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Energy Research and Development Division

FINAL PROJECT REPORT

Scale-Up of Advanced Deconstruction and Conversion Technologies for Producing Biofuels from Woody Biomass

Gavin Newsom, Governor

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The authors thank the California Energy Commission for the opportunity to demonstrate a novel biomass conversion technology that can help meet the state's goals for reducing greenhouse gas emissions.

PREFACE

The California Energy Commission's (CEC) Energy Research and Development Division supports energy research and development programs to spur innovation in energy efficiency, renewable energy and advanced clean generation, energy-related environmental protection, energy transmission and distribution and transportation.

In 2015, then-Governor Edmund Brown, Jr. released Executive Order B-32-15, the California Sustainable Freight Initiative, which established targets to improve freight system efficiency by 25 percent by 2030 and to transition into zero emission technologies. Per the establishment of initiatives and California's continued efforts to meet Federal Ambient Air Quality Standards, state agencies have created several funding programs to improve air quality with specific focus on the modernization of California's transportation system. With funding from the Budget Act of 2016, the California Energy Commission has implemented a Low Carbon Fuel Research and Development Program, supporting the advancement of low-carbon drop-in fuels production derived from California's biomass feedstocks through the development of intermediate fuels. Renewable biofuels, fuels produced from biomass sources, can be produced to be fully compatible with existing infrastructure and vehicle technologies which can minimize the overall cost of adoption. These intermediate fuels will be used for sustainable low-carbon fungible biofuels production for consumption in California's transportation sector in an effort to further reduce harmful emissions being generated by goods movement vehicles used throughout the state.

Scale-up of Advanced Deconstruction and Conversion Technologies for Producing Biofuels from Woody Biomass is the final report for the FRD-17-004 project conducted by Lawrence Berkeley National Laboratory. The information from this project contributes to the Energy Research and Development Division's Low Carbon Fuel Research and Development Program.

For more information about the Energy Research and Development Division, please visit the CEC's research website (www.energy.ca.gov/research/) or contact the CEC at 916-327-1551.

ABSTRACT

This project successfully developed, optimized, and scaled an innovative ionic liquid pretreatment technology to convert waste woody biomass to fermentable sugars in hydrolysate at 83 percent yields and further achieve an overall carbon conversion efficiency from biomass to fuel of nearly 80 percent. The conversion process required no solid-liquid separations, which reduces complexity and eliminates intermediate materials being lost. This project demonstrated scale-up from prior lab scale (around 2 liters) to a working volume of 680 liters in a 1,600-liter industrial-level fermenter, an important validation of commercial feasibility and scalability. The project team engineered a yeast strain to make advanced automotive and aviation biofuels (for example, isoprenol), which builds on the project accomplishments to establish the foundation for a broad variety of advanced biofuels made using the same woody biomass feedstock and processing technologies. The project identified paths forward to continue developing these biomass conversion approaches in future research, with a pilot plant based on this conversion technology being the next major step to commercializing new advanced biofuels made from California's waste woody biomass.

Keywords: Biofuels, ionic liquids, fermentation, cellulosic ethanol, scale-up, biomass pretreatment

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EXECUTIVE SUMMARY

Introduction

Fossil fuels from oil, coal, and natural gas provide more than 80 percent of the energy used in the United States. The nation's transportation infrastructure operates almost exclusively on petroleum-derived fuels, and petroleum provides 90 percent of the chemical products made in the United States. California accounts for about 10 percent of the nation's total consumption of petroleum, only second to Texas. Burning these fossil fuels contributes to climate change, while producing certain chemicals and materials from petroleum takes a great deal of energy and produces environmental pollution. Developing renewable transportation biofuels and bioproducts is important to meeting the goal of statewide carbon neutrality by 2045.

Plant matter from nonfood crops could provide a large portion of alternative fuels and products to meet California's clean energy goals. An estimated 47 million bone dry tons of lignocellulose (made up of cellulose, hemicellulose, and lignin) is available in California and could provide a large and strategic domestic source of nearly carbon-neutral, specialty biofuels and renewable chemicals. This requires addressing barriers such as the lack of scalable and sustainable energy crops; difficulty in separating and breaking down the plant matter into usable form; lack of a robust pretreatment technology for any feedstock; and, the high cost of the enzymes used to produce fermentable sugars. There is also a pressing need for an efficient method to producing alternative fuels for automotive, aviation, and diesel applications, as well as renewable bioproducts for a host of applications, that can displace petroleum-derived products.

Project Purpose

The mission of the Joint BioEnergy Institute is to perform cutting-edge science and develop new technologies that will provide dedicated, resource-efficient energy crops and efficient processes to convert nearly all of the carbon in those crops into specialty biofuels and bioproducts at prices that are competitive with petroleum fuels and chemicals and also reduce greenhouse gas emissions by 90 percent relative to petroleum products. The biofuels community also recognizes that a biorefinery must produce both biofuels and bioproducts to be competitive in the marketplace.

The project used California Energy Commission (CEC) funding to support a specific research effort coordinated with proposed United States Department of Energy funding of the Joint BioEnergy Institute. The research focused on optimizing the breakdown of sugars from California-relevant feedstocks and conversion to either cellulosic ethanol or isopentenol, a leading candidate for low-temperature combustion engines, as well as a drop-in gasoline blendstock (biofuels that function like petroleum fuels and can be used in the existing transportation infrastructure). The scale-up work was done at facilities in place at Aemetis, a California-based biofuels company. There, the researchers addressed issues around an optimized conversion technology across large production volumes to demonstrate how to achieve a fuel cost of $2.50/gallon within 7-9 years after project initiation. One challenge to achieving California's energy goals is transferring promising laboratory results to enable the production of advanced biofuels, and then demonstrating that these technologies are

commercially viable and meet the state's required levels of greenhouse gas emission reductions in accordance with California's Low Carbon Fuels Standard.

Project Approach

This project leveraged state-of-the-art monitoring capabilities at the Joint BioEnergy Institute and used a sophisticated laboratory management system with redundant backups to ensure data and information storage and integrity. Lawrence Berkeley National Laboratory technical and program management project team worked closely with CEC staff to ensure that the project was on time, on budget, and made expected progress toward technical goals. The subcontract with Aemetis was put into place as a Collaborative Research and Development Agreement between Lawrence Berkeley National Laboratory and Aemetis. The team's technical approach to efficiently convert California woody biomass wastes to biofuels and bioproducts of immediate value leveraged three critical technical approaches and was demonstrated at scale in collaboration with Aemetis Inc. Specifically the team conducted work on the following three tasks:

- Task 1: Biomass deconstruction (Lead: Dr. John Gladden, Sandia National Laboratories-Livermore). Included optimizing the Joint BioEnergy Institute-pioneered ionic liquid-based deconstruction of the lignocellulosic material.
- Task 2: Strain development (Lead: Dr. Taek Soon Lee, Lawrence Berkeley National Laboratory). Included developing a microbial strain to convert carbon (specifically C6/C5 sugar streams) to the gasoline replacement and platform chemical, isopentenol.
- Task 3: Scale-up and commercial demonstration (Lead: Jeff Welch, Aemetis). Included collaboration with Aemetis Inc. to evaluate both the ionic liquid deconstruction process and biofuel production at a commercial scale.

Project Results

CEC funding enabled a pioneering accomplishment of scientific and industrial significance in biomass conversion to biofuels through a public-private partnership between Lawrence Berkeley National Laboratory, Sandia National Laboratories, and Aemetis. This project successfully used ionic liquid pretreatment technology to convert waste woody biomass to fermentable sugars in hydrolysate (a substance that is produced from the chemical breakdown of a compound when reacting with water). The hydrolysate was then converted into cellulosic ethanol with overall fermentation efficiency exceeding 90 percent and achieved overall conversion efficiency from biomass to fuel of nearly 80 percent. The project team was able to scale-up from prior lab scale (2-liter fermentation) to a working volume of 680 liters in a 1,600-liter industrial-level fermenter. This process is an important validation of commercial feasibility and scalability. The team also engineered a yeast strain to make advanced automotive and aviation biofuels, which builds on this project's accomplishments to establish the foundation for a broad variety of advanced fuels made using the same feedstock and processing technologies. The researchers identified paths forward to continue developing these biomass conversion approaches, with a pilot plant being the next major step to commercializing new biofuels made from California's waste woody biomass.

Technology/Knowledge Transfer/Market Adoption (Advancing the Research to Market)

Transfer of technology from this project includes intellectual property developed prior to the project and protocols developed under CEC funding. Lawrence Berkeley National Laboratory and Aemetis, Inc. are in negotiations to license three United States patents on background intellectual property, and close cooperation among the parties throughout this project facilitated transfer of operating protocols and conditions. Knowledge transfer to the broader scientific and business community has occurred through publications, conference presentations (for example, the Symposium on Biomaterials, Fuels and Chemicals and the Advanced Bioeconomy Leadership Conference), and training of undergraduate and post-Doctoral students under this funding. Market transfer will occur in collaboration with Aemetis, the industry partner for this project. Aemetis is currently evaluating opportunities beyond the scope of this project that will require separate funding to design, build, and operate an integrated pilot plant to bring the new pretreatment technology to market. Currently, Lawrence Berkeley National Laboratory (representing the Joint BioEnergy Institute and the Advanced Biofuels and Bioproducts Process Development Unit at the United States Department of Energy) and Aemetis are negotiating an option agreement where Aemetis will be provided legal access to Joint BioEnergy Institute intellectual property to proceed with commercialization. This will create opportunities for Aemetis to commercialize a technology and engineering package to the industry with a focus on approximately 220 corn grain ethanol plants currently operating nationwide.

Benefits to California

The project provides the following quantitative benefits to California:

- Commercially relevant demonstration of an advanced biofuel pathway for producing ethanol and isopentenol with the potential to reduce greenhouse gas emissions by about 70 percent relative to petroleum.
- Demonstration of an advanced deconstruction technology with the potential to generate sugar yields of 90 percent from a wide range of California-relevant, nonfood feedstocks.

The development of technologies for producing advanced biofuels as drop-in replacements for current petroleum transportation fuels will benefit California in a variety of ways including reducing carbon dioxide emissions, providing technologies to support formation of new companies, and educating the next generation of the biotechnology workforce. The results of this research project and Joint BioEnergy Institute's further research will provide technologies that will significantly reduce greenhouse gas emissions to the target of 40 percent below 1990 levels by 2030 as called for in former Governor Edmund G. Brown, Jr.'s Executive Order B-30-15. Achieving this goal will help reduce the likelihood of major climate events such as droughts and rising sea levels, as well as improve air quality across the state. Successful implementation of the Joint BioEnergy Institute technology will also overcome important economic factors that currently prevent widespread adoption of cellulosic biomass-derived biofuels, thus enabling small start-up companies to build competitive businesses without significant investment capital and catalyze partnerships between public and private entities. Additionally, the Joint BioEnergy Institute will train hundreds of students and early career scientists in the biotechnology field, helping California drive the 21st century bioeconomy.

CHAPTER 1: Introduction

Joint BioEnergy Institute

Fossil fuels derived from oil, coal, and natural gas provide 81 percent of the energy consumed in the United States, with the established United States transportation infrastructure operating nearly exclusively on petroleum-derived hydrocarbons. Additionally, petroleum provides 90 percent of the commodity and specialty chemicals and materials made in the U.S. Burning fossil fuels also contributes to climate change, and the production of many chemicals and materials from petroleum requires significant energy inputs and pollutes the environment. The development of alternative, renewable transportation biofuels and bioproducts is therefore critical to the energy, environmental, and economic security of the nation.

Lignocellulosic biomass (plant material made up of cellulose, hemicellulose, and lignin) from nonfood crops could provide a large fraction of those alternative fuels and products. There are approximately one billion dry tons of lignocellulosic biomass available annually in the U.S. with the potential to provide a significant and strategic renewable domestic source of nearly carbon-neutral, specialty (drop-in and/or fungible) biofuels and renewable chemicals. Mobilizing this strategic renewable carbon resource to enable the bioeconomy of the U.S. requires addressing significant roadblocks. These include the lack of scalable and sustainable energy crops; difficulty in separating and breaking down lignocellulose into targeted intermediates at high yields; lack of a robust feedstock agnostic pretreatment technology; and expense of enzymes used to produce fermentable sugars and other targeted intermediates. There is also a pressing need for efficient microbial routes to advanced fuels for automotive, aviation, and diesel applications, and renewable bioproducts for a host of applications that can displace petroleum-derived products.

The Joint BioEnergy Institute (JBEI, www.jbei.org) is a pioneer in the development of advanced biofuels centrally located in Emeryville, California. JBEI's vision is that lignocellulosic biomass can be converted into economically viable, carbon-neutral, specialty biofuels, all the organic chemicals currently derived from petroleum, and many other useful bioproducts that cannot be efficiently produced from petroleum. JBEI's mission is to enable this vision through cutting edge science and development of new technologies to provide the nation and the world with dedicated, resource-efficient, energy crops and efficient processes to convert nearly all of the carbon in those crops into specialty biofuels and bioproducts at prices that are competitive with petroleum-derived fuels and chemicals with a 90 percent reduction in green-house gas emissions relative to petroleum-derived products. Informed by technoeconomic and lifecycle analysis, JBEI's proposed research and development program will provide technologies that, when fully realized and scaled in an integrated biomass-to-biofuels-and-bioproducts process, enable the realization of:

- Gasoline, diesel, and jet fuel replacements at less than $4 per gallon without a bioproduct, gasoline, diesel and jet fuel replacements at ~$2.50 per gallon when bioproducts are co-produced with the fuel.

- Drop-in, commodity bioproducts (production of a million tons per year or more) that can compete with the same petroleum-derived molecules and that reduce biofuel prices.
- Novel bioproducts that cannot be efficiently produced from petroleum, have desirable properties, and reduce biofuel selling prices.

Doing so will reduce the nation's dependence on fossil fuels, the amount of carbon added to the atmosphere, and contamination of the environment, while also providing the scientific tools and knowledge required to transform the bioenergy marketplace. JBEI's approach and integrated high-risk, high payoff approach to science aligns with and supports the United States Department of Energy (USDOE) and California strategic plans for renewable energy, bioenergy, mission innovation, and sustainability.

To achieve these goals and metrics, JBEI advanced the fundamental science required to realize 1) engineered plants that have up to a 250 percent increase in the ratio of six-carbon to five-carbon sugars, radically altered lignin optimized for biofuel and bioproduct synthesis, and low susceptibility to pests; 2) an integrated, feedstock agnostic deconstruction process using renewable and biocompatible ionic liquids that liberates 90 percent of the sugars and lignin-derived intermediates suitable for biological conversion; 3) engineered microorganisms that can produce the fuels and bioproducts at industrially relevant titers, rates and yields (no less than 20 grams per liter [g/L], 2 g/L per hour [g/L/hr], 75 percent of theoretical for biofuels and 10 g/L, 1 g/L/hr, and 50 percent of theoretical for bioproducts) from a combined aromatic-sugar stream; 4) integrated technologies that enable the science needed to achieve these performance metrics and advance the bioenergy community; and 5) technoeconomic and life-cycle models of the processes to continuously evaluate the impact of scientific discoveries on the cost and greenhouse gas reductions of fuels and to indicate where scientific breakthroughs are needed to reduce fuel cost and greenhouse gas emissions. The results generated by the proposed USDOE research program will develop the technologies demonstrated with Aemetis with CEC funding.

Project Structure

The research team's technical approach to efficiently convert California agricultural and hardwood wastes to biofuels and bioproducts of immediate value leveraged three critical technical approaches and was demonstrated at scale in collaboration with Aemetis Inc. The researchers conducted work on the following three tasks:

- Task 1: Biomass deconstruction (Lead: Dr. John Gladden, Sandia National Laboratories-Livermore). Optimized the JBEI pioneered ionic liquid (IL) based deconstruction of the lignocellulosic material.
- Task 2: Strain development (Lead: Dr. Taek Soon Lee, Lawrence Berkeley National Laboratory). Developed a microbial strain that can convert the carbon (specifically C6/C5 sugar streams) to the gasoline replacement and platform chemical, isopentenol.
- Task 3: Scale-up and commercial demonstration (Lead: Jeff Welch, Aemetis). Collaborated with Aemetis Inc. to evaluate both the IL deconstruction process and biofuel production at a commercially relevant scale.

CHAPTER 2:
Project Approach

Ionic Liquid Pretreatment

Pretreatment of lignocellulosic biomass with ILs has been demonstrated to be effective at reducing the recalcitrance to enzymatic hydrolysis for a wide variety of plants. This process liberated fermentable sugars that can be converted to biofuels. In this project, various California based woody biomass were studied for their potential to release fermentable sugars after IL pretreatment and enzymatic hydrolysis. Two IL pretreatment methods were studied: 1) the neat IL pretreatment method using 100 weight (wt) percent ethanolamine acetate combined with an early separation approach for IL recovery, and 2) the aqueous IL method using 10 wt percent choline lysinate in water in a one-pot bioprocess that combined all the steps of pretreatment, hydrolysis, and fermentation in one unit operation. To optimize this IL-based process on California woody biomass, the researchers tested different process conditions at the bench scale:

- Temperature = [140, 160]°C
- Time = [3, 6] hour
- Biomass Type = [pine (P), fir (F), almond (A), walnut (W)]
- Feedstock Blend = [1/1/1/1, 1/1/1/0, 2/2/1/0] A/W/P/F
- Enzyme loading = [20, 30] milligram (mg) protein/gram (g) biomass
- Enzyme composition = [9/1, 7/3] cellulase/hemicellulase
- Scale = [1, 30] g biomass
- Reactor = [Batch, Stirred]

Materials

Aemetis, Inc. (Cupertino, California, U.S.) provided pine, fir, walnut, and almond that was used as received, all California woody biomass obtained from Paddock Inc. in Oakdale, California. The almond and walnut wood waste came from local orchards, while the pine wood came from forest thinning. The biomass was dried for 24-48 hour in a 40ºC oven. Subsequently, it was a knife-milled with a 2 mm screen (Thomas-Wiley Model 4, Swedesboro, NJ). The resulting biomass was then placed in leak-proof bags and stored in a cool dry place. Additionally, a small portion of the pine and fir were further sieved (mesh #270 with ~ 50 micron opening) to obtain a separate fraction with smaller particle sizes. The following chemicals were purchased from Sigma Aldrich (St. Louis, MO) and used as received: hydroxyethylamine, also known as, ethanolamine (ACS reagent, 99.0 percent purity), acetic acid (ACS reagent, 99.7 percent purity). Novozymes' (Davis, CA) cellulase and hemicellulase complexes Cellic® CTec3 and Htec3 were used as received.

Synthesis of Ionic Liquids

Synthesis of Aprotic Ionic Liquid (Choline Lysinate)

Researchers weighed lysine monohydrate (0.4 mol, 65.68 g) into a 500 milliliter (mL) round bottom flask and dissolved it in 100 mL deionized water at room temperature to obtain a clear solution (light lime-yellow). Then the flask was mounted on an ice-bath (3-5˚C) and N2 was purged for 20-30 mins. Next 46 wt percent of choline hydroxide in water (0.4 mol, 105.15 g) was added dropwise to lysine solution while maintaining the temperature of the ice-bath (3-5 ˚C). The mixture was stirred for 48 hours at room temperature. Excess water was removed under reduced pressure and the mixture was added to acetonitrile/methanol (9:1, volume to volume ratio (v/v)) to remove the excess starting materials. Finally, the solvents were removed under reduced pressure and the mixture was freeze-dried to get the final product (Yield~ 95 percent, light orange). The product, thus obtained, was characterized by nuclear magnetic resonance spectrometry (NMR) using deuterium labeled dimethylsulfoxide (DMSO-d6) as an external lock solvent.

