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High-efficiency conversion of ionic liquid-pretreated woody biomass to ethanol at pilot scale

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ABSTRACT: With a diverse and widely distributed global resource base, woody biomass is a compelling organic feedstock for conversion to renewable liquid fuels. In California, woody biomass comprises the largest fraction of underutilized biomass available for biofuel production, but conversion to fuels is challenged both by recalcitrance to deconstruction and by toxicity towards downstream saccharification and fermentation due to organic acids and phenolic compounds generated during pre-treatment. In this study, we optimize pretreatment and scale-up of an integrated one-pot process for deconstruction of California woody biomass using the ionic liquid cholinium lysinate [Ch][Lys] as a pretreatment solvent. By evaluating the impact of solids loading, solids removal, yeast acclimatization, fermentation temperature, fermentation pH, and nutrient supplementation on final ethanol yields and titers, we achieve nearly full conversion of both glucose and xylose to ethanol with commercial C5-utilizing *Saccharomyces cerevisiae*. We then demonstrate process scalability in a 680 L pilot-scale fermentation, achieving >80 % deconstruction efficiency, >90 % fermentation efficiency, 27.7 g/L ethanol titer, and >80 % ethanol distillation efficiency from the IL-containing hydrolysate post-fermentation. This fully integrated process requires no intermediate separations and no intermediate detoxification of

the hydrolysate. Using an integrated biorefinery model, current performance results in a minimum ethanol selling price of $8.8/gge. Reducing enzyme loading along with other minor process improvements can reduce the ethanol selling price to $3/gge. This study is the largest-scale demonstration of ionic liquid pretreatment and biofuel conversion known to date, and the overall biomass-to-ethanol efficiencies are the highest reported to date for any ionic liquid-based biomass to biofuel conversion.

KEYWORDS: woody biomass, ionic liquid, ethanol, scale-up, pilot scale, carbon footprint, technoeconomic analysis

INTRODUCTION

Production of renewable and affordable second-generation biofuels is a critical component in the broader strategy for reducing fossil fuel consumption in the transportation sector. Renewable liquid biofuels can improve energy security and mitigate the risks associated with GHG emissions and climate change.[1] Current first-generation biofuels are produced primarily from food crops with high concentrations of readily accessible sugar, starch, or lipid - including corn, sugarcane, and seed oils. In recent decades, greater emphasis has been placed on second-generation biofuels produced from non-food biomass including agricultural and forest residues, dedicated energy crops, and the lignocellulosic fraction of municipal and industrial solid waste.[2] Cellulosic biofuels can be produced from agricultural, forestry, and other organic residues that do not require any new cultivation of land, and from high biomass-yielding crops cultivated on marginal land not suitable for food production.[3] Cellulosic biofuels can reduce GHG emissions by 60% or more relative to gasoline, depending on the selected feedstock, and can achieve net-negative emissions after accounting for soil carbon sequestration and/or electricity export credits.[4,5]

Woody biomass is one of the most important sources of bioenergy globally and is obtained from a diverse and widespread resource base including short-rotation woody crops, timber and milling residues, municipal solid waste, and non-merchantable stems.[6,7] In California, woody biomass comprises the largest fraction of biomass potentially available for energy production, with forestry and mill residues estimated to reach nearly 34 million bone dry tonnes annually by 2050, plus an additional 4 million bone dry tonnes of orchard and vineyard residues.[8] As compared to herbaceous biomass, woody biomass can be collected throughout the year, reducing the

need for long-term storage. On average, woody biomass also has a higher cellulose to hemicellulose ratio relative to typical herbaceous feedstocks, is more dense, and has a lower ash content, reducing dead load in transportation and conversion.[9] In addition to compositional benefits, utilizing forest biomass as a feedstock into a biorefinery can provide an array of cost-effective public benefits, decreasing the frequency and scale of catastrophic wildfires while creating jobs in rural communities. In California alone, 1.5 million dead trees were felled between 2016 – 2019 during forest management, but due to structural integrity concerns only a small portion of the cost to fell trees could be recovered.[10]

