs and sensitivity effects were calculated. Nth plant production costs revealed the potential for profitable and cost competitive renewable diesel pricing. In addition, the model predicted the cost advantaged nature of a yeast strain capable of fermenting both C6 and C5 sugars to farnesene.

The Amyris IBR pilot plant project validated that renewable domestic feedstocks, consisting of syrup and biomass based lignocellulosic sugars, can be converted to the advanced biofuel compound farnesane and to alternative chemical molecules such as Amyris's plasticizer compound Flexasene 1011.

Overall we successfully demonstrated that the Amyris fermentation process could be adapted to utilize glucose derived from high-impact feedstocks. The key drivers for viable economic production of advanced biofuels are: the availability of cheap sugar feedstocks which have high sugar concentration, low solids, and absence of fermentation inhibitors; strains that are engineered for high productivity and maximum yield; and ultimately strains that are engineered for consumption of C5 sugars.