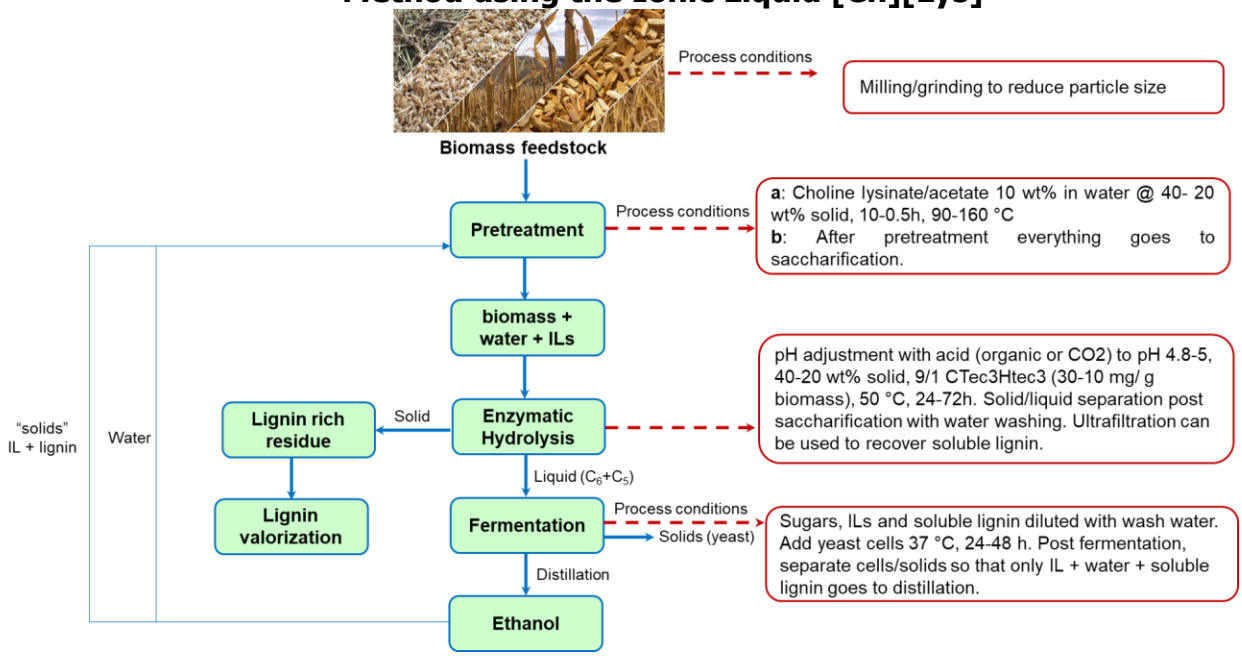
Synthesis of Protic Ionic Liquid (Ethanolammonium Acetate)

The researchers synthesized ethanolammonium acetate ([EOA][OAc]) by the equimolar addition of the acid and bases as neat reagents (based on the stoichiometric requirements) to eliminate both the need for solvent and the introduction of incidental water. The [EOA][OAc] protonic ionic liquid (PIL) was synthesized using a round-bottom flask equipped with two addition funnels — one for the acid and one for the base. The reagents (ethanolamine and acetic acid) were slowly added into the flasks and homogenized with a magnetic stirring bar. Researchers then mounted the flask in an ice/water bath (~4 ˚C) to prevent heat buildup during the reaction. After complete addition, the reaction was allowed to come to completion for 24 hours. The final product was characterized with NMR using DMSO-d6 as an external lock solvent.

Biomass Pretreatment and Enzymatic Hydrolysis

The research team carried out biomass pretreatment at a solid loading of 15 wt percent in a one-pot configuration. Typically, 0.75 g of biomass was mixed thoroughly with 4.25 g of IL in a pressure tube, followed by heating in an oil bath at 140-160 °C for 3-6 hours. Post pretreatment, 10 molar (M) HCl was added to adjust the pH of the biomass slurry to 5. Subsequently, 20-30 mg protein/g of biomass of commercial enzyme mixtures, Cellic CTec3 and HTec3 (9:1 v/v) was added to the biomass slurry to carry out saccharification at 50 °C for 72 hours at 48 revolutions per minute (rpm) in a rotary incubator. After hydrolysis, liquid samples were collected and centrifuged at 12,000 rpm for 2 minutes and the supernatant was filtered using 0.45 µm centrifuge filters before performing sugar analysis, as described below. Figure 1 provides the process schematic.

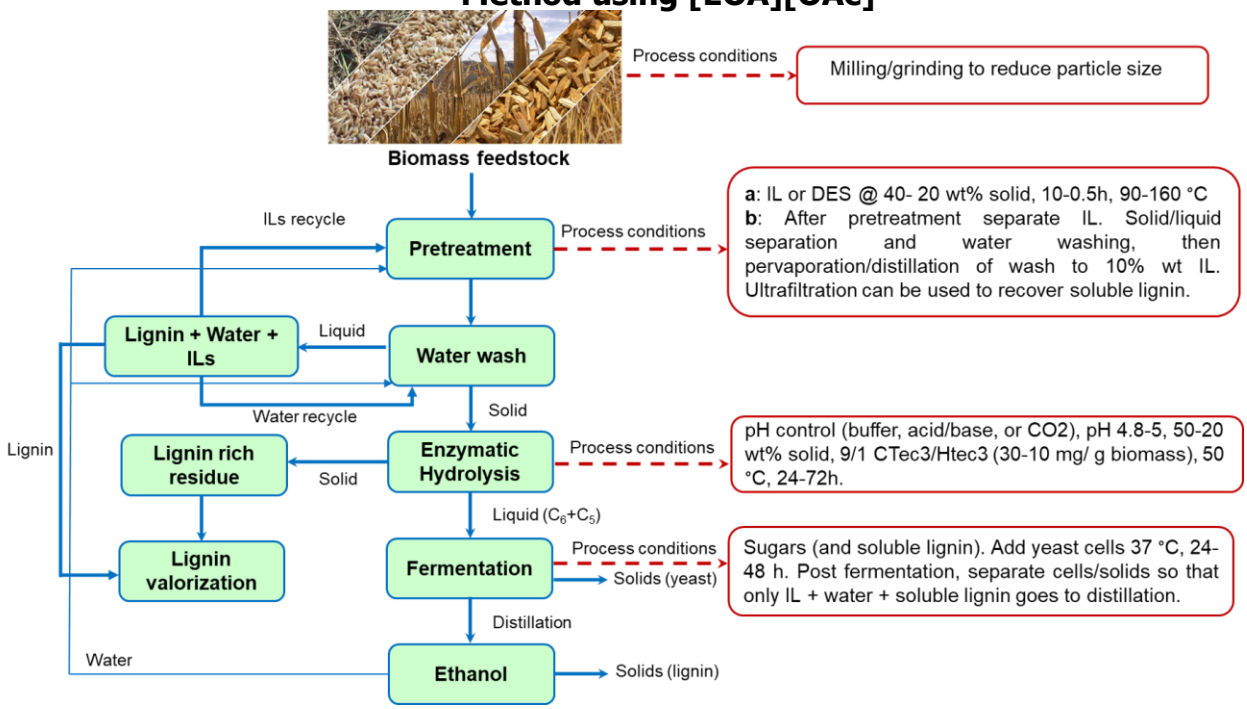
Figure 1: Process Flow Diagram Describing the One-Pot Ionic Liquids Pretreatment Method using the Ionic Liquid [Ch][Lys]



Source: Lawrence Berkeley National Laboratory

For the PIL, the pretreatment was carried out using the conventional method that involves early separation (or washing) to remove the IL after pretreatment (Figure 2).

Figure 2: Process Flow Diagram Describing the Neat Ionic Liquids Pretreatment Method using [EOA][OAc]



Source: Lawrence Berkeley National Laboratory

In a typical experiment, researchers loaded biomass and ionic liquid into an ace pressure tube (50 mL, Ace Glass Inc., Vineland, New Jersey) and homogenized it. The solid loading was controlled at 15 wt percent solids (based on a 1g biomass scale) and heated in an oil bath set to 140 ºC for 3 hours. After cooling for 30 minutes, researchers washed the mixture with deionized water at least five times (using a total of 200 mL water). The solid fraction was recovered via centrifugation, then lyophilized for complete water removal. For enzymatic hydrolysis, the 0.15 g of the recovered biomass was loaded into a test-tube at 1.5 wt percent solids loading. The liquid fraction contained 50 vol percent of a 0.1 M citrate buffer (pH 5), 1 vol percent sodium azide and 20 mg protein/g biomass using a 9/1 mixture of the CTec3/Htec3 and completed with deionized water to attain the desired solid loading. The researchers subsequently incubated the mixture at 50 ºC for 72 hours at 50 rpm in a rotary incubator (Enviro-Genie, Scientific Industries, Inc.) The amount of sugars released were quantified using HPLC after the incubation was completed. Thereafter, the following additional conditions were studied sequentially to optimize the process yields for high sugar release: scale [1, 30] g, solid loading [15, 30, 50] wt percent, biomass size [0.05, 2] mm, temperature [140, 160] ºC, time [3, 6] h, enzymes [20, 30] Ctec3/HTec3 [9/1, 7/3].

The research team analyzed the composition of the untreated sorghum was performed to determine the glucan, xylan, and klason lignin following the two-step acid hydrolysis procedure. In summary, 300 mg of the dry biomass was exposed to 3 mL of 72 percent w/w H_2SO_4 at 30 ºC for 1 hour, followed by secondary hydrolysis at 4 percent w/w H_2SO_4 at 121 ºC for 1 hour. After the two-step acid hydrolysis, acid-insoluble lignin was obtained by filtering the hydrolysates through filter crucibles. Klason lignin was determined by subtracting the weight of oven-dried residual solids (105 ºC) and the ash content (575 ºC). Monomeric sugars (glucose and xylose) were determined by high-pressure liquid chromatography (HPLC) using an Agilent 1200 series instrument equipped with a refractive index detector and Bio-Rad Aminex HPX-87H column, coupled with a guard column assembly. Product separation was obtained at 60 °C with 4 mM H_2SO_4 as a mobile phase at a flow rate of 0.6 mL/min.

Optimization of Ionic Liquid Pretreatment

After the bench-scale optimization, the research team scaled up the one-pot ionic liquid pretreatment and saccharification process to 10 L and 210 L sequentially. The 10 L scale experiments were carried out in a 10 L Hastelloy C276 Parr vessel. Two biomass solid loading conditions were evaluated (15 percent and 25 percent solid loading) with a 3 Kg final working weight. A mixture of pine, almond and walnut (1:2:2) were used as substrate. Pretreatment conditions for both experiments were: 10 percent wt. [Ch][Lys], 160 °C, 50 rpm for 3 hours. After pretreatment, the reaction was cooled to room temperature and adjusted to pH 5 using 50 percent (w/w) H_2SO_4. Saccharification was conducted at 50 °C with agitation at 50 rpm for 72 h. Enzyme loading for each process was 30 mg/g biomass with CTec3:HTec3 ratio of 9:1.

The researchers conducted the one-pot pretreatment and enzymatic saccharification at 210 L scale in an Andritz 210L Hastelloy C276 pressure reactor (AG, Graz, Austria) with a helical impeller using three solid loading (19 percent, 22 percent and 25 percent) and two working volumes (75 Kg and 90 Kg). Pine, almond and walnut (1:2:2) were premixed with 10 percent wt. [Ch][Lys] and water and heated to 160 °C for 3 hours at 45 rpm. After IL pretreatment, the contents were cooled to 50 °C and adjusted to pH 5.0 with 50 percent (v/v) H_2SO_4. Subsequently, 30 mg/g biomass of CTec3 and HTec3 (9:1 ratio) was added to the pretreated

biomass and saccharification was carried out at 50 °C at 45 rpm for 72 h. To prevent bacterial contamination, researchers added 50,000 U/L Penicillin and 50 mg/L Streptomycin after harvest and the hydrolysate was stored at 4 °C. For both scale-ups, researchers took samples at regular intervals that were analyzed by HPLC.

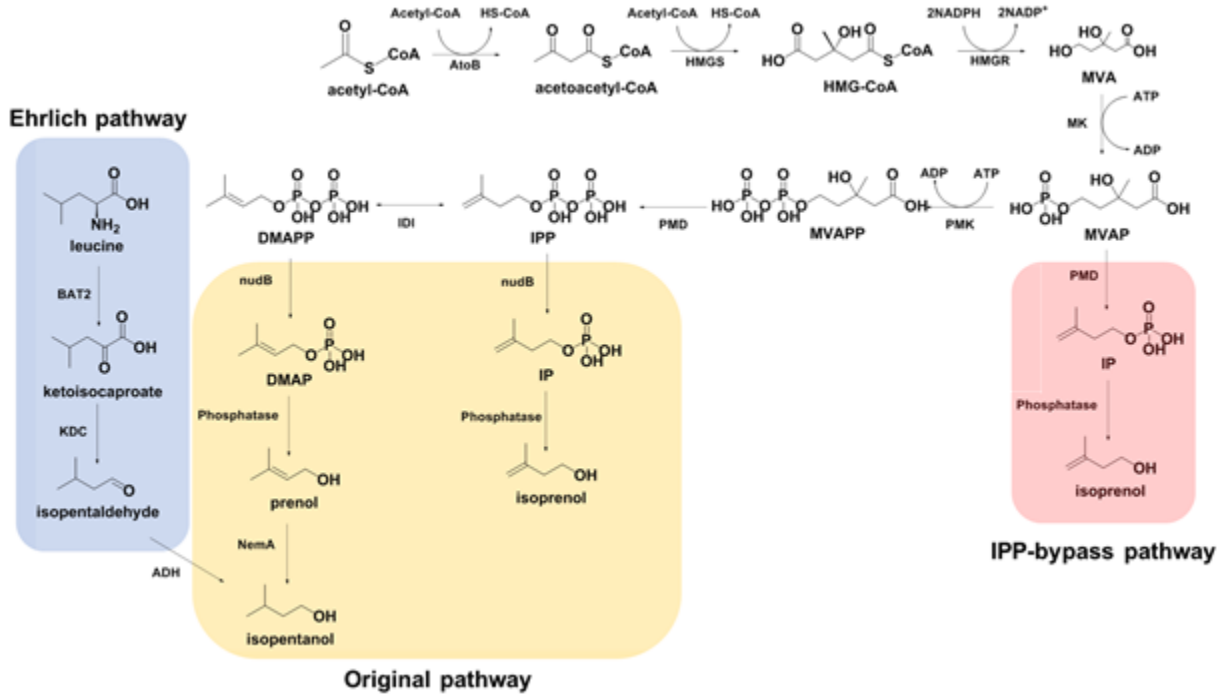
Strain Engineering

For microbial production of isoprenol, the mevalonate (MVA) pathway has been engineered and optimized in *Escherichia coli* (*E. coli*), and recently, in *Corynebacterium glutamicum* (*C. glutamicum*) (Withers et al., 2007; George et al., 2014; Chou and Keasling, 2012; Liu et al., 2013; Sasaki et al., 2019; Kang et al., 2016; Kang et al., 2019). A heterologous MVA pathway was constructed to produce isoprenol in E. coli by overexpressing 7 genes. Isopentenyl diphosphate (IPP), a universal precursor of isoprenoid biosynthesis, is accumulated via both the MEP and MVA pathways and dephosphorylated to isopentenyl monophosphate (IP) by a promiscuous activity of E. coli endogenous phosphatase NudB (Fig. 1). Finally, isoprenol is produced via hydrolysis of IP by various endogenous phosphatases such as apg, qyab, and aphA. The C. glutamicum also was engineered to produce isoprenol via a heterologous MVA pathway (Withers et al., 2007; George et al., 2014; Chou and Keasling, 2012; Sasaki et al., 2019; Kang et al., 2016; Kang et al., 2019). The isoprenol was produced by combining three approaches: deletion genes of poxB and ldhA, media optimization from sorghum biomass hydrolysate and employment of NADH-dependent HmgR. However, the accumulation of IPP via engineered MVA pathway has caused growth inhibition, reduced cell viability, and plasmid instability, and resulted in low yield and titer in the producing host. To overcome these limitations, a new pathway that avoids IPP formation for isoprenol production was designed (**Error! Reference source not found.**) (Kang et al., 2016). Using promiscuous activities of two enzymes, phosphome-valonate decarboxylase from S. cerevisiae (PMDsc) and an aphA, in a novel "IPP-bypass pathway" was developed for isoprenol production, and the 3.7 g/L isoprenol titer was reached in batch condition using minimal medium (M9) and a titer of 10.8 g/L was recently reported by optimization of fed-batch fermentation process (Kang et al., 2019).

The biotechnology industry widely uses *S. cerevisiae* in the biotechnology industry due to its inherent safety, industrial robustness, ease of genetic manipulation, and generally regarded as safe (GRAS) for large-scale operation. Due to these advantages, the yeast cell factories were used to produce branched-chained higher alcohols (Generoso et al., 2015), biofuels derived from terpenoids such as isoprene and farnesene, and pharmaceutical terpenoids such as amorphadiene (Zhang, Nielsen and Liu, 2017). The Ehrlich pathway on S. cerevisiae is a well-known route to produce branched-chain higher alcohols such as isobutanol, isopentanol, and 2-methyl-1-butanol. Biological branched-chain higher alcohols production starts from decarboxylation of keto acids, which are the precursors of several amino acids such as leucine, isoleucine, or valine, forming aldehydes or ketones that can be further reduced to branched-chain higher alcohols by alcohol dehydrogenases. Also, *S. cerevisiae* has been employed to produce biofuels derived from terpenoids. S. cerevisiae was directly evolution coupled with perturbation of gal regulon for 3.7 g/L isoprene production. And FPP-overproducing platform and bisabolene synthases led to bisabolene titer >900 mg/L. Furthermore, with successful examples of engineering yeast for industrial-scale terpenoid production, such as β-farnesene

production with titers exceeding 130 g/L in the bioreactor and amorphadiene production with titers exceeding 40 g/L in the bioreactor.

Figure 3 Schematic Diagram of Original and Isopentenyl Diphosphate-Bypass Pathway for Isoprenol Production



The mevalonate pathway in *S. cerevisiae* consists of 7 reactions to convert acetyl-CoA into IPP and DMAPP. Dephosphorylation of these compounds by NudB, a promiscuous E. coli phosphatase, produces isoprenol. The IPP-bypass pathway was proposed in this study: direct decarboxylation of mevalonate diphosphate (MVAP) followed by dephosphorylation of isopentenyl monophosphate (IP). The PMD has promiscuous activity toward nonnative substrates such as MVAP. The Ehrlich pathway is a well-known route to produce branched-chain higher alcohols such as isobutanol, isopentanol, and 2-methyl-1-butanol. One of branched-chain amino acids as leucine is converted to isopentanol on Ehrlich pathway Abbreviations: HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA; MVA, mevalonate; MVAP, mevalonate phosphate; MVAPP, mevalonate pyrophosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl diphosphate; AtoB, acetoacetyl-CoA thiolase; HMGS, HMG-CoA synthase; HMGR, HMG-CoA reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; PMD, mevalonate pyrophosphate kinase; IDI, isopentenyl pyrophosphate isomerase; NudB, *E. coli* native phosphatase.

Source: Lawrence Berkeley National Laboratory

In this study, the researchers engineered *S. cerevisiae* for improved biosynthesis of isoprenol. The team engineered both the original MVA pathway and the IPP-bypass pathway to improve isoprenol production via the IPP-bypass pathway, and engineered the strains by deleting a promiscuous endogenous kinase that could divert the pathway flux by generating IPP from isoprenyl phosphate (IP), a key intermediate of IPP-bypass pathway, and from isoprenol.

In this work, the project team mostly focused on the proof of concept of the yeast isoprenol production and the development of more efficient yeast strains for isoprenol production using simple sugar as a carbon source. Because the pathway behavior in hydrolysate has not been explored in this production host, researchers will need more in-depth study about the effect of hydrolysate to the pathway for isoprenol production that will take more time and effort after preparation of the strain that produces high titer of isoprenol. Therefore, within the scope of

this project, the research team did not explore the production of isoprenol using biomass derived hydrolysate.

Strains and Plasmids

The researchers used *S. cerevisiae* CEN.PK2-1C (MATa; ura3-52; trp1-289; leu2-3_112; his3Δ1; MAL2-8C; SUC2) as the background strain for all constructs, and *E. coli* DH1 was used to propagate the recombinant plasmids. *S. cerevisiae* CEN.PK2-1C genome was used for construction of all linear DNA and plasmids. All chemicals were purchased from Sigma Aldrich (Sigma-Aldrich, U.S.).

Design, Quantification, and Quantitation of the Original Pathway and Isopentenyl Diphosphate-Bypass Pathway

To produce isoprenol via the original MVA pathway, the researchers integrated 5 pathway genes (*EfmvaE*, *EfmvaS*, *ERG8sc*, *ERG12sc,* and *ERG19sc*) on the genome. All of the target genes, promoters and terminators were amplified from the *S. cerevisiae* genome. *EfmvaE* and *EfmvaS* were integrated with the URA selection marker on the URA locus and expressed under the control of galactose promoter (pGal). *ERG12sc* and *ERG19sc* were integrated with histidine (HIS) selection marker on the leucine (LEU) locus and expressed under the control of pGal. *ERG8sc* was integrated with the tryptophan (TRP) selection marker on Gal1 locus and expressed under the control of pGal. To use galactose promoter, *GAL1*, *GAL7*, and *GAL10* were deleted from the genome. For integration and deletion of target genes, the linear DNAs including 500 base pair (bp) homologous arms (HAs), selection marker, target genes with promoter and terminator were assembled using Gibson assembly kits (NEB, England). The homologous recombinase was used to integrate the assembled linear DNA on the target region. The NudB was expressed on the high copy (2 micron) plasmid with the LEU selection marker.

For the construction of isopentenyl diphosphate-bypass pathway (IBP) strains, the research team integrated 4 pathway genes (*EfmvaE*, *EfmvaS*, *ERG12sc,* and *ERG19sc*) on the genome. All target genes, promoters and terminators were amplified from the *S. cerevisiae* genome. *EfmvaE* and *EfmvaS* were integrated with the URA selection marker on the URA locus and expressed under the control of galactose promoter (pGal). *ERG12sc* and *ERG19sc* were integrated with histidine (HIS) selection marker on the leucine (LEU) locus and expressed under the control of pGal. To use galactose promoters, *GAL1*, *GAL7*, and *GAL10* were deleted and the TRP selection marker was inserted into the *GAL1* locus. The PMD was expressed on the high copy (2 micron) plasmid with the LEU selection marker.

For the isoprenol production, the researchers individually inoculated a single colony of the original pathway strain and IBP strain in glass tubes containing 5 mL YPD supplemented 2 percent glucose at 30ºC and shaken at 200 rpm overnight. The cells were inoculated to the initial OD 0.02 in 50 mL YPD medium supplemented with 2 percent glucose. After 12 h, the integrated genes were induced by adding galactose.

The cells were first adapted in the Delft medium by serially diluting cell cultures in a fresh Delft medium. Briefly, each single colony was inoculated in YPD overnight and diluted 50-fold (v/v) in Delft medium. The adapted cells were diluted 50-fold (v/v) in a fresh Delft medium again, and the final adapted cells were inoculated to initial OD 0.02 in 50 mL Delft medium

supplemented with 2 percent glucose. After 12 hours, integrated genes were induced by adding galactose.

For isoprenol quantification and quantitation, the research team combined the cell culture (1 mL) with an equal volume of ethyl acetate (1 mL) containing 1-butanol (30 mg/L) as an internal standard and vigorously mixed for 10 min. The cell cultures and ethyl acetate were separated by centrifugation at 14,000 x g for 5 min. A 500 µL of ethyl acetate layer was analyzed by gas chromatography – flame ionization detection (GC-FID, Thermo Focus GC) equipped with a DB-WAX column (15-m, 0.32mm inner diameter, 0.25-µm film thickness, Agilent, USA), and the oven temperature program was as follow: started at 40 ºC, a ramp of 15 ºC/min to 100 ºC, a ramp of 40 ºC/min to 230 ºC and held at 230 ºC for 3 min.

Deletion of the 5-phosphomevalonate Kinase and Choline Kinase

The researchers analyzed the target region based on the Saccharomyces Genome Database (SGD, https://www.yeastgenome.org/). The CRISPR/Cas9 system was used to construct strains for isoprenol production. For CRISPR/Cas9, pCut plasmids were derived from a yeast episomal shuttle vector and have a 2-micron origin of replication and an uracil selection marker (Reider Apel, d'Espaux et al. 2017). The Cas9 is driven by the ADH1 promoter and CYC1 terminator. The 20 bp single guide RNA (sgRNA) on the target gene is controlled under a tyrosine promoter and an SNR52 terminator. The researchers used Benchling web tool (https://www.benchling.com/) to design primers to create donor DNA fragments with 500 bp flanking regions homologous to the respective target site. CEN.PK2-1C genomic DNA served as a template to generate all flanking regions, promoters, and terminator fragments. The deletion was screened by growing recombinants on an SC agar plate without URA. The deletion of target genes, 5-phosphomevalonate kinase (PMK or ERG8) and choline kinase (CK), were confirmed using specific primers. Primers were designed based on the sequence flanking the target region to amplify the junction sequence.

Validation of Phosphatases for Isoprenol Production

To improve IP hydrolysis to isoprenol, the research team tested 15 previously reported phosphatases for their promiscuous activity; 4 phosphatases amplified from *E. coli* DH1 include aminoglycoside-3-phosphotransferase (AphA), glucose-1-phosphatase (Agp), fructose-1-phosphate phosphatase (YqaB) (Kang, George et al. 2016), and alkaline phosphatase (PhoA), and 11 phosphatases from *S. cerevisiae* CEN.PK2-1C include sugar alcohol phosphatase (PYP1) (Xu, Lu et al. 2018), phosphatidate phosphatase (PAH1) (Han, Wu et al. 2006), glycerol-3-phosphatases (GPP1 and GPP2), serine/threonine phosphatase (GLC7), acid phosphatases (PHO3 and PHO5), alkaline phosphatase (PHO8 and PHO13), lipid phosphate phosphatase (LPP1) (Faulkner, Chen et al. 1999), and diacylglycerol phosphate phosphatase (DPP1) (Faulkner, Chen et al. 1999). The Gibson assembly was employed to construct plasmids. For the construction of the phosphatase expression vectors, the phosphatase genes were individually cloned on the pRS425. All of phosphatases expression was controlled under constitutive GK1 promoter and ADH1 terminator. This plasmid contains the 2-micron origin and the LEU as a selectable marker in yeast.

Fermentation Process Optimization

Novozymes engineered the xylose utilizing strain, Saccharomyces cerevisiae NZ 22273, used for this study and maintained it in a 25 percent (w/v) glycerol stock solution at -80 °C. Cell growth was performed in two steps. The first seed culture was grown in 250 mL baffled flasks containing 50 mL YPD media (10 g/L yeast extract, 20 g/L1 peptone, 20 g/L glucose and 10 g/L xylose) using defrosted cell suspension from glycerol stock. Cells grown in the YPD media were then used to inoculate seed 2 in a 50:50 mixture of YPD and filtered hydrolysate using a 10 percent (v/v) inoculum size. In both steps, the cells were incubated at 30 °C or 35 °C at 220 rpm for 24 h. The YPD media and hydrolysate were filtered sterilized with 0.2 mm pore filters and 100,000 U/L Penicillin and 100 mg/L Streptomycin were added prior inoculation. Microbial growth was measured by optical density at 600 nm in a spectrophotometer (Thermo Scientific™ GENESYS™ 10S UV-Vis Spectrophotometer).

The researchers carried out small scale fermentation in 100 mL sealed glass bottles with 80 mL working volume. Each bottle was aseptically batched with 72 mL filtered/unfiltered hydrolysate and 8 mL inoculum from Seed 2. This step aimed to evaluate the effect of temperature, pH, media supplementation, solid separation, strain acclimatization and the effect of inhibitors in the fermentation media. The fermentations were performed at 30 °C or 35 °C, pH 5 or pH 5.5, 100 rpm for 7 days. 50,000 U/L Penicillin and 50 mg/L Streptomycin were added prior fermentation. Filtered hydrolysate was prepared by centrifuging the hydrolysate at 4000 relative centrifugal force (xg) for 20 min to remove the solids and sterilizing through a 0.2 mm filter prior to use.

Scale-Up at Aemetis

Following process optimization, the research team performed a 5L-scale fermentation in a Sartorius fermenter (Sartorius BIOSTAT® B, Germany) to evaluate critical bioprocess parameters reproducibility. No solids separation was performed, and the unfiltered hydrolysate was pasteurized at 70 °C for 30 min. Each vessel was batched with 2.7 L of hydrolysate and 300 mL of seed 2 was used as inoculum, for a final volume of 3 L. Fermentation conditions were as follows: temperature 30 °C or 35 °C; pH 5 or pH 5.5, 300 rm, 5 days fermentation time.

The project team demonstrated a 1500L-scale fermentation in an ABEC fermenter (ABEC, Springfield, MO) with a final working volume of 680 L and a 10 percent (v/v) inoculum size. The seed train for this process was completed in two stages. First, 2 L shake flasks containing 1 L yeast extract peptone dextrose (YPD) media each were inoculated with hydrated yeast (target: 10^7 viable cells/ml). This step was conducted in a benchtop orbital shaker (CERTOMAT® BS-1) at 35 °C, 200 rpm for 24 h. Then, 7 L of seed culture from the first step was used to inoculate a 150-L bioreactor (ABEC, Springfield, MO) containing 63 L of a 50:50 mixture of YPD and filtered hydrolysate. YPD and hydrolysate were pumped through a 0.2 µm filter into the bioreactor and 100,000 U/L Penicillin and 100 mg/L Streptomycin were added prior inoculation. The cells were incubated at 35 °C, 100 rpm with a 30 L/min air flow rate. Samples were taken periodically to monitor sugar consumption. After 10 h, 68 L of acclimated cells was transferred to the fermentation tank containing 615 L of unfiltered hydrolysate, totalizing 680 L.

Researchers transferred unfiltered hydrolysate into the 1500 L tank and pasteurized at 70 °C for 5 min to avoid inhibitor formation. After pasteurization, the tank was cooled to room temperature and 100,000 U/L Penicillin and 100 mg/L Streptomycin were added to prevent bacterial growth. The pH was then adjusted to 5.5 with 10 M NaOH. Off-gas exhaust from the fermenter was connected to a cold trap to account for ethanol evaporation. The fermentation was carried out at 35 °C, 80 rpm for 5 days. Samples were taken in regular intervals and analyzed by HPLC.

CHAPTER 3:
Project Results

Ionic Liquid Pretreatment

Biomass Composition and Extractives

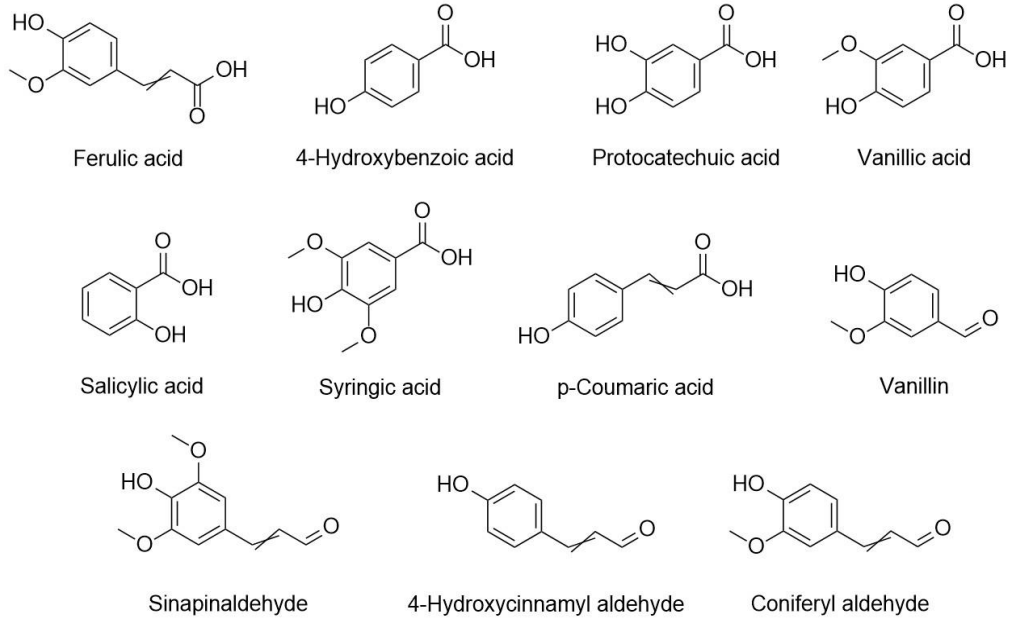
The research team determined the compositional analysis (Table 1) of the biomass following an established National Renewable Energy Laboratory (NREL) protocol. The extractive contents for almond, pine, fir, and walnut were 383.8, 175.4, 343.1, 340.0 g/kg of biomass, respectively. The extracts are predominantly recovered using ethanol and water as extraction solvents. The extractives in woody biomass (softwood and hardwood) are typically composed of free sugars, terpenoids, fatty acids, phenolics. The following are the free phenolics found within the extracts recovered from the woody biomass (Figure 4).

Table 1: Chemical Composition of the Four Woody Biomass Types Studied

Biomass	Extractives (g/kg of biomass)	Glucan (g/kg of biomass)	Xylan (g/kg of biomass)	Klason Lignin (g/kg of biomass)	Ash (g/kg of biomass)
Almond	383.8±3.8	222.6±2.3	110.6±1.2	200.3±2.4	2.0±0.2
Pine	175.4±5.0	330.5±9.5	139.9±4.2	274.3±4.1	1.4±0.1
Fir	343.1±3.4	284.6±3.3	120.2±2.1	234.2±1.9	8.0±0.1
Walnut	340.0±3.4	264.9±3.5	105.7±2.5	200.0±1.5	7.0±0.1

Source: Lawrence Berkeley National Laboratory

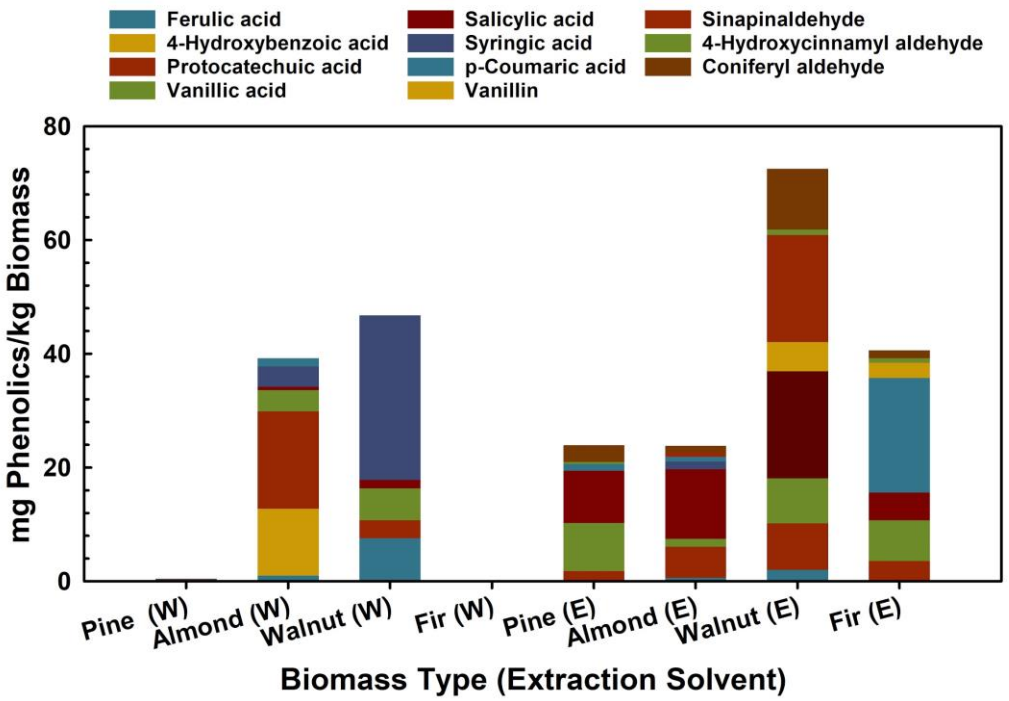
Figure 4: Chemical Structures of the Ethanol and Water Extractives



Source: Lawrence Berkeley National Laboratory

Figure 5 shows the composition for the extractives recovered from the biomass using ethanol and water. The water extracts yielded 0.5, 39.2, 46.8, 0.0 mg/kg total phenolics for pine almond, walnut, and fir, respectively. Similarly, the ethanol extract yielded 23.9, 23.9, 72.6, 40.6 mg/kg total phenolics for pine almond, walnut, and fir, respectively. The softwood (pine and fir) yielded a negligible amount of phenolics from water, while the main extracts were retrieved using ethanol as a solvent. The ethanol extract was predominantly protocatechuic acid, vanillic acid, salicylic acid and p-coumaric acid with trace amounts of syringic acid, p-Coumaric acid, sinapine aldehyde, and coniferyl aldehyde. On the other hand, the hardwoods generated large amounts of phenolics with both water and ethanol. The water extracts were predominantly 4-Hydroxybenzoic acid, protocatechuic acid, vanillic acid, and syringic acid for almond, while walnut comprised of ferulic acid, protocatechuic acid, vanillic acid, and syringic acid. Similarly, the ethanol extract consisted mainly of protocatechuic acid and salicylic acid for almond, whereas walnut extractives composed of ferulic acid, protocatechuic acid, vanillic acid, salicylic acid, and coniferyl aldehyde. For additional extractive characterization different solvents, as well as, different analytical techniques could be explored to truly reveal the full composition of free sugars, terpenoids, fatty acids etc. that are present within the raw biomass.

Figure 5: Extractive Composition of the Phenolic Content for Four Woody Biomass Types using Water (W) and Ethanol (E)



Source: Lawrence Berkeley National Laboratory

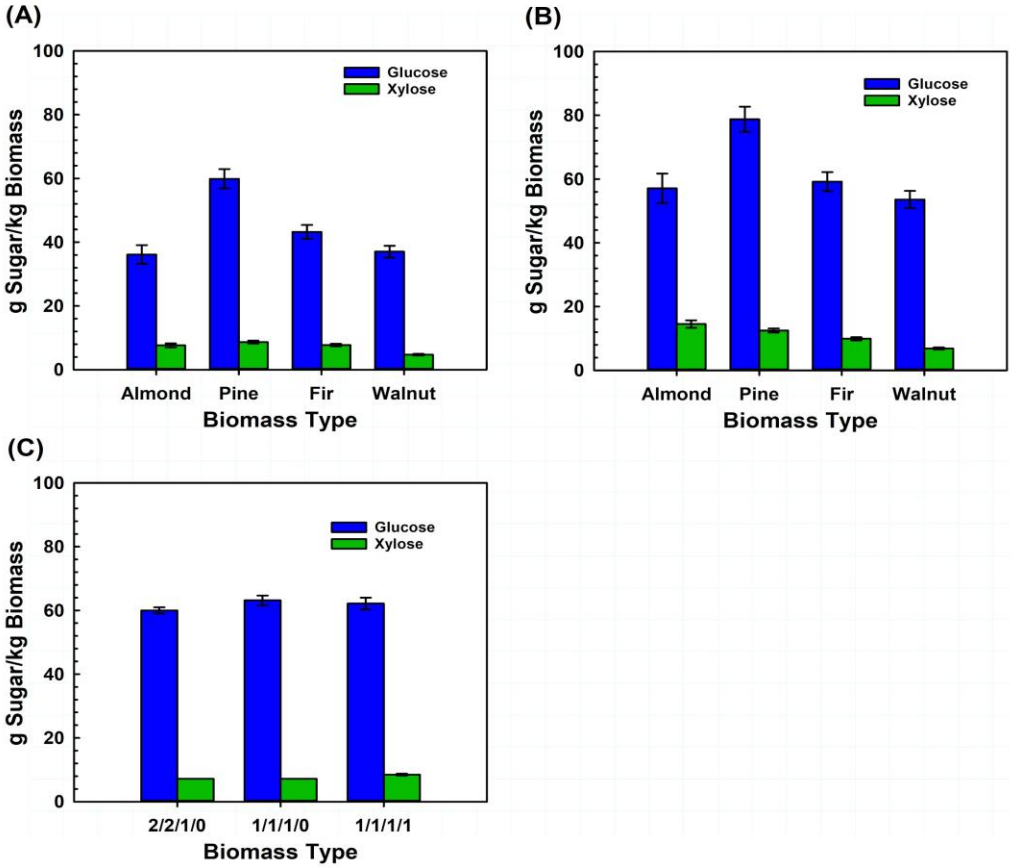
Among the biomass surveyed, researchers obtained the highest glucan content of 330.5 g/kg from pine, while almond had lowest glucan content of 222.6 g/kg of biomass. Xylan content of almond (110.6 g/kg of biomass) and walnut (105.7 g/kg of biomass) were very similar, while pine (139.9 g/kg of biomass) and fir (120.2 g/kg of biomass) were significantly different. Combining both glucan and xylan, the total fermentable sugars for the four biomasses ranged 333.2-470.4 g/kg of biomass. In addition to cellulose and hemicellulose, lignin is another vital

building block of the plant cell wall that accounts for approximately 10–30 percent of the biomass. Lignin content for almond, pine, fir, and walnut were 200.3, 274.3, 234.2, 200.0 g/kg of biomass, respectively. Presence of higher lignin content provides potential of upgrading lignin from the biomass toward value added products such as phenols, activated carbons, composites, energy storage materials, and antimicrobial agents, to name a few. Overall, results illustrate that woody biomass is a promising feedstock for production of biofuels and platform chemicals; however, differences in composition could lead to variability in the process design and product streams.

Fermentable Sugar Yields from Woody Biomass using Ionic Liquids

As a base line, the research team determined the glucose and xylose yield from the four untreated biomasses by performing enzymatic hydrolysis at 20 and 30 mg protein/g biomass of enzyme (Figure 6).

Figure 6: Glucose and Xylose yields from Enzymatic Hydrolysis of Untreated Raw Biomass



(A) 20 mg protein/g biomass of enzyme; (B) 30 mg protein/g biomass of enzyme, as well as (C) mixed biomass using 30 mg protein/g biomass of enzyme.

Source: Lawrence Berkeley National Laboratory

Glucose yields (Figure 6A) for almond, pine, fir, and walnut at 20 mg enzyme protein/ g biomass were 36.1, 59.9, 43.3, 37.0 g/kg of dry biomass, respectively. Under similar enzymes loading xylose yield for almond, pine, fir, and walnut were 7.7, 8.7, 7.7, 4.8 g/kg of dry biomass, respectively. Total sugar released for all the biomass varied between 41.8-68.6 g/kg

of dry biomass, with lowest and highest total sugar yields obtained from fir and pine, respectively. Similarly, glucose yield (Figure 6B) for almond, pine, fir, and walnut at 30 mg enzyme protein/ g biomass were 57.1, 78.8, 59.2, 53.6 g/kg of dry biomass, respectively. Under similar enzymes loading xylose yield for almond, pine, fir, and walnut were 14.5, 12.5 9.9, 6.9 g/kg of dry biomass, respectively. Total sugar released for all the biomass varied between 60.5-91.3 g/kg of dry biomass, with lowest and highest total sugar yields obtained from fir and pine, respectively.

To improve the fermentable sugar released, researchers pretreated all the biomasses with [Ch][Lys] and [EOA][OAc] with three different pretreatment severity factors (SF) of 3.4, 4.0, and 4.3 corresponding to different reaction time and temperature. The selection of different pretreatment time for both the pretreatment corresponds to the severity factor $Log\ R_o$ calculated as described by equation 1:

$$\boldsymbol{Log\ R_0\ =\ Log\ [t\ \times exp\ (\frac{(T-100)}{14.75})]}\ (1)$$

Where t is the treatment time (min) and T is the reaction temperature (˚C).

Impact of [Chy][Lys] on Biomass Pretreatment Efficiency

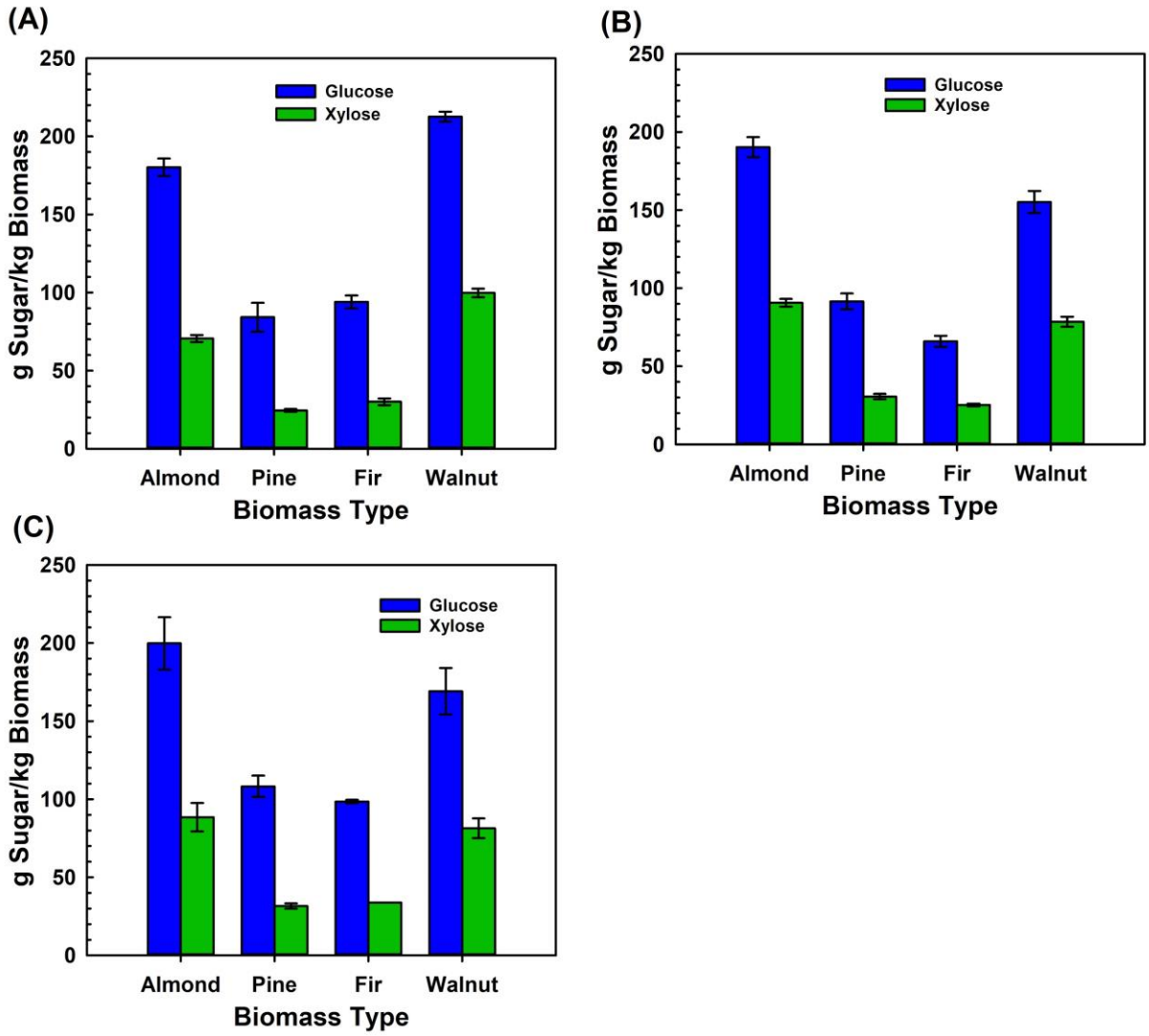
Unlike traditional biomass conversion technology, one-pot conversion of lignocellulosic biomass to fuels can reduce the operating cost by consolidating three (pretreatment, saccharification, and fermentation) unit operations and reduces the energy input for the mass transfer between reactors. The research team's previous results have shown that cholinium lysinate [Ch][Lys], a biocompatible IL, has proven its efficacy in biomass pretreatment due to the greater hydrogen bond basicity for the IL with [Lys]-anions as compared with acetate ILs. In addition, lignin solubilization and low toxicity to the enzymes or microbes makes cholinium lysinate an ideal candidate for one-pot approach. Similarly, previous results have shown that IL:water mixture can be as effective as pure IL for fractionating plant materials and extracting lignin.

Figure 7 A, B, and C show glucose and xylose yields from enzymatic hydrolysis of [Ch][Lys] pretreated biomass at 140 and 160 ˚C, using a 20-30 mg of enzyme protein/g of starting biomass. Figure 7A illustrates the glucose and xylose yields from all the four biomasses at 140 °C, 3 h, (SF = 3.4) and 20 mg protein/g biomass of enzyme. Results show that under the SF of 3.4, glucose yield for almond, pine, fir, and walnut were 180.3, 84.2, 94.1, 212.7 g/kg of dry biomass, respectively. Similarly, xylose yield for almond, pine, fir, and walnut were 70.6, 24.6, 30.1, 99.8 g/kg of dry biomass, respectively. Further increasing the pretreatment severity to 4.0 (Figure 7B) and keeping 20 mg protein/g biomass of enzyme loading constant lead to an increase in the fermentable sugar release for almond and pine, while a decrease was noticed for fir and walnut. Total sugar released from all the four biomasses varied between 91.4-281.1 g/kg of dry biomass, with pine showing the highest 8.8 percent increase in total sugar released, whereas fir showed a 29.8 percent decrease in total sugar released. Glucose yield for almond, pine, fir, and walnut were 190.4, 91.6, 66.1, 155.2 g/kg of dry biomass, respectively, while xylose yield for almond, pine, fir, and walnut were 90.7, 30.6, 25.3, 78.5 g/kg of dry biomass, respectively.

Keeping the pretreatment severity (4.0) constant, an increased enzyme loading of 30 mg protein/g biomass led to a significant increase in glucose yield for almond and pine (Figure 7C). Glucose yield for almond, pine, fir, and walnut were 199.8, 108.2, 98.5, 169.1 g/kg of dry

biomass, respectively, while xylose yield for almond, pine, fir, and walnut were 88.5, 31.5, 33.7, 81.3 g/kg of dry biomass, respectively. Taken together, pretreatment at 160 °C, 3 hours, (SF= 4) and 30 mg protein/g biomass of enzyme was selected as the best condition to maximize fermentable sugars from almond, pine, and fir, which was used as a benchmark for further optimization experiments.

Figure 7: Glucose and Xylose Yields from Enzymatic Hydrolysis of [Ch][Lys] Pretreated Samples



A) 140 ℃, 3 h, and 20 mg protein/g biomass of enzyme; B) 160 ℃, 3 h, and 20 mg protein/g biomass of enzyme; C) 160 ℃, 3 h, and 30 mg protein/g biomass of enzyme.

Source: Lawrence Berkeley National Laboratory

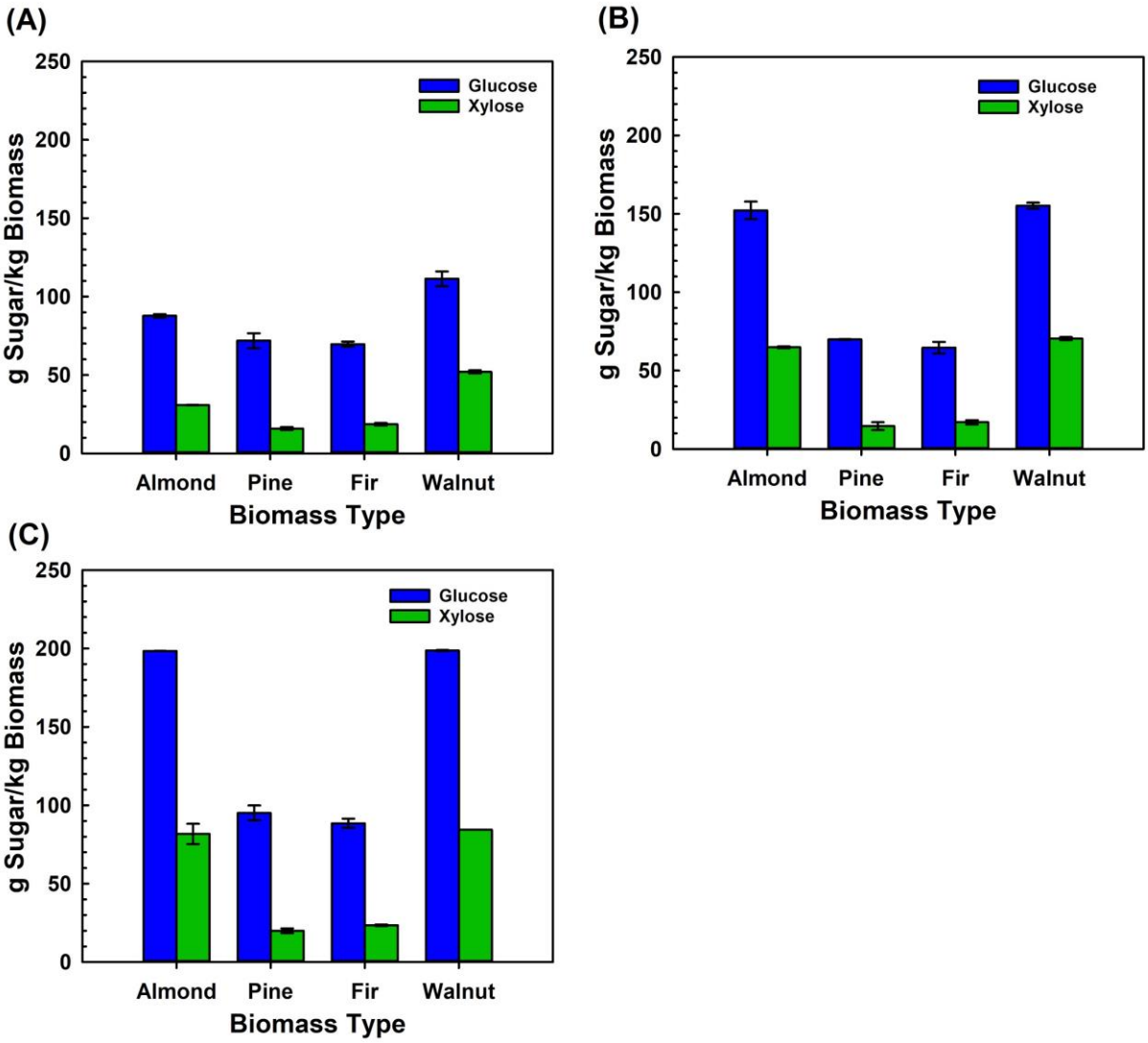
Impact of Ethanolammonium Acetate [EOA][OAc] on Biomass Pretreatment Efficiency

The transformation of biomass into liquid biofuels has been demonstrated using specific ionic liquids such as 1-ethyl-3-methylimidazolium acetate ([C_2C_1Im][OAc]) and cholinium lysinate ([Ch][Lys]). While these ILs have been recognized as effective pretreatment solvents, the use of protic ionic liquids (PILs) for biomass pretreatment is a cost competitive option that has

been reported to reduce ethanol selling process by 40 percent. In particular, the biocompatible PIL ethanolammonium acetate ([EOA][OAc]) is associated with a low cost (~$1 per kg) and has been used for the integrated biofuel production without pH adjustments, water-wash and solid–liquid separations.

Figure 8 shows the glucose and xylose yields from the enzymatic hydrolysis of [EOA][OAc] pretreated biomass at 140 and 160 ˚C, using a 20-30 mg of enzyme protein/g of starting biomass.

Figure 8: Glucose and Xylose Yields from Enzymatic Hydrolysis of [EOA][OAc] Pretreated Samples



A) 140 ℃, 3 h, and 20 mg protein/g biomass of enzyme; B) 160 ℃, 3 h, and 20 mg protein/g biomass of enzyme; C) 160 ℃, 3 h, and 30 mg protein/g biomass of enzyme.

Source: Lawrence Berkeley National Laboratory

Figure 8A illustrates the glucose and xylose yields from all the four biomasses at 140 ˚C, 3 hours, (SF=3.4) and 20 mg protein/g biomass of enzyme. The results show that under the SF of 3.4, glucose yield for almond, pine, fir, and walnut were 87.8, 71.9, 69.7, 111.4 g/kg of dry biomass, respectively. This represents a 20-300 percent increase in glucose yields (compared

to the raw biomass), indicating that the use of the ILs for pretreatment was effective at deconstructing the biomass and reducing recalcitrance. However, the results depict that almond and walnut responded quicker and easier to the pretreatment yielding 140 percent and 200 percent increases in glucose, respectively. On the other hand, the increase in glucose yields for pine and fir were only 20 percent and 60 percent, respectively. Similarly, xylose yield for almond, pine, fir, and walnut were 30.9, 15.9, 18.7, 52.0 g/kg of dry biomass, respectively.

Despite the improvement in sugar release, neither of the processes employed had fully released the expected sugars theoretically present within the biomass (see Table 1). Therefore, the research team carried out additional optimization. Further increasing the pretreatment severity to 4.0 and keeping 20 mg protein/g biomass of enzyme loading constant increased the fermentable sugar release for almond and walnut, while there was negligible change for pine and fir. Glucose yield for almond, pine, fir, and walnut were 152.3, 69.9, 64.6, 155.3 g/kg of dry biomass, respectively, while xylose yield for almond, pine, fir, and walnut were 65.0, 14.6, 17.1, 70.4 g/kg of dry biomass, respectively. Total sugar released from all the four biomasses varied between 81.8-225.7 g/kg of dry biomass, with almond and walnut showing the highest increase in total sugar released with and average improvement of 92 percent and 37 percent, respectively.

Keeping the pretreatment severity (4.0) constant, the researchers employed an increased enzyme loading of 30 mg protein/g biomass that led to a significant increase in glucose yield for almond and walnut (Figure 8C). Glucose yield for almond, pine, fir, and walnut were 198.5, 95.1 88.5, 198.7 g/kg of dry biomass, respectively, while xylose yield for almond, pine, fir, and walnut were 81.7, 20.0, 23.4, 84.4 g/kg of dry biomass, respectively. This indicates an average of 31 percent total sugar increase (amongst all four biomasses), as a result of the increased enzyme loading. Based on these results, pretreatment at 160 °C, 3 h, (SF= 4) and 30 mg protein/g biomass of enzyme was selected as the best condition to maximize fermentable sugars from almond, pine, and fir, and was used as a benchmark for further optimization experiments.

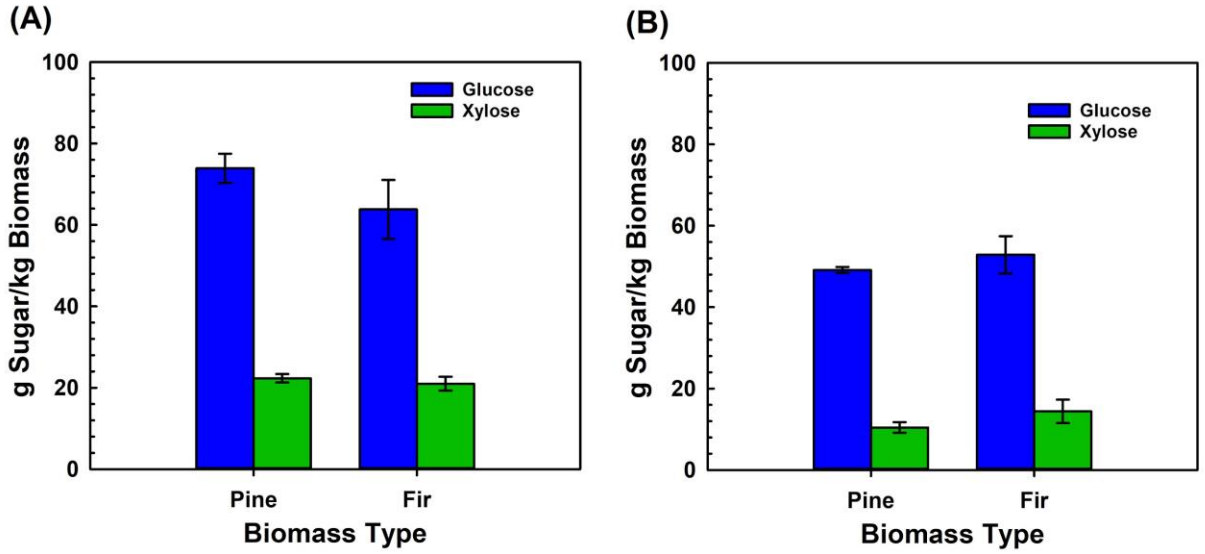
Overall, [Ch][Lys] and [EOA][OAc] pretreatment on woody biomass were effective in releasing fermentable sugars. Results show that both the ILs were able to unlock the maximum sugar releasing threshold for almond and walnut. However, under those conditions pine and fir showed little or no change in total sugar yield. Hence, further optimization to experimental conditions were performed to improve the sugar yields for pine and fir.

Optimization of Sugar Released from Pine and Fir

The researchers carried out further process optimization out for the softwood (Pine and Fir) to improve fermentable sugar release. Figures 9A-B show the glucose and xylose yields from the enzymatic hydrolysis of [Ch][Lys] and [EOA][OAc] pretreated biomass at 160 °C, 6 h, (SF=4.3) using 30 mg of enzyme protein/ g of starting biomass. Total sugar yield for pine and fir in presence of [Ch][Lys] IL at SF of 3.4 were 108.8 and 124.2 g/kg of dry biomass, respectively (Figure 8). When SF was increased to 4.0, total sugar yield for pine and fir increased to 139.7 and 132.2 g/kg of dry biomass, respectively (Figure 8). However, further increasing the SF to 4.3 (Figure 9A), resulted in a decrease in total sugar yield. Under the highest SF, glucose yield for pine and fir were 73.9 and 63.8 g/kg of dry biomass, respectively, while xylose yield for pine and fir were 22.3 and 21.0 g/kg of dry biomass, respectively. For

the [EOA][OAc] pretreated biomass, the results show that glucose yield for pine and fir, were 49.2, 52.9, g/kg of dry biomass. Considering the factor of time, this represents a 29 percent decrease in glucose yields for pine and 17 percent decrease for fir. This indicates that the increasing the pretreatment time up to 6 hours was ineffective at further deconstructing the biomass and reducing recalcitrance. Instead, the increased pretreatment severity led to a loss in released polysaccharides and also sugars (likely due to the possibility of breaking down the sugars and polysaccharides into degradation products). Similarly, the results show that xylose yield for pine and fir, were 10.5, 14.4 g/kg of dry biomass, which also represents a 29 percent and 17 percent decrease in xylose yields, respectively.

Figure 9: Glucose and Xylose Yields from Enzymatic Hydrolysis of Samples Treated at 160 ˚C, 6 h, and 30 mg protein/g biomass of Enzyme



(A) [Ch][Lys]; (B) [EOA][OAc] pretreated samples.

Source: Lawrence Berkeley National Laboratory

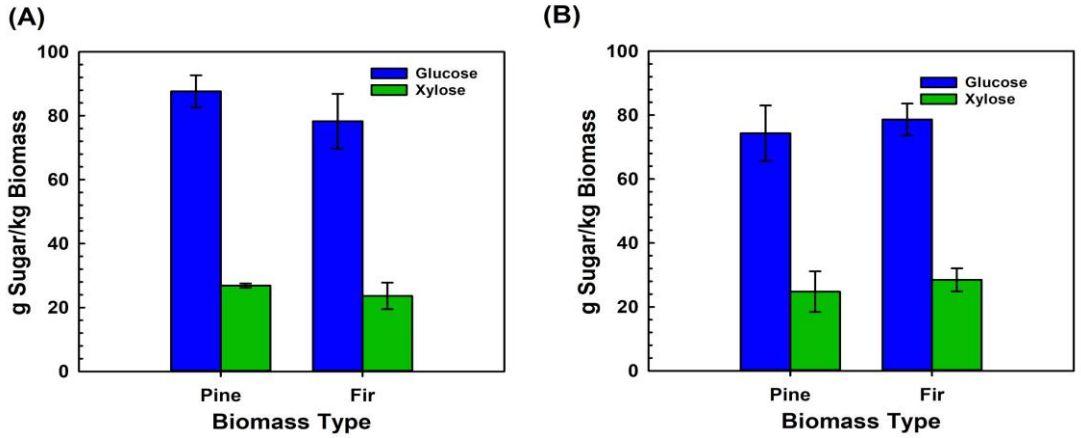
Impact of Enzyme Loading

Enzyme doses and pretreatment conditions need to be optimized to make the conversion process economically viable. Although high yields can be realized by applying high enzyme loadings post biomass pretreatment, from a commercial standpoint the lowest possible amount of enzyme must be used to maximize fermentable sugar release. Therefore, the research team investigated effects of enzyme loadings on saccharification on the optimized pretreatment conditions for IL pretreatment of pine and fir. Figure 10 shows the glucose and xylose yields from the enzymatic hydrolysis of [Ch][Lys] and [EOA][OAc] pretreated biomass at 160 ˚C, 3 hours, using ~ 27 mg of enzyme protein/ g of starting biomass at a ratio of 7/3 Ctec3/HTec3 (by keeping the cellulase constant and increasing the xylanase amount). Results for the [Ch][Lys] pretreated biomass (Figure 10A) illustrate that total sugar yield for pine and fir were 114.5 and 101.9 g/kg of dry biomass, respectively. Interestingly, the total sugar yield decreased for pine (21 percent) and fir (29 percent) compared to corresponding low xylanase loading.

Under the high xylanase loading conditions, xylose yield for pine and fir were 26.8 and 23.7 g/kg of dry biomass, showing little or no change compared to the lower xylanase loading.

Similarly, with constant cellulase loading glucose yield for pine and fir were 87.7 and 78.3 g/kg of dry biomass, which represents 24 percent and 25 percent decrease in yields for pine and fir, respectively. For the [EOA][OAc] pretreated biomass, the results show that glucose yield for pine and fir, were 74.3, 78.6 g/kg of dry biomass. Considering the factor of enzyme ratio, this represents a 21 percent and 18 percent decrease in glucose yields for pine and fir respectively (when compared to the analogous low xylanase loading. Similarly, the results show that xylose yield for pine and fir, were 24.8, 28.5 g/kg of dry biomass, which represents a 24 percent and 21 percent increase in xylose yields for pine and fir, respectively. Taken together, results indicate that the increase in xylanase was effective at improving the overall xylose yield. However, this also led to a reduction in the cellulase efficiency.

Figure 10: Glucose and Xylose Yields from Enzymatic Hydrolysis of Samples Treated at 160 ˚C, 3 h, and 27 mg protein/g biomass of with a 7/3 Ctec3/Htec3 Enzyme Ratio



(A) [Ch][Lys]; (B) [EOA][OAc] pretreated samples.

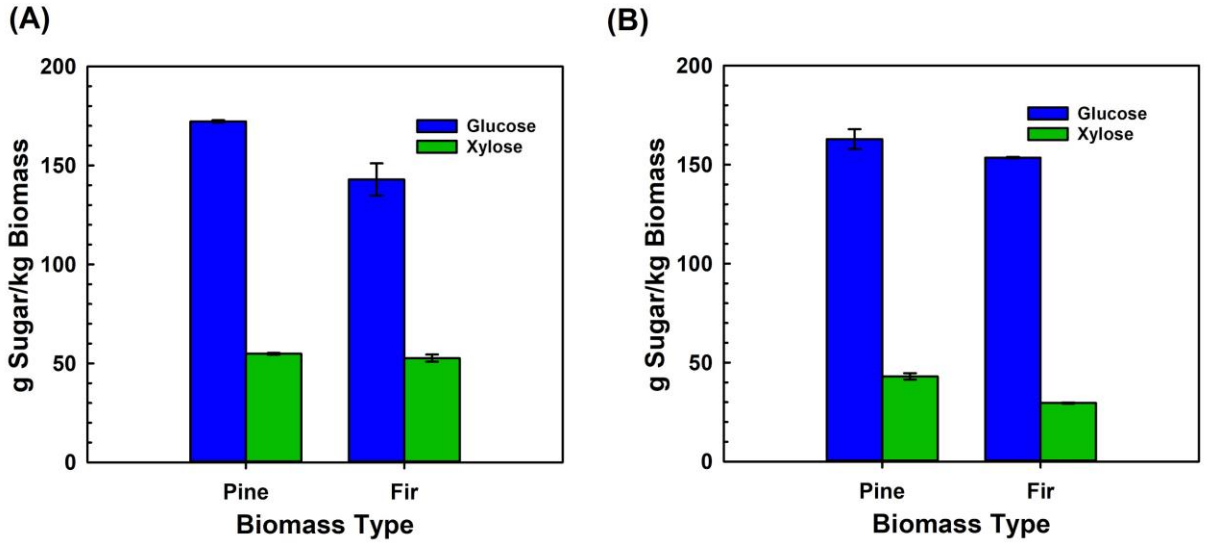
Source: Lawrence Berkeley National Laboratory

Impact of Particle Size

Biomass particle size substantially affects pretreatment and subsequent enzymatic hydrolysis. Particle size reduction enhances the effective surface area to volume ratio, facilitating enzyme accessibility into the active sites of substrate. Therefore, the researchers reduced particle size for pine and fir to 50 µm to improve the amount of fermentable sugars released. Figure 11A shows the glucose and xylose yields from the enzymatic hydrolysis of [Ch][Lys] pretreated biomass at 50 µm, 160 °C, 3 h, using 30 mg of enzyme protein/ g of starting biomass at a ratio of 9/1 Ctec3/HTec3. Results show a significant increase of total sugar yield for pine (172.3 g/kg of dry biomass) and fir (142.9 g/kg of dry biomass), which accounts for an increase of 62 percent for pine and 48 percent for fir compared to results under similar conditions at 2mm particle size. The glucose yield for pine was 172.3 g/kg of dry biomass (59 percent increase), while xylose yield saw a significant increase of 74 percent (54.9 g/kg of dry biomass). The research team also noticed similar increasing trends for fir pretreated biomass, with glucose yield of 142.9 g/kg of dry biomass (45 percent) and xylose yield of 52.7 g/kg of dry biomass (56 percent). For the [EOA][OAc] pretreated biomass, the results show that glucose yield for pine and fir, were 162.9, 153.6 g/kg of dry biomass. Considering the factor of enzyme ratio, this represents a 71 percent and 73 percent increase in glucose yield for pine

and fir, respectively. Similarly, the results show that xylose yield for pine and fir, were 43.1, 29.6 g/kg of dry biomass, which represents a 115 percent and 26 percent increase in xylose yield for pine and fir, respectively. Overall, the decrease in particle size was effective at further deconstructing the biomass and reducing recalcitrance, thereby improving the sugar yields. The reduction in particle size improves mass transfer and allows the ILs to penetrate the biomass' cell wall for easier lignin extraction. Nevertheless, biomass comminution is an energy intensive strategy that is not worth exploring. Instead, the researchers anticipate an improvement in yield (based on mass transfer) when a stirred tank reactor is used versus a stagnant reactor without agitation. Lastly, the results show that pine more than fir proved to be more amenable to process improvements.

Figure 11: Glucose and Xylose Yields from Enzymatic Hydrolysis of Samples with 50 µm particle size at 160 °C, 3 h, and 30 mg protein/g biomass of Enzyme



(A) [Ch][Lys]; (B) [EOA][OAc] pretreated samples.

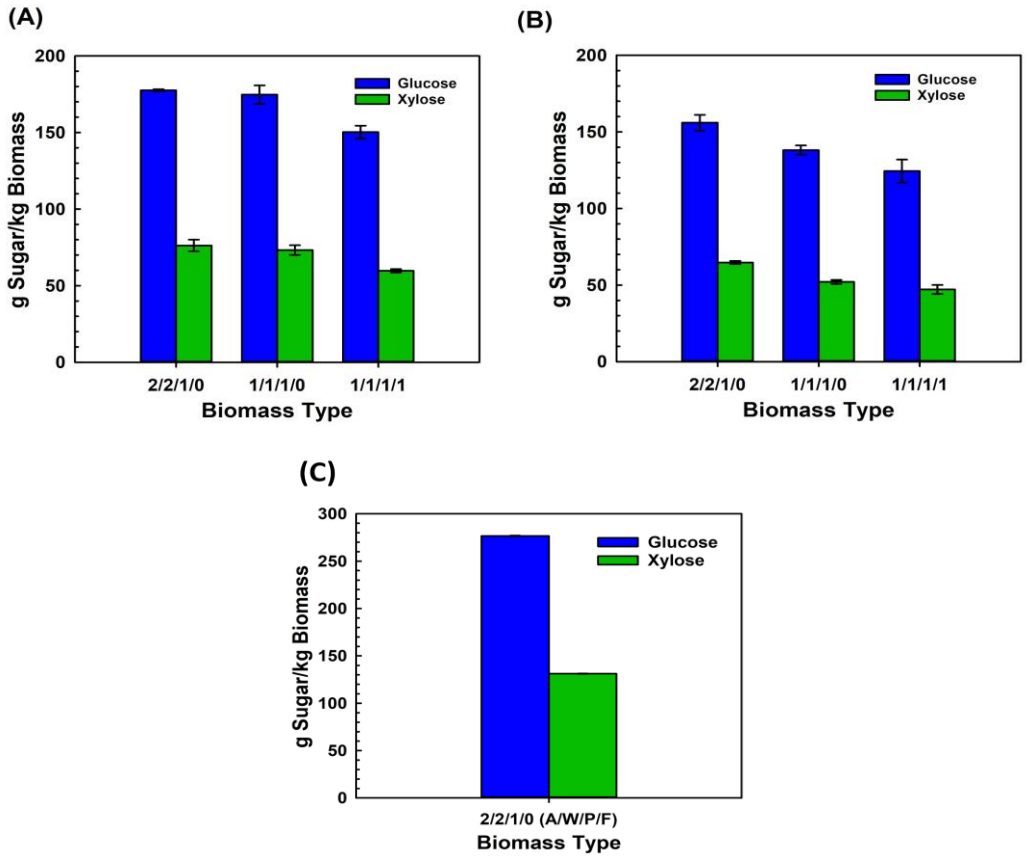
Source: Lawrence Berkeley National Laboratory

Mixed Biomass and Intermediate Scale-Up

Effective and efficient functioning of a biorefinery to maintain productivity and profitability hinges on the ability to convert readily available and affordable feedstocks. The available feedstocks in a biorefinery will most likely be diverse and mixed composition, which will vary as a function of time and price. Hence, it is highly desirable for the biorefineries to process mixed biomass feedstocks with minimal adverse impact on overall performance including sugar and fuel titers. In this study, researchers studied three different mixed biomass by varying the weight fraction of almond (A), walnut (W), pine (P) and fir (F). Figure 12A shows the effect of mixed biomass on glucose and xylose yields from the enzymatic hydrolysis of [Ch][Lys] pretreated samples under optimal conditions. The glucose and xylose yields for a mixture containing equal fractions of biomass types (1/1/1/1) of the four biomasses were 150.3 and 59.7 g/kg of dry biomass, respectively. Interestingly, when fir was eliminated for the mixture and keeping equal fractions (1/1/1/0) for almond, pine, and walnut, a significant increase in glucose (174.8 g/kg of dry biomass) and xylose (73.3 g/kg of dry biomass) yields were obtained. However, further changing the mixture comprising 20 percent pine and 40 percent

of both almond and walnut saw little or no change in glucose (177.6 g/kg of dry biomass) and xylose (76.3 g/kg of dry biomass) yield. For the [EOA][OAc] pretreated biomass (Figure 12B), the results show that glucose yield for mixed biomass were 156.0, 138.2, 124.5 g/kg of dry biomass for 2/2/1/0, and 1/1/1/0 and 1/1/1/1 A/W/P/F. Similarly, the results show that xylose yield was 64.8, 52.1, 47.1 g/kg of dry biomass. Although, the overall sugars released were ~12 percent less than the expected amount (based on the ratios for the pure biomass streams). This is likely due to the heterogeneity that occurs when mixing biomass as large particles. Nevertheless, once this slight reduction has been accounted for, the response was very close to that of a linear correlation between biomass ratios and sugar yields.

Figure 12: Glucose and Xylose Yields from Enzymatic Hydrolysis of 2 mm particle sized mixed biomass (Almond/Walnut/Pine/Fir) at 160 ˚C, 3 h, and 30 mg protein/g biomass of Enzyme



(A) [Ch][Lys]; (B) [EOA][OAc] pretreated samples; (C) larger scale Parr reactor (30g-scale) via [Ch][Lys].

Source: Lawrence Berkeley National Laboratory

Combined results show that both ILs were effective in pretreating biomass mixture of almond, walnut, pine, and fir. Under optimal pretreatment conditions, biomass mixture of 20 percent pine and 40 percent of both almond and walnut resulted in highest fermentable sugar release. At this mixture total sugar yield for [Ch][Lys] and [EOA][OAc] were 253.8 and 299.6 g/kg of dry biomass, respectively. Despite higher sugar yields from [EOA][OAc], researchers carried out further process scale-up using [Ch][Lys] because of its biocompatible nature, which provides a platform to consolidate processes into the one-pot system. Figure 12C demonstrates the 30 g (mixed biomass) scale experiment (under optimal condition) using

[Ch][Lys]. Total sugar yield of 407.9 g/kg of dry biomass was obtained, which accounts for 60 percent increase compared to small scale (Figure 12A). Glucose and xylose yield for the mixed biomass were 276.7 g/kg of dry biomass (55 percent increase) and 131.2 g/kg of dry biomass (72 percent increase), respectively. This observed increase in fermentable sugar yields is attributed to better mixing of the biomass in the reactor vessel leading to improved mass transfer.

Scale-Up of Ionic Liquid Pretreatment and Enzymatic Saccharification

Biomass Procurement for Scale-Up Evaluation

The research team obtained three types of California woody biomass from Paddock Inc. in Oakdale, California to assess potential in large-scale deconstruction and fermentation studies (Figure 13). Almond and walnut wood waste was procured from local orchards, with pine wood obtained from forest thinning. Each of the three biomass types was broken to mulch with a commercial wood chipper and subsequently milled to 5mm particle size prior to pretreatment (Figure 13). Biomass was pretreated without further drying.

Figure 13: Biomass collection in Oakdale, California

Final milled biomass comparison for walnut (bottom left), pine (bottom center), and almond (bottom right).

Source: Lawrence Berkeley National Laboratory

Table 2 summarizes compositional analysis of dry biomass. Following successful results with mixed biomass feedstocks during bench-scale process development, researchers used a mixture of 40 percent almond, 40 percent walnut, and 20 percent pine by dry weight for subsequent scale-up experiments.

Table 2: Compositional Analysis of Three California Woody Biomass Types Evaluated in this Study

Composition (%)	Almond	Walnut	Pine
Extractives	15.94 ± 0.30	12.42 ± 1.10	7.12 ± 0.34
Glucan	38.54 ± 0.311	26.29 ± 0.704	29.82 ± 0.566
Xylan	16.056 ± 0.115	9.921 ± 0.268	13.48 ± 0.281
Klason Lignin	21.63 ± 0,145	17.63 ± 0.951	24.74 ± 0.072
Ash	8.13 ± 1.207	19.23 ± 2.304	0.0 ± 0.07

Source: Lawrence Berkeley National Laboratory

Initial Scale-Up Results

Following bench-scale pretreatment optimization, the research team tested scale-up of the ionic liquid pretreatment process in both 8L and 210L pretreatment vessels at the USDOE Advanced Biofuels and Bioproducts Process Development Unit (ABPDU) at LBNL (Figure 14). The 210L vessel is designed with large ports and a helical impeller to enable effective mixing at up to 25 percent dry solids loading. Three pretreatment conditions were tested in 75kg batches — 19 percent, 22 percent, and 25 percent solids loading — with run conditions and sugar yields summarized in Table 3.

As expected, sugar concentrations following saccharification were highest for the highest solid loading condition, resulting in a maximum of 57.2 g/L glucose and 31.9 g/L xylose in the final hydrolysate. Higher sugar concentrations came at the expense of glucan conversion, which declined from 76.9 percent at 19 percent solids loading to 71.8 percent at 25 percent solids loading. This decline in saccharification efficiency is likely due to reduced mixing effectiveness in the highest solids loading condition, resulting in a dead zone for mixing at the base of the vessel. Xylan conversion was near complete, exceeding 97 percent in each of the three conditions. Overall pretreatment efficiency was highest at 19 percent solids loading, at 82.8 percent combined glucan and xylan conversion. For each of the three conditions, xylan conversion was near-complete after 24 hours of saccharification, while glucan conversion improved marginally from hours 24 to 72 (Figure 14).

Due to the higher efficiency at 19 percent solids loading, materials handling challenges pumping unfiltered hydrolysate, and toxicity effects during fermentation at 25 percent solids loading (Figure 15), the researchers opted to move forward with 19 percent dry solids loading for the remainder of the scale-up work to ensure a successful and efficient fermentation campaign. In the future, these materials handling and toxicity challenges could be addressed with custom mixing and pumping equipment during commercial scale-up, potentially resulting in higher sugar concentrations and proportionally higher ethanol titers. To evaluate larger-scale fermentation in the 680L pilot campaign, researchers prepared a composite batch of unfiltered hydrolysate by aggregating multiple Andritz campaigns, with the final sugar concentrations in the composite hydrolysate approximating the 19 percent solids loading condition.

Table 3: Pretreatment and Saccharification Conditions and Efficiencies in the 210L Andritz Pretreatment Vessel at Advanced Biofuels and Bioproducts Process Development Unit

Pretreatment condition	19% dry solids	22% dry solids	25% dry solids
Working volume (kg)	75	75	75
Pretreatment time (min)	180	180	180
Pretreatment temp (°C)	160	160	160
Saccharification time (hrs)	72	72	72
Saccharification temp (°C)	50	50	50
IL loading (%)	10%	10%	10%
Solids loading (%)	19%	22%	25%
Enzyme loading (mg/g biomass)	30	30	30
Pine (%)	20%	20%	20%
Walnut (%)	40%	40%	40%
Almond (%)	40%	40%	40%
Final glucose (g/L)	47.0	51.4	57.2
Final xylose (g/L)	23.7	28.4	31.9
Total glucose + xylose (g/L)	70.7	79.8	89.1
Glucan conversion (%)	77.7%	73.6%	71.8%
Xylan conversion (%)	95.5%	98.0%	97.6%
Combined glucan+xylan conversion (%)	83.0%	80.7%	79.3%

19 percent solids loading condition represents the average of three campaigns. This condition was used as the basis for fermentation process optimization in shake flasks and for the 600L fermentation campaign.

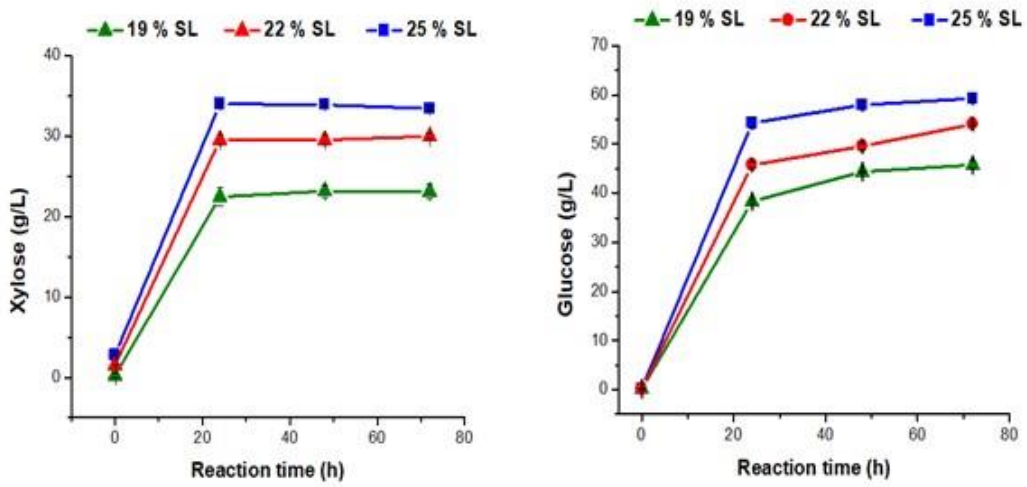
Source: Lawrence Berkeley National Laboratory

Figure 14: Vessels Used for Pretreatment Process Optimization at Scale

The 8L (left), and the 210L Andritz vessel used for scale-up of pretreatment and saccharification (right). This vessel has a 140L working volume and is equipped with a helical impeller for high solids mixing.

Source: Lawrence Berkeley National Laboratory

Figure 15: Glucose and Xylose Release During Enzymatic Saccharification in the 210L Andritz vessel at 19 percent, 22 percent, and 25 percent Solids Loading



Source: Lawrence Berkeley National Laboratory

Scale-Up at Aemetis

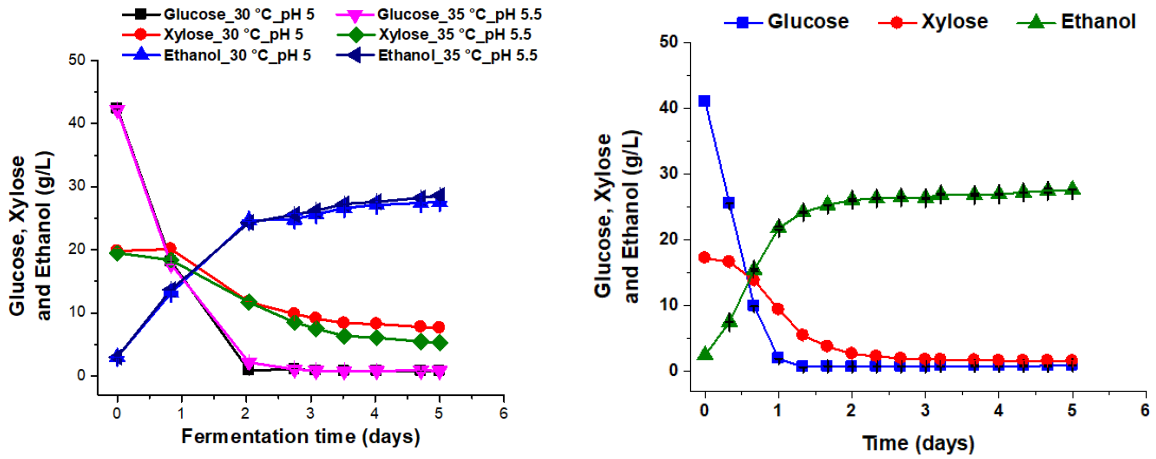
Following fermentation optimization in shake flasks, the researchers executed technology transfer with Aemetis to evaluate the process at pilot scale. Initial technology transfer was performed at 5L scale (Figure 16). Fermentations at 5L-scale demonstrated complete glucose consumption after two days, with 70-80 percent xylose consumption after 5 days of fermentation. Troubleshooting after the 5L campaign revealed two potential inhibitory factors – harsh pasteurization conditions for the whole slurry hydrolysate at 5L scale resulted in formation of toxic byproducts, and more rapid growth of the seed 2 culture in 5L experiments resulted in inoculation with stationary phase yeast. For the subsequent pilot scale fermentation pasteurization hold time was reduced to 5 minutes, and the seed 2 culture was intensively monitored to ensure inoculation in late-log phase growth.

Following the 5L technology transfer campaign, researchers demonstrated the process at 680L pilot scale in a 1,500L ABEC fermenter (Figure 16), for fermentation of a 680L batch of unfiltered hydrolysate produced over multiple deconstruction campaigns in the 210L Andritz pretreatment vessel at ABPDU. The seed train for this process included 7L shake flask cultivation in YPD media, followed by 65L bioreactor cultivation in an aerated 150L ABEC bioreactor, with an acclimatization media consisting of 50 percent YPD and 50 percent sterile-filtered hydrolysate. The seed culture was monitored for glucose and xylose depletion, and at 14 hours of cultivation time 65L of acclimated seed was inoculated into 615kg of unfiltered hydrolysate, for a total batch volume of 680L.

The research team encountered numerous logistical challenges during the scale-up process that will inform future commercialization efforts. Most notably, the high solids nature of the material challenged pumping and mixing capabilities at the pilot plant. Future plants will need to carefully account for material transport considerations to further intensify the solids loading of the process and prevent failure of pumps and agitators. In addition, silica particles from the biomass tended to settle at the bottom of the fermentation vessel, resulting in abrasion and subsequent failure of the agitator seal assembly. While the impact of the seal failure on the

fermentation was limited to accumulation of 50kg of clean steam condensate in the vessel, this vessel geometry should be avoided in future campaigns in favor of top-mounted agitation.

Figure 16: Sugar Consumption and Ethanol Production at 5L and 680L Scale



Glucose consumption, xylose consumption, and ethanol production in 5L bioreactor fermentations (left), as well as in the final fermentation, conducted with an initial volume of 680L unfiltered hydrolysate in a 1500L bioreactor (right).

Source: Lawrence Berkeley National Laboratory

Figure 17: Pilot Scale Evaluation of the Ethanol Conversion Process

Pilot scale evaluation included seed 1 cultivation in shake flasks (left), seed 2 cultivation in a 150L aerated bioreactor (center), and the final 680L ethanol fermentation in a 1,500L agitated bioreactor (right).

Source: Lawrence Berkeley National Laboratory

With the seed train and pasteurization improvements introduced following the 5L fermentation, the pilot scale campaign achieved near complete glucose utilization after 24 hours, with >90 percent xylose utilization after 72 hours (Figure 16). The fermentation achieved a final titer of 27.6 g/L after 120 hours, with a fermentation efficiency of 93.3 percent as a function of the initial glucose and xylose present in the fermenter. When coupled to the deconstruction efficiency of 83.0 percent, this result yields an aggregate deconstruction and fermentation efficiency of 77.4 percent (Table 4).

Table 4: Final Deconstruction and Fermentation Efficiencies from Deconstruction Runs at 75kg Working Volumes and Fermentation at 680L Working Volume

Deconstruction and Fermentation Efficiencies	Value
Working volume per batch (kg)	680
Solids loading (%)	19%
Pine (%)	20%
Walnut (%)	40%
Almond (%)	40%
Final glucose (g/L)	47.0
Final xylose (g/L)	23.7
Total glucose + xylose (g/L)	70.7
Glucan conversion efficiency (%)	77.7%
Xylan conversion efficiency (%)	95.5%
Combined glucan + xylan conversion efficiency (%)	83.0%
Fermentation ethanol titer (g/L)	27.7
Fermentation glucose consumption (%)	97.7%
Fermentation xylose consumption (%)	90.9%
Fermentation efficiency: C5 + C6 conversion to ethanol (%)	93.3%
Combined carbon efficiency: Deconstruction and fermentation (%)	77.4%

Source: Lawrence Berkeley National Laboratory

Strain Engineering for Isoprenol Production

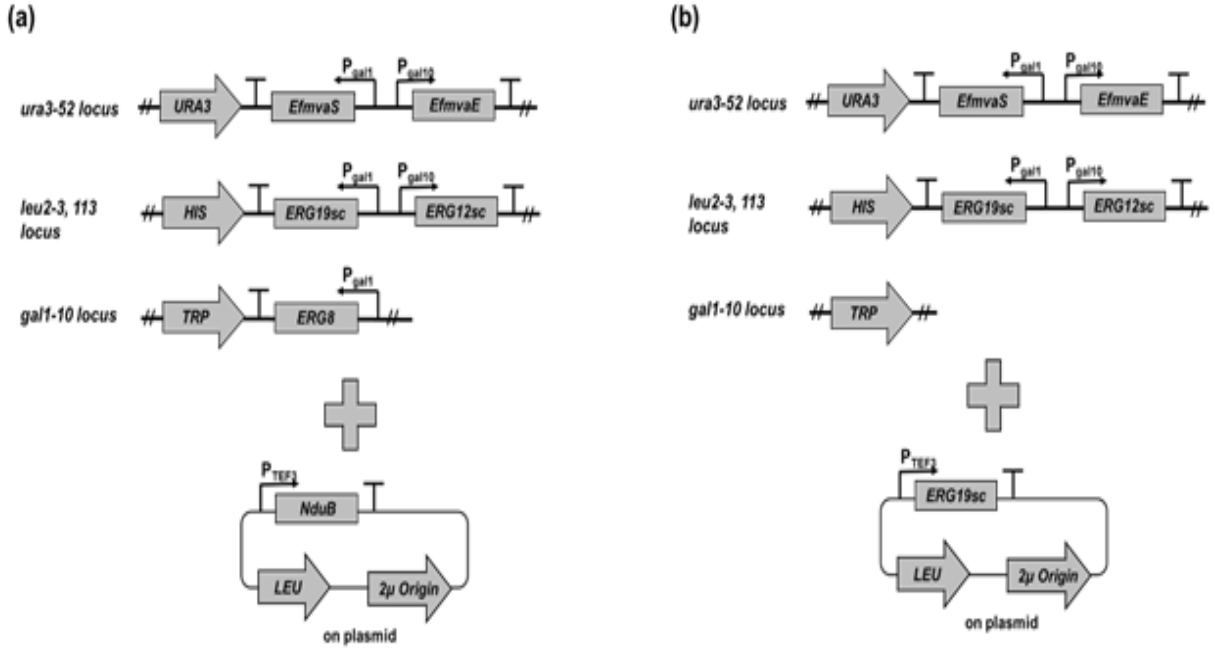
Design of the Isoprenol Pathway using the Original Mevalonate (MVA) Pathway

To produce isoprenol in *S. cerevisiae*, the research team first conceived the pathway using the endogenous MVA pathway. To improve accumulation of IPP in the cytosol for isoprenol production, researchers employed truncated HMG-CoA reductase (HMGR) and downregulated the expression of ERG20, farnesyl pyrophosphate synthetase that consumes IPP and DMAPP. In that work, however, isoprenol was produced only at a very low titer (40 µg/10 mL weight cell volume) from the engineered *S. cerevisiae*, and they explained this low titer is due to the toxicity of isopentenols, including isoprenol and prenol, by disrupting cell membranes rapidly.

In the first strategy for isoprenol production in yeast, the research team also designed the pathway for isoprenol production in *S. cerevisiae* via the original MVA pathway (Figure 18). The IPP is an important metabolite in *S. cerevisiae* as it is used for the synthesis of sterols, ubiquinone, dolichols, and isoprenoids, but researchers assumed that the cytosolic IPP level may not be high enough for high-level production of isoprenol. To increase IPP pools, the team overexpressed MVA pathway enzymes by adding more copies of pathway genes either native or from different species. Researchers integrated 5 genes (*EfmvaE* and *EfmvaS* from *Enterococcus faecalis*, and *ERG8sc*, *ERG12sc* and *ERG19sc* from *S. cerevisiae*) on the host genome (Figure 18a). Instead of using NudF for the hydrolysis of IPP to isoprenol, the team

used two step hydrolysis by a promiscuous phosphatases NudB and AphA from *E. coli*. Promiscuous phosphatase NudB was expressed on a 2-micron high-copy plasmid under a TEF promoter.

Figure 18: Construction of Isoprenol Production Pathway



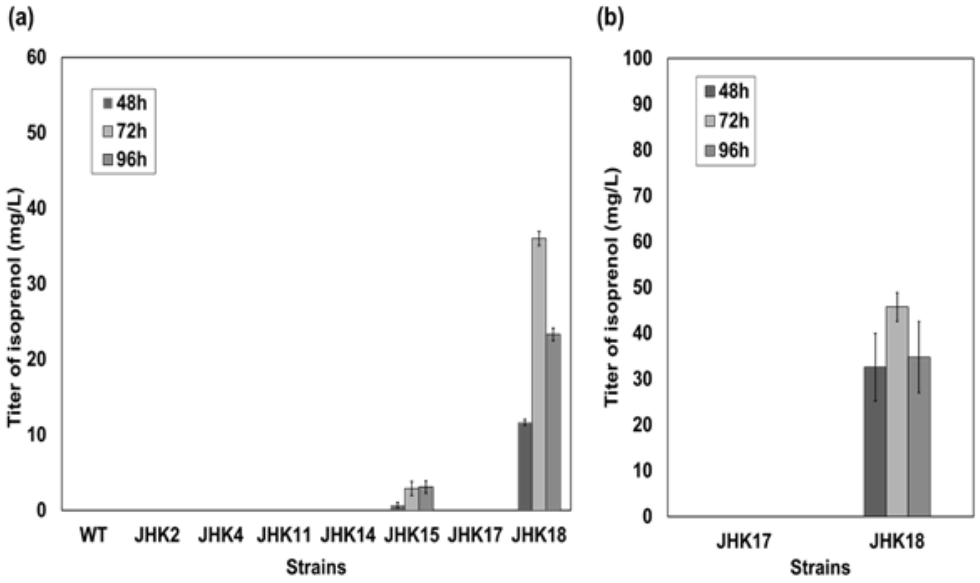
(a) To construct the original MVA pathway, the 5 MVA pathway genes (*EfmvaE*, *EfmvaS*, *ERG8sc*, *ERG12sc,* and *ERG19sc*) were integrated on the genome under the control of galactose promoters. NudB was expressed on the high-copy plasmid under the control of TEF3 promoter. (b) To construct the IPP-bypass pathway, the 4 pathway genes (*EfmvaE*, *EfmvaS*, *ERG12sc*, and *ERG19sc*) were integrated on the genome under the control of galactose promoters. The *ERG19sc* (or PMD) was expressed on the high-copy plasmid under the control TEF3 promoter.

Source: Lawrence Berkeley National Laboratory

Initial Assessment of Isoprenol Production with MVA Pathway

The wild type strain did not produce isoprenol in the YPD medium at any detectable level regardless of NudB overexpression on plasmid (WT and JHK2 in Figure 19a). The strain (JHK11) that overexpresses the top portion of the MVA pathway from *Enterococcus faecalis* (*EfmvaE* and *EfmvaS*) and NudB did not produce isoprenol in the YPD either. This result indicated that the IPP is not produced enough to produce isoprenol just by overexpression of the top portion. Two more genes (*ERG12sc* and *ERG19sc*) were overexpressed from the genome, and this strain (JHK15) started to produce isoprenol but the titer was still very low (less than 4 mg/L) (Figure 19a). When all 5 genes of the MVA pathway were integrated on the genome, this strain (JHK18) produced 36 mg/L of isoprenol which was the highest isoprenol titer for the original MVA pathway achieved in the YPD medium (Figure 19a).

Figure 19: Isoprenol Production via the Original MVA Pathway



(a) Isoprenol titers from the strains in the YPD medium supplemented with 2 percent glucose and 2 percent galactose (b) Isoprenol titer from two strains (JHK17 and JHK18) in the Delft medium supplemented with 2 percent glucose and 2 percent galactose. Error bars represent one standard deviation from the biological replicates. WT, wild type; JHK2, wild type with *NudB* expression on the plasmid; JHK4, 2 genes (*EfmvaE* and *EfmvaS*) integrated on the genome with pRS425; JHK11, integration of 2 genes (*EfmvaE* and *EfmvaS*) on the genome and expression of *NudB* on the plasmid; JHK14, integration of 4 genes (*EfmvaE*, *EfmvaS*, *ERG12sc*, and *ERG19sc*) on the genome with pRS425; JHK15, integration of 4 genes (*EfmvaE*, *EfmvaS*, *ERG12sc*, and *ERG19sc*) on the genome and expression of *NudB* on the plasmid; JHK17, integration of 5 genes (*EfmvaE*, *EfmvaS*, *ERG8sc*, *ERG12sc*, and *ERG19sc*) on the genome with pRS425; JHK18, integration of 5 genes (*EfmvaE*, *EfmvaS*, *ERG8sc*, *ERG12sc*, and *ERG19sc*) on the genome and expression of *NudB* on the plasmid.

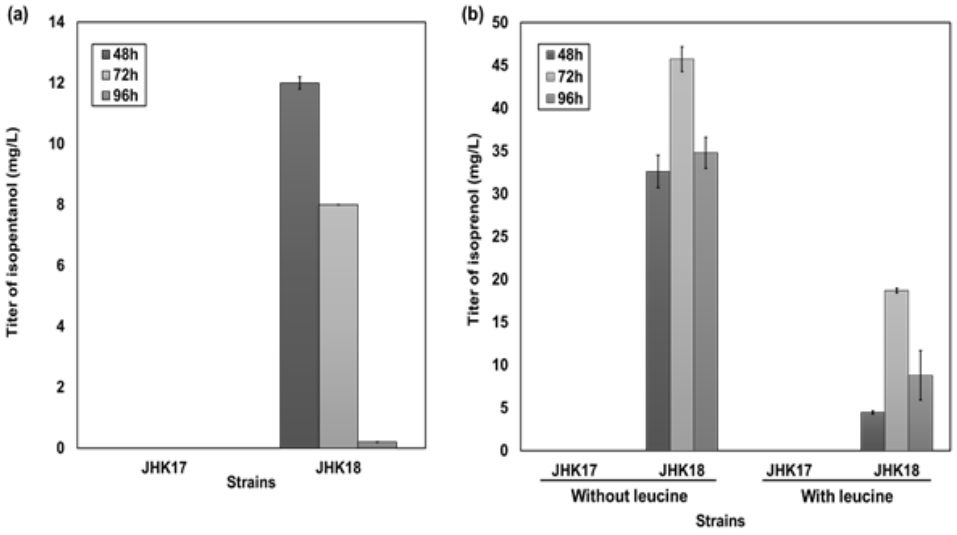
Source: Lawrence Berkeley National Laboratory

As this strain includes endogenous IPP isomerase (IDI) that can produce DMAPP from IPP, the researchers expected that this strain could also produce prenol, an isomer of isoprenol produced from DMAPP. But interestingly, the research team did not detect prenol and could suggest the following explanation why prenol was not produced. First, the cytosolic DMAPP level may not be high enough for the production of prenol in a detectable level. It is reasonable as the ratio of IPP and DMAPP is regulated by IDI and in nature the ratio is maintained to much more IPP than DMAPP. Second, even though a small amount of prenol was produced, prenol could be converted to isopentanol by promiscuous reductases on *S. cerevisiae*.

To confirm isopentanol production from DMAPP, the researchers cultured the best isoprenol producing strain (JHK18) in the YPD in a minimal medium (Delft medium). A minimal medium was used because leucine, which exists in the YPD (from the yeast extract), can be converted to isopentanol via the Ehrlich pathway. As predicted, the strain JHK18 produced 12 mg/L of isopentanol from the Delft medium while the control strains (JHK17) did not produce any isopentanol (Figure 20a). This result supports the hypothesis that a small amount of prenol that was produced from DMAPP could be converted to isopentanol by unknown promiscuous reductases.

Interestingly, the isoprenol production increased in the Delft medium compared to the production titer from the YPD medium. The strain JHK18 produced 45 mg/L isoprenol in the Delft medium at 72 hr (Figure 20b). To explain a lower titer in the YPD medium compared to the titer in the Delft medium, the research team hypothesized that branch-chain amino acids such as leucine, isoleucine, and valine in the YPD medium may inhibit endogenous alkaline phosphatases and negatively affect the hydrolysis of IPP. There are several literatures that report the effect of branched-chain amino acids on the alkaline phosphatases activity in a mammalian system even though the mechanism of the leucine effect in *S. cerevisiae* has not been verified. To test the leucine effect in yeast, researchers cultured the cell in the Delft medium supplemented with 1 g/L leucine. With excess leucine the strain JHK18 produced 18 mg/L of isoprenol, a 2.5-fold decreased isoprenol production (Figure 20b), and this result supports the hypothesis that the excess leucine from yeast extract may inhibit isoprenol production in the YPD. As the research team confirmed this leucine effect in *S. cerevisiae*, they decided to use the Delft medium in all the isoprenol production work in *S. cerevisiae*.

Figure 20: Isopentanol Production and the Leucine effect for Isoprenol Production using Delft Medium



(a) isopentanol production of the original MVA pathway strains (b) the leucine effect for isoprenol production via the original MVA pathway. The strains were cultured in the 250 mL flask supplemented with 2 percent glucose and 2 percent galactose either with 1 g/L leucine or without leucine. Error bars represent one standard deviation from three biological replicates.

Source: Lawrence Berkeley National Laboratory

Design of the IPP-Bypass Pathway for Isoprenol Production

As the original MVA pathway strain (JHK18) produced only low titer of isoprenol, the researchers also designed a recently reported an IPP-bypass pathway (IBP), to improve isoprenol production. The IBP is designed based on the promiscuous activity of PMD (ERG19) toward the nonnative substrate MVAP and can save one ATP and one enzyme (PMK or ERG8) for isoprenol production compared to the original MVA pathway.

To construct the IBP in *S. cerevisiae*, the team integrated four MVA pathway genes (2 heterologous genes (*EfmvaE*, *EfmvaS*) from *Enterococcus faecalis* and 2 endogenous yeast genes (*ERG12sc* and *ERG19sc*)) on the yeast genome under galactose promoters, Gal1 and

Gal10 (Figure 3.13). As a promiscuous activity of ERG19sc is known to be rate-limiting in the *E. coli* IBP, the researchers overexpressed ERG19sc on a high copy 2-micron plasmid under a strong TEF3 promoter.

Because the IBP requires the accumulation of MVAP for the production of IP by ERG19sc, the researchers needed to either knock out or knock down the activity of the endogenous enzyme PMK (or ERG8sc) whose native substrate is MVAP. The ERG8sc, however, is an important gene in the endogenous MVA pathway and a ERG8sc-knockout strain would not be able to grow unless there is another route to provide IPP, a universal precursor of isoprenoids. Therefore, the team introduced an alternative route to provide IPP after knocking out *ERG8sc* in the genome using an archaeal IP kinase (IPK) from *Methanothermobacter thermautotrophicus* that has shown to phosphorylate IP to IPP in *E. coli*.

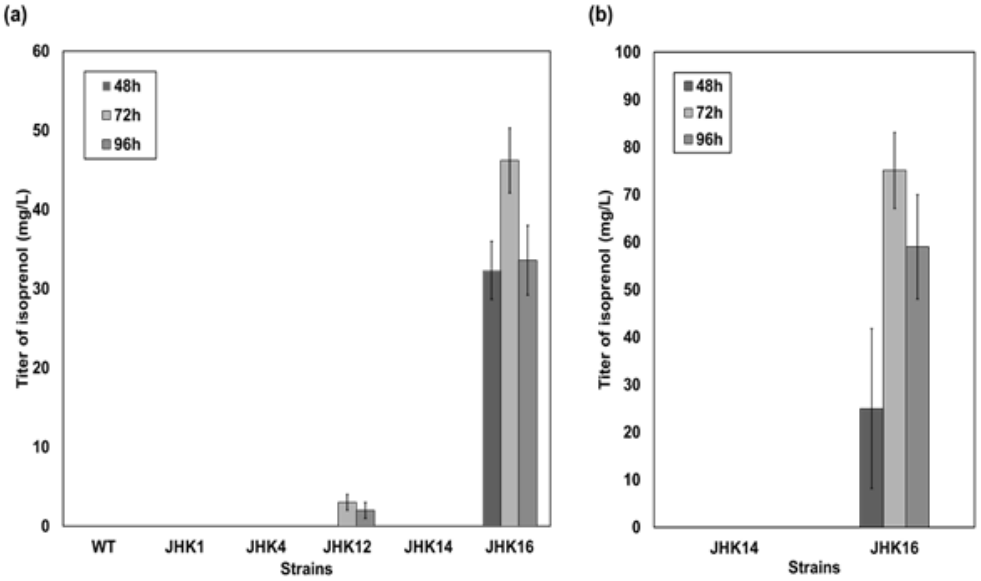
Initial Assessment of Isoprenol Production via IPP-Bypass Pathway

Based on the results in the previous section for the original pathway strains, the researchers cultured the IBP strain in both the YPD and the Delft medium. As shown in Figure 21, the wild type strain with ERG19sc overexpressed on plasmid (JHK1) did not produce any detectable level of isoprenol. When the top portion of the MVA pathway from *E. faecalis* (*EfmvaS* and *EfmvaE*) was overexpressed from the genome as well as ERG19sc on plasmid, this IBP strain (JHK12) started to produce isoprenol at less than 5 mg/L titer. When the other two pathway genes (*ERG12sc* and *ERG19sc*) were also integrated and overexpressed from the genome, this strain (JHK16) produced 45 mg/L of isoprenol in the YPD after 72 hour (Figure 21a) and 75 mg/L of isoprenol in the Delft medium after 72 hour (Figure 21b), which are 1.8-fold and 1.3-fold improvements over the titers from the original MVA pathway strain, respectively.

The IBP strain (JHK16) is more efficient in isoprenol production than the original MVA pathway strains probably because MVAP, a native substrate of ERG8sc (or PMK) and a new substrate of ERG19sc, is mostly used for the isoprenol production via the IPP-bypass pathway when ERG19sc was highly overexpressed. On the other hand, MVAP was mostly used for IPP production in the original MVA pathway as IPP is used for the synthesis of various isoprenoids and neutral lipids for the growth. It is noteworthy that the ERG19sc overexpression is enough for re-directing the flux from the native MVA pathway for IPP production to the IPP-bypass pathway for isoprenol production even with a valid MVA pathway as the research team did not knock-out or knock-down the original MVA pathway by deletion of *ERG8sc*.

With the confirmation that the IPP-bypass pathway works well and seems to be better than the original MVA pathway for isoprenol production in *S. cerevisiae*, the researchers continued to use the IBP strain for further engineering to improve isoprenol titer.

Figure 21: Isoprenol Production via the IPP-bypass Pathway



(a) Isoprenol titers in the YPD medium supplemented 2 percent glucose and 2 percent galactose. (b) Isoprenol titers of JHK19 and JHK20 in the Delft medium supplemented 2 percent glucose and 2 percent galactose. Error bars represent one standard deviation from three biological replicates. WT, wild type; JHK1, wild type with pRS425; JHK4, integration of 2 genes (EfmvaE and EfmvaS) on the genome with pRS425; JHK12, integration of 2 genes (EfmvaE and EfmvaS) on the genome with pERG19sc; JHK14 integration of 4 genes (EfmvaE, EfmvaS, ERG12sc, and ERG19sc) on the genome with pRS425; JHK16, integration of 4 genes (EfmvaE, EfmvaS, ERG12sc, and ERG19sc) on the genome with pERG19sc.

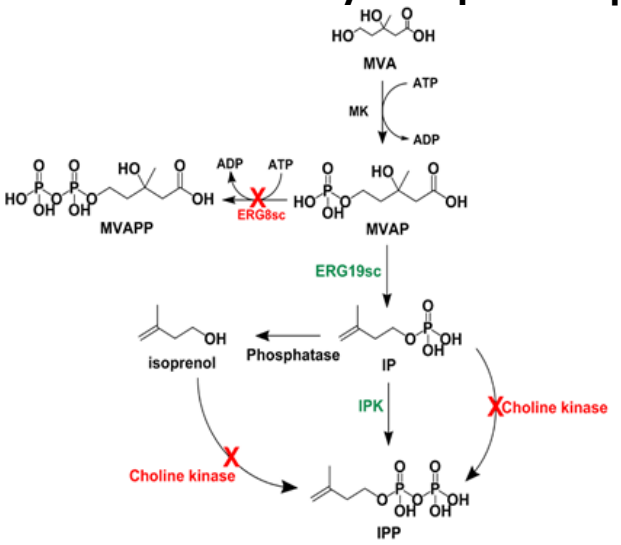
Source: Lawrence Berkeley National Laboratory

Knockout of 5-phosphomevalonate Kinase (*ERG8sc*) and Choline Kinase (*CK*)

To achieve a high level of the MVAP, a substrate of the rate-limiting enzyme (ERG19sc) in the IPP-bypass pathway, the researchers could either increase the MVAP formation by ERG12sc (mevalonate kinase) or decrease the MVAP consumption by ERG8sc (mevalonate phosphate kinase) in the MVA pathway. The team first attempted to decrease unwanted consumption of MVAP by knocking out *ERG8sc* in the IBP strain (Figure 22). As *ERG8sc* is an essential gene to produce IPP in *S. cerevisiae*, the *ERG8sc* knockout strain would not grow in the medium unless the researchers provided an alternate route to IPP. To supply IPP for growth in the *ERG8sc* knockout strain, the team employed IP kinase (IPK) from *Methanothermobacter thermautotrophicus* for phosphorylation of IP to IPP in the IPP-bypass pathway.

As expected, the *ERG8sc* knockout strain with heterologous *IPK* (JHK27) could grow in the Delft medium. However, this strain only produced isoprenol very little, as shown in Figure 23. Surprisingly, the researchers observed that the *ERG8sc* knockout strain (JHK26) also started to grow slowly even without IPK expression (Figure 23a). This observation suggested that there is a putative kinase that can phosphorylate either MVAP, IP, or even isoprenol to produce IPP in the *ERG8sc* knockout strain. The team constructed the *CK* knockout strain to prevent a potential conversion of IP and isoprenol to IPP. The isoprenol titer of 130 mg/L was achieved in the Delft medium for 72 hour by the *CK* knockout strain (JHK22), a twofold improvement over the control strain (JHK16) before knocking out choline kinase (Figure 23d).

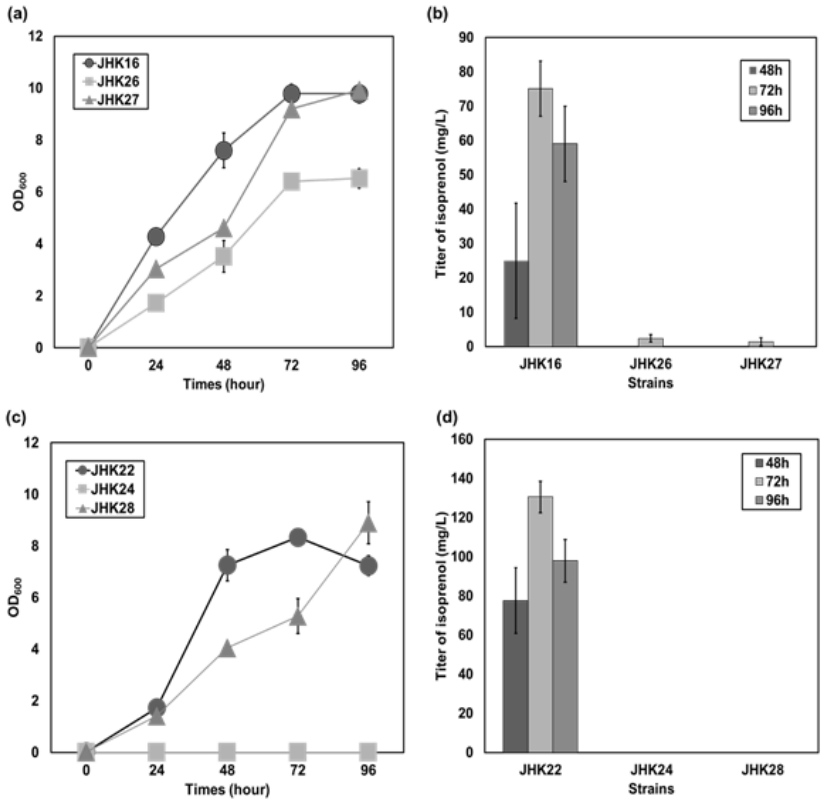
Figure 22: Modification of MVA Pathway to Improve Isoprenol Production



***ERG8sc* (*PMK*) was deleted and IP kinase (*IPK*) was integrated on the genome. ERG19sc was overexpressed on the plasmid. The choline kinase (*CK*) is deleted to improve isoprenol production.**

Source: Lawrence Berkeley National Laboratory

Figure 23: Effect of Deletion of *CK* and *ERG8sc* on Isoprenol Production

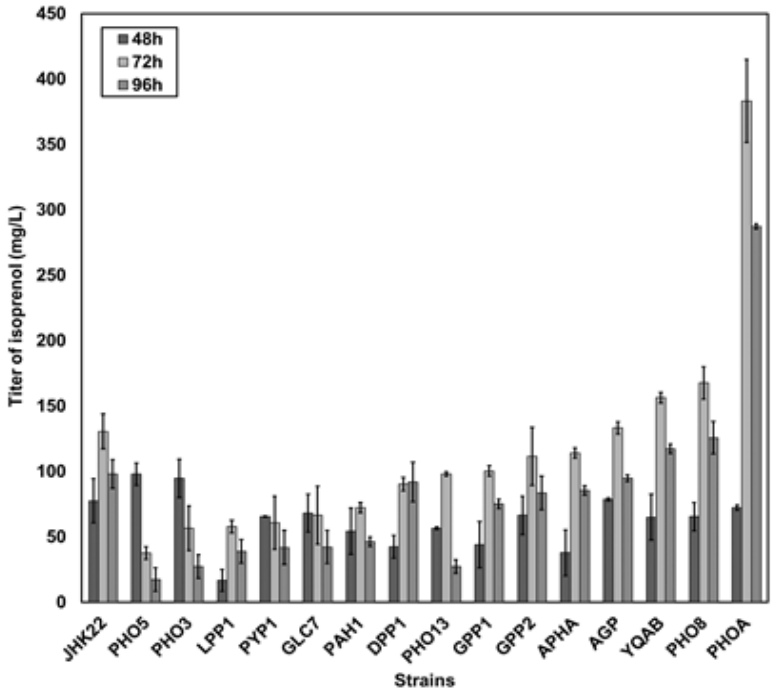


(a) The growth rate and (b) the isoprenol titer of three strains without *CK* deletion (control IBP strain (JHK16), Δ*ERG8sc* strain (JHK26), and Δ*ERG8sc* strain with *IPK* integration (JHK27)). (c) The growth rate and (d) the isoprenol titer of three strains that *CK* is deleted (control IBP strain (JHK22), Δ*ERG8sc* strain (JHK24), and Δ*ERG8sc* strain with *IPK* integration (JHK28)). The strains were cultured in the Delft medium supplemented with 2 percent glucose and 2 percent galactose at 30ºC and 200 rpm.

Source: Lawrence Berkeley National Laboratory

This result supports the hypothesis that the CK could phosphorylate IP and isoprenol to IPP in *S. cerevisiae*. As expected, the strain JHK24, in which both *CK* and *ERG8sc* were knocked out, did not grow either in the YPD (data not included) or in the Delft medium (Figure 23c). Also as expected, the double knockout strain with an *IPK* integration (JHK28) grew slowly with a long lag and log phase but it did not produce any isoprenol (Figure 23c and Figure 23d). This is probably due to a low efficiency of the IP conversion to IPP by IPK and the researchers concluded that the recovered production of IPP via modified MVA pathway (that is, Δ*CK* Δ*ERG8sc* with IPK expression) is probably enough for survival but not enough for isoprenol production as it seems to be difficult to control ratio of IP for isoprenol production and IPP for cell survival. Therefore, the team concluded to use the *CK* knockout strain without knocking out *ERG8sc* (JHK22) as the IBP background strain for further modification and engineering to produce isoprenol.

Figure 24: Isoprenol Production Via IPP-Bypass Pathway with Phosphatase



JHK22 is the control strain and all the other strains include each phosphatase on the plasmid in addition to the control strain.

Source: Lawrence Berkeley National Laboratory

Validation of Promiscuous Phosphatases for Isoprenol Production

Without overexpressing any additional phosphatase for IP hydrolysis, the CK knockout strain could still produce isoprenol using the endogenous phosphatases but the titers were still low. To improve IP hydrolysis in yeast isoprenol strain, the researchers tested 15 phosphatases from *E. coli* and *S. cerevisiae* which were previously reported for promiscuous phosphatase activities. Researchers achieved the most interesting result in the strain that overexpressed PhoA, an alkaline phosphatase from *E. coli*. When PhoA was overexpressed in the strain PHOA, the isoprenol titer increased significantly and reached 380 mg/L which is about 300 percent of that of the control (**Error! Reference source not found.**).

CHAPTER 4: Technology/Knowledge/Market Transfer Activities

Transfer of technology from this project includes intellectual property developed prior to the project and protocols developed under CEC funding. LBNL and Aemetis, Inc. are in negotiations to license three U.S. patents on background intellectual property, and close cooperation among the parties throughout this project facilitated transfer of operating protocols and conditions. Knowledge transfer to the broader scientific and business community is occurring through publications, conference presentations, and training of undergraduate and post-Doctoral students under this funding. Market transfer is occurring in collaboration with Aemetis, the industry partner for this project. Next steps in future research include further work to design, build, and demonstrate a pilot plant, leading to opportunities for Aemetis to commercialize a technology and engineering package to the industry--with focus on the approximately 220 corn grain ethanol plants currently operating nationwide.

Ionic Liquid Pretreatment

Substantial progress was made in this project on IL pretreatment of mixed-waste California woody biomass to enable conversion into biofuels. This is an important scientific achievement that is currently being disseminated through multiple peer-reviewed publications and conference presentations to the scientific and industrial community and commercialized by Aemetis.

Technology Transfer

JBEI has established patent portfolios on a wide range of IL-based pretreatment technologies. Selected patent applications relevant to processes demonstrated in this project are being optioned for license to Aemetis for commercialization, and include the following patents:

- LBNL reference 2014-139: Method to overcome the ionic liquid-cellulase pH mis-match for one-pot pretreatment and saccharification process enabling the use of biocompatible ionic liquids and commercial enzyme cocktails": U.S. application
- LBNL reference 2014-167: "Ammonium based Ionic Liquids for lignocellulosic processing": U.S. application
- LBNL reference 2015-143: "High Gravity, Fed-batch Ionic Liquid Based Process for Biomass to Fuels/Chemicals": U.S. application

This aspect of the project was largely a demonstration of technology that had previously been demonstrated at laboratory scale at JBEI, and as a result no new intellectual property was disclosed under this project. No new intellectual property will be included in the option agreement being negotiated. In addition to legal intellectual property, steps to transfer JBEI and ABPDU technology to Aemetis have been active throughout this project. Aemetis technical and business personnel have been included and engaged in biweekly progress calls throughout the duration of the project, and fermentation scientists from LBNL's ABPDU were on-site during the 670 liter fermentation.

Knowledge Transfer

Aemetis and JBEI/ABPDU scientists are engaging in multiple approaches to transfer knowledge from this project to the scientific and industrial community:

- Publications: JBEI, ABPDU, and Aemetis are preparing three manuscripts for publication in the scientific literature, including a planned publication on bench-scale pretreatment (including distillable ionic liquids), with proof-of-concept fermentation, and an additional planned publication on pretreatment scale-up, fermentation optimization, fermentation scale-up, and techno-economic analysis. The current plan is to publish these two together in the same journal to coordinate publicity around the publications.
- Conferences: Due to coronavirus-caused cancellations, conference presentations are not currently planned to present results of the pretreatment/fermentation, but submissions will be made once conferences are rescheduled. The Symposium on Biomaterials, Fuels and Chemicals (SBFC) will be evaluated as a good venue to present these results. Additional presentations at trade conferences with a focus on addressing the industrial community, such as the Advanced Bioeconomy Leadership Conference or BIO Impact, will also be considered. A media outreach will be conducted, concurrent with release of the manuscripts and the CEC final report. Internal presentations will be made at the JBEI Annual Meeting.
- Post-Docs/students trained: Four postdocs at JBEI and ABPDU and one intern at ABPDU in the Science Undergraduate Laboratory Internship (SULI) program participated in and received training under this project.

Market Transfer

Aemetis is evaluating opportunities to design, build, and operate an integrated pilot plant for extended periods as the major next step to bring this new pretreatment technology to market. Experimental next steps to design and build an effective pilot plant are outlined in Chapter 5 under "Next Steps/Recommendations". Certain of these steps are expected to be conducted in partnership between JBEI/ABPDU and Aemetis, Inc., and funding to accomplish these is being investigated. Aemetis will take the lead to obtain financing and coordinate design, construction, and operation of the pilot plant. An option agreement being negotiated between LBNL (representing JBEI and ABPDU) and Aemetis that will provide Aemetis with legal access to JBEI intellectual property to proceed with commercialization.

Upon successful operation of the pilot plant, Aemetis intends to commercialize the technology through internal use and by licensing the technology, engineering design packages, and operational assistance to the industry. With approximately 220 corn ethanol plants in the U.S., and with the ethanol industry eager to find new revenue and profit sources, there are highly leveraged opportunities to decrease the carbon intensity of the fuel supplies in California and the nation by better using woody waste feedstocks, in addition to adapting this technology to other lignocellulosic feedstocks.

Strain Engineering and Fermentation

This project achieved scientifically significant advances in strain engineering. First, the project demonstrated a new C6/C5 strain to produce high yields of ethanol on hydrolyzed woody waste biomass. Second, the project made substantial productivity advances in engineering the

isoprenol pathway into yeast, using sophisticated metabolic pathway engineering and multi-omics technologies. Fermentation at 670 liter working volume scale of a C6/C5 strain that produced high yields of cellulosic ethanol from woody waste hydrolysate without filtration made using ionic liquid pretreatment technology is a pioneering accomplishment of this project.

Technology Transfer

Prior to this project, JBEI had previously established patent portfolios on metabolic pathway engineering for isoprenol production. These patents remain available for license, and LBNL is in discussions for their commercialization across the industry. Fermentation technology demonstrated in this project is primarily established know-how and best practices through the establishment of standard operating procedures and protocols shared with Aemetis. There is no JBEI or LBNL intellectual property to license. No new intellectual property pertaining to fermentation was developed under this project.

Knowledge Transfer

- Publications: JBEI, ABPDU, and Aemetis have submitted two manuscripts for publication regarding use of the C6/C5 strain to convert woody waste hydrolysate to ethanol. JBEI scientists are preparing a manuscript on engineering the isoprenol pathway into S. cerevisiae.
- Conferences: Due to coronavirus-caused cancellations, conference presentations are not currently planned to present results of either ethanol production from hydrolysate with the C6/C5 yeast strain or the isoprenol pathway engineering. As described above, conference presentations will be evaluated once coronavirus meeting and travel restrictions are eased.
- Post-Docs/students trained: Three post-docs were trained under this funding.

Market Transfer

Novozymes is commercializing its C6/C5 yeast for broad applications to make ethanol, and this project's demonstration of lignocellulosic ethanol made from woody hydrolysates, coupled with Aemetis' plans to commercialize pre-treatment/fermentation technology demonstrated in this project, can make the combined technology package available broadly throughout the industry. In parallel, LBNL has an active program to develop isoprenol as an automotive fuel and as an intermediate to high-performance aviation fuels. The results from this program demonstrate productivity improvements for a new microbial host that can be grown on broadly available biomass feedstocks and will provide a high degree of leverage for introducing advanced biofuels.

CHAPTER 5:
Conclusions and Recommendations

CEC funding enabled a pioneering accomplishment of scientific and industrial significance in biomass conversion to biofuels through a public-private partnership between the LBNL, Sandia National Laboratories, and Aemetis. This project successfully used IL pretreatment technology to convert waste woody biomass to fermentable sugars in hydrolysate, converted hydrolysate into cellulosic ethanol with overall fermentation efficiency exceeding 90 percent, and achieved overall conversion efficiency from biomass to fuel of nearly 80 percent. The project successfully scaled-up from prior lab scale (2 L fermentation) to a 670 L industrial-level fermenter, validating the commercial feasibility of this technology. The team also engineered a yeast strain to make advanced automotive and aviation biofuels, which builds on this project's accomplishments to establish the foundation for a broad variety of advanced fuels made using the same feedstock and processing technologies. Paths forward to continue developing these biomass conversion approaches are identified, with a pilot plant being the next major step to commercializing new biofuels made from California's waste woody biomass.

Pretreatment

Over the course of this project, the research team has demonstrated an effective system for IL pretreatment of California woody biomass, followed by fermentation of C5 and C6 sugars to ethanol at high conversion efficiency and with no intermediate separations. Of the four biomass types tested, almond and walnut demonstrated superior deconstruction efficiency, resulting in the highest sugar yields. The recalcitrance of pine (and fir) to deconstruction was prominent (low sugar yields), but the research team found that this recalcitrance could be effectively mitigated by blending pine with almond and walnut. This mixed feedstock approach is well-suited to the diverse and often seasonal woody biomass feedstocks available in California and indicates potential for additional process improvements via further optimization of biomass blending characteristics.

The project used widely available commercial equipment to mill the waste biomass collected from farms and commercial forestry during scale-up of the process, indicating that the process demonstrated in this project is compatible with existing biomass collection infrastructure. Likewise, use of commercial cellulase and hemicellulase enzymes indicates compatibility with existing saccharification technologies. The researchers used enzymes for this project in a standard formulation, but the commercial supplier can generate tailored formulations to further increase the efficiency of enzymatic deconstruction of California woody biomass if this process were to be commercialized.

Following process optimization, the researchers demonstrated deconstruction efficiency of 83 percent for combined cellulose and hemicellulose, while validating the technology at scale exceeding 100 L. Integration with fermentation proved challenging due to inhibitors formed in pretreatment - the resulting toxicity was mitigated primarily via yeast acclimatization and adjustment of fermentation parameters, to achieve high sugar conversion at high pretreatment solids loading. Moving forward, further co-optimization of pretreatment and fermentation may enable selective reduction in inhibitors. Pretreatment can be optimized via

feedstock selection and control over pretreatment conditions. Fermentation can be optimized by adapting the yeast strain to the inhibitors using adaptive laboratory evolution. The combined approach will enable high efficiency biomass deconstruction and fermentation, pushing this technology closer to commercialization.

Biofuels

Cellulosic Ethanol

Fermentation of the hydrolysates resulting from the one-pot pretreatment and saccharification process required no additional separations to remove ILs, residual lignin, or inhibitors produced during biomass pretreatment. Elimination of separation steps reduces process complexity and eliminates intermediate losses in the overall process. The process was compatible with commercial yeast, engineered for xylose utilization, and required no additional nutrient inputs to achieve near-complete sugar utilization during ethanol fermentation. Yeast acclimatization in IL hydrolysate, cultivation temperature, and starting pH were identified as critical parameters for mitigation of hydrolysate toxicity. During scale-up of the process to 670L fermentation volume, the researchers demonstrated 98 percent glucose consumption, 91 percent xylose consumption, maximum ethanol titer of 27.7 g/L, and an overall fermentation efficiency of 93 percent. During process scale-up, the project team achieved 77 percent end-to-end efficiency for the process, from cellulose and hemicellulose in the raw feedstock to fermented ethanol, setting a strong benchmark for conversion of recalcitrant woody biomass to finished fuels. When taken as a whole, this process effectively expands the use of IL-based pretreatment to woody biomass and demonstrates the potential of this technology at pilot scale with commercially relevant feedstocks, processing equipment, saccharification enzymes, and yeast strains.

Moving forward, the researchers propose further scale-up of this technology to achieve a fully integrated demonstration-scale process, operating for longer periods at high efficiency and high intensity, and enabling enhanced exploration of feedstock effects under industrially relevant conditions. Validation at demonstration scale would enable use of fit-for-purpose equipment, including continuous flow pretreatment reactors better suited for high-solids mixing. Such reactors would enable hydrolysate production with higher sugar content, while the improved mixing could further improve process economics and decrease hydrolysate toxicity by reducing pretreatment hold times. To achieve ethanol fermentation at higher titers, strain improvement techniques could be employed to overcome hydrolysate toxicity, including tolerance engineering and adaptive laboratory evolution. Use of a dedicated facility would also allow exploration of simultaneous saccharification fermentation configurations, enabling further consolidation of unit operations, further increases in process intensity, and an associated reduction in overall process cost.

Isoprenol

Recently, isoprenol (3-methyl-3-butene-1-ol) has gained much interest from the biofuels and bioproducts sector. In this study, the researchers reported a successful metabolic engineering of *S. cerevisiae* for the production of isoprenol. Engineering of the original MVA pathway was first attempted for isoprenol production by overexpression of MVA pathway and promiscuous phosphatase NudB. The original pathway strain could produce about 45 mg/L of isoprenol in

the Delft medium, which is a significant improvement from the previously reported titer, but the titer was still not high enough for any further application probably due to the difficulty in IPP accumulation for isoprenol production as IPP is the key intermediate for various other isoprenoid production in yeast.

To overcome this limitation, the researchers developed the IPP-bypass pathway for isoprenol production by taking advantage of the promiscuous activity of ERG19 (PMD) toward the nonnative substrate MVAP, and the initial IPP-bypass pathway strain produced isoprenol at the titer of 75 mg/L in the Delft medium. The attempt to accumulate MVAP by knocking out ERG8 (PMK) was not successful, but the researchers identified a key endogenous kinase (choline kinase, CK) that negatively affects isoprenol production in yeast. Therefore, the team constructed the *CK* knockout strains and produced 130 mg/L isoprenol after 72 h. Using metabolomics data, researchers identified that the pathway bottleneck is the last step, a hydrolysis of IP to isoprenol, and to improve IP hydrolysis selected and tested 15 phosphatases that have been reported for promiscuous activity and found that the expression of PhoA from *E. coli* significantly improves isoprenol titer. The team achieved 380 mg/L of isoprenol titer using the *CK* knockout strain with ERG19 and PhoA overexpressed on plasmid. With further pathway optimization and host engineering for biomass hydrolysate utilization, yeast will be able to provide a valuable platform for isoprenol and related C5 compounds production in a commercially viable way.

Next Steps and Recommendations

This project demonstrated, for the first time and at a significant demonstration scale, the IL-based deconstruction of mixed woody waste biomass and its conversion to cellulosic ethanol. The project also established the first steps towards developing yeast as an effective microbial host to make isoprenol, which is a promising renewable automotive fuel candidate and key intermediate for high-performance aviation fuels. CEC funding for this project built a substantial foundation for continued development of renewable ethanol and automotive/ aviation fuels from waste woody biomass, thereby establishing opportunities to more productively use California's woody waste material, provide value to removing fire-prone deadwood from California's forests, mitigate climate change by reducing California's fuel carbon intensity, and delivering superior fuel products to California's consumers.

The most important recommended next step to implement these technologies at full commercial scale and realize these benefits for California is to build an integrated pilot plant and demonstrate continuous operations for 6-12 months in a commercial environment. The target feedstock volume for the pilot plant is one ton of sugar produced from waste woody biomass per day. The pilot plant would further fine-tune technologies to maintain and increase high efficiency conversion and evaluate feedstock effects under industrial conditions. Successful demonstration at pilot scale would then enable Aemetis, this project's corporate partner, to obtain financing to build a commercial-scale facility that would most likely be co-located at its existing ethanol plant in Keyes, California.

The pilot plant is the key next step for commercial implementation, and prior to its design, additional technology investigations should be undertaken to build the most effective pilot plant possible. These investigations include:

1. Feedstock handling and preparation: The researchers ground waste woody feedstocks to 5 mm for this project, and continued exploration is needed to explore the relationship between biomass particle size and system-level yields and productivity, while minimizing the amount of grinding and processing required to simplify operations and reduce operational costs while maintaining yields.
2. Pretreatment technology: Cholinium lysinate was used as the IL to make hydrolysate for the 670-liter fermentation of this project and is an excellent candidate for further scaling to the proposed pilot plant. However, a distillable ethanolamine acetate pretreatment technology was also evaluated at lab scale in the project and showed initial promise as well as potential advantages that could make it even more efficient for commercial scale pretreatment of woody biomass. It would be prudent to continue evaluation of the ethanolamine acetate technology and demonstrate it at larger scale at LBNL's ABPDU before deciding on the pilot plant operating technology and design. A comprehensive comparison and evaluation of these two options for commercial-scale pretreatment of woody biomass would guide continued commercialization to the most efficient commercial operations possible.
3. Continuous-flow pretreatment reactors: Hydrolysate for this project was made under batch conditions, but developing a continuous-flow pretreatment reactor would have multiple advantages — especially at commercial scale — including a) better high-solids mixing; b) hydrolysates with higher sugar content; c) improved process economics; d) simplified operations; and e) decreased hydrolysate toxicity to the microbial host, since pretreatment holding times would be reduced so there would be less opportunity for toxicity formation.
4. Process engineering of unit operations: Consolidating and streamlining unit operations would increase process intensity, simplify overall operations, reduce capital costs, and reduce fuel product manufacturing costs. Evaluating simultaneous saccharification and fermentation configurations represents a major opportunity to optimize process operations.
5. Strain improvement for cellulosic ethanol: The C6/C5 ethanol yeast used in this project is an important technology advance for increasing fuel yields on biomass carbon, since the yeast consumes and converts both six- and five-carbon sugars (from cellulose and hemicellulose), thereby addressing a major challenge for commercial feasibility. Continued optimization of the C6/C5 strain can include increasing tolerance to hydrolysate toxicity by tolerance engineering, longer term acclimatization, and/or adaptive laboratory evolution.
6. Strain improvement for isoprenol: Continued engineering of the yeast strain engineered with the isoprenol pathway has potential to enable high-performance, renewable automotive and jet fuel to be made from California's woody waste streams. Approaches to continue improving isoprenol productivity include continued pathway engineering (especially guided by multi-omics analysis and pathway modeling), engineering for hydrolysate toxicity tolerance, and potential transfer of the pathway into a C6/C5 yeast strain to increase biomass carbon utilization.
7. TEA/LCA analyses: Figorous techno-economic and life cycle analyses will better predict the economic value created by fuels made from waste woods, where the greatest gains

can be obtained from operational improvements, and compliance with California regulatory requirements and targeted reductions in fuel carbon intensity.

8. Additional feedstocks: Exploring pretreatment and hydrolysis of additional biomass feedstocks can expand opportunities for year-round operations of a waste-to-fuel facility. Evaluating additional feedstocks would comprise both technical evaluation of pretreatment and conversion to fuel, as well as feasibility of long-term supply agreements.

CHAPTER 6:
Benefits to Ratepayers

JBEI's vision is to convert lignocellulosic biomass into economically viable, carbon-neutral, specialty biofuels. JBEI's mission is to achieve this vision with cutting edge science and new technologies to provide energy crops - and processes to convert carbon in those crops into biofuels and bioproducts at competitive prices - while also reducing greenhouse gas emissions by 90 percent compared to petroleum-derived products. Informed by the research team's technoeconomic and lifecycle analysis, JBEI's ultimate goal is to provide technologies that, when fully realized and scaled in an integrated biomass-to-biofuels-and-bioproducts process, will enable the realization of:

- Gasoline, diesel, and jet fuel replacements at less than $4 per gallon without a bioproduct.
- Gasoline, diesel, and jet fuel replacements at around $2.50 per gallon when bioproducts are coproduced with the fuel.
- Drop-in, commodity bioproducts (production of a million tons per year or more) that can compete with the same petroleum-derived molecules and that reduce biofuel prices.
- Novel bioproducts that cannot be efficiently produced from petroleum, have desirable properties, and reduce biofuel selling prices.

Doing so will reduce the nation's dependence on fossil fuels, the amount of carbon added to the atmosphere, and contamination of the environment while providing the scientific tools and knowledge required to transform the bioenergy marketplace. In addition, JBEI's work will provide California with a valuable outlet for woody biomass wastes in the form of low-carbon biofuels and environmentally friendly production processes for various chemicals that would otherwise be produced from petroleum. Development of technologies such as those demonstrated in this project capable of producing advanced biofuels as drop-in replacements for current petroleum-derived transportation fuels will benefit California in a variety of ways including reducing carbon dioxide emissions into the atmosphere, providing technologies to support nascent company formation, and educating the next generation of the biotechnology workforce. Achieving these goals will help reduce the likelihood of major climate events such as droughts and rising sea levels, as well as improve air quality across the state. Successful implementation of JBEI technology in the marketplace will also overcome important economic factors that currently prevent widespread adoption of cellulosic biomass-derived biofuels, thus enabling small start-up companies to build competitive businesses without significant investment capital and catalyze partnerships between public and private entities. The results achieved in this project (>80 percent deconstruction efficiency, >90 percent fermentation efficiency and scale up, and >95 percent glucose and >90 percent xylose consumption) are a critical step in their commercial development and deployment.

Potential Quantitative Benefits to California

The results from this project will have the following quantitative benefits to California:

- Demonstration of an advanced biofuel pathway with the potential to reduce GHG emissions by greater than or equal to 70 percent, relative to petroleum.
- Demonstration of an advanced deconstruction technology with the potential to generate sugar yields of 90 percent from a wide range of California-relevant, nonfood woody biomass feedstocks.

California Impacted Market Segments

The market sectors that will be impacted by the technologies developed in this project include: light-duty vehicles, which will be impacted by the introduction of biomass-derived drop-in replacements for gasoline; medium- and heavy-duty vehicles, which will be impacted by the introduction of biomass-derived drop-in replacements for diesel; and aviation, which will be impacted by the introduction of biomass-derived drop-in replacements for jet fuel.

LIST OF ACRONYMS

Term	Definition
[Ch][Lys]	Cholinium Lysinate
[C_2C_1Im][OAc]	1-ethyl-3-methylimidazolium acetate
[EOA][OAc]	Ethanolammonium Acetate
µm	Micrometer
1H-NMR	Proton nuclear magnetic resonance spectroscopy
A	Almond
ABPDU	Advanced Biofuels and Bioproducts Process Development Unit
ACS	American Chemical Society
ATP	Adenosine triphosphate
Bp	Base pair
C	Centigrade
C6/C5	Glucose to xylose ratio
CK	Choline Kinase
Ctec3	Cellulase enzyme mixture
EH	Enzymatic Hydrolysis
DMAPP	dimethylallyl diphosphate
DMSO-d6	Dimethyl sulfoxide
DOE	US Department of Energy
F	Fir
g	gram
g/kg	Grams per kilogram
g/L	Grams per liter
g/L/hr	Grams per liter per hour
GRAS	Generally Recognized as Safe
H_2SO_4	Sulfuric Acid
HA	Homologous arm
HCl	Hydrogen Chloride
HmgR	3-hydroxy-3-methylglutaryl-coenzyme A reductase

Term	Definition
HMGR	HMG-CoA reductase
HMGS	HMG-CoA synthase
HPLC	High-pressure liquid chromatography
HTec3	Hemicellulase enzyme mixture
IBP	IPP-bypass Pathway
IDI	isopentenyl pyrophosphate isomerase
IL	Ionic Liquid
IP	isopentenyl monophosphate
IPK	Isopentenyl Kinase
IPP	Isopentenyl Diphosphate
JBEI	Joint BioEnergy Institute
Kg	Kilogram
LBNL	Lawrence Berkeley National Laboratory
LCFS	Low Carbon Fuel Standard
LEU	Leucine
Mg	milligram
Mg/g	Milligram per gram
Mg/kg	Milligrams per kilogram
MK	Mevalonate kinase
mm	Millimeter
MVA	Mevalonate
MVAP	Mevalonate diphosphate
MVAPP	Mevalonate pyrophosphate
NADH	Nicotinamide adenine dinucleotide (NAD) + hydrogen (H)
NREL	National Renewable Energy Laboratory
P	Pine
pH	Potential of hydrogen/power of hydrogen
PIL	Protic Ionic Liquid
PMD	Mevalonate pyrophosphate kinase

Term	Definition
PMK	Phosphomevalonate kinase
Rpm	Revolutions per minute
SBFC	Symposium on Biomaterials, Fuels and Chemicals
SF	Severity factors
SULI	Science Undergraduate Laboratory Internship
TEF	Translation elongation factor
TRP	Tryptophan
URA	Uracil
v/v	Volume/volume ratio
W	Walnut
w/v	Weight/volume ratio
w/w	Weight/weight ratio
wt	Weight
xg	Relative centrifugal force
YPD	Yeast extract peptone dextrose
µL	Microliter

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