While woody biomass is an attractive target for biofuel conversion, high energy inputs for size reduction, high lignin content, cellulose crystallinity, acetate content, and the presence of toxic extractables can challenge deconstruction and bioconversion of woody biomass as compared to herbaceous biomass.[11] This recalcitrance and toxicity necessitates development of novel upstream approaches. Currently, chemical pretreatments including dilute acid, alkaline, organosolv, steam explosion, lignin oxidation, and sulfite pretreatment (SPORL) have been employed to overcome recalcitrance of lignocellulose. Physical pretreatments, including wood chipping, grinding, and milling, are principally used for size reduction. Common challenges in commercial pretreatment include high energy consumption, sugar loss during intermediate separations, cellulose decrystallization ineffectiveness, efficient recovery of pretreatment chemicals, and wastewater treatment.[9,12,13]

Ionic liquid (IL) pretreatment has a number of potential benefits over alternative pretreatment techniques, including the nonflammability of the extraction solvent, chemical and thermal stability, excellent solvation ability, negligible vapor pressure, and widely tunable properties enabling deployment across a wide array of feedstock types and downstream applications[13–15]. ILs containing cholinium cations and amino acid anions ([Ch][AA]), known as “Bionic Liquids”, have a higher selectivity for lignin removal and lower sugar loss compared to non-renewable ILs. Bionic Liquids are prepared from naturally occurring, renewable starting materials, and are therefore more biocompatible to enzymes and microorganisms than acetate-based ILs.[14,15] These characteristics enable their use in one-pot systems combining pretreatment, saccharification, and fermentation with no intermediate separations.[16,17] Separation-free processes require fewer unit operations and can maintain high solids loading throughout, reducing capital

costs. Eliminating separation and washing steps also eliminates intermediate sugar losses and production of wastewater, reducing operating costs.[18] To date, separation-free processing of ionic liquid-pretreated biomass has been demonstrated with herbaceous biomass for conversion of C6 sugars to ethanol[16] and for conversion of C5 and C6 sugars to bisabolene.[17] More recently, in a work described by Das et. al[19], conversion of [Ch][Lys]-pretreated woody biomass was demonstrated at bench scale using a blend of almond, walnut, and pine wood, achieving a maximum sugar yield of >90 % and an ethanol titer of 18 g/L.

In this study, we build on the proof of concept demonstrated by Das et al.[19] by optimizing pretreatment and scale-up of an integrated one-pot process for deconstruction of California woody biomass and for efficient fermentation of the resulting hydrolysate, documenting strategies to overcome biomass recalcitrance and toxicity towards bioconversion. In particular, we focus on achieving efficient woody biomass deconstruction at high solids loading, and on adapting fermentation conditions to overcome hydrolysate toxicity and achieve full conversion of both glucose and xylose in the resulting intensified hydrolysates. By stacking these techniques, we achieve across the board improvements in pretreatment efficiency, C5 and C6 concentrations, fermentation efficiency, and final ethanol titer. We then demonstrate efficient ethanol distillation and recovery directly from a from whole slurry IL-containing fermentation for the first time to date. This work represents the first pilot scale demonstration of a one-pot ionic liquid conversion process from feedstock through to distilled ethanol, and the biomass to ethanol efficiencies of the resulting process are the highest reported to date for any ionic liquid-based biomass to biofuel conversion.

EXPERIMENTAL SECTION

Feedstock and enzymes: This study was conducted using three Californian woody biomass types obtained from Paddock Inc. in Oakdale, CA. Almond and walnut wood waste were procured from orchards in Oakdale, CA, and pine wood was obtained from forest thinning in Tuolumne County, CA. Each of the three biomass types were cut into mulch with a commercial wood chipper and subsequently milled in a Haybuster hammermill to 1/4” screen size prior to pretreatment (Figure 1).

Figure 1. Milled California woody biomass: almond (left), walnut (center) and pine (right)

Enzymatic hydrolysis and saccharification was performed using Novozymes, Inc. Cellic® CTec3 and Cellic® HTec3 commercial enzymes. The total protein content was determined by Bradford method using bovine serum albumin (BSA) as protein standard.[20] Total cellulase activity measured as filter paper units (FPU) and xylanase activity were determined by the methods described by Ghose[21] and Rodrigues et al.[22], respectively. One FPU/mL refers to 1 umol of reducing sugar released per minute per mL of the enzyme mixture used and one unit of xylanase activity (U) was defined as the amount of enzyme required to release one mol of xylose per minute under assay conditions. Compositional analysis of dry biomass is summarized in table 1. The high ash content in almond and walnut is thought to originate from residual silica accumulated during storage in the field following biomass milling.

Table 1. Compositional analysis of Almond, Walnut and Pine

	Almond	Walnut	Pine
Extractives (%)	15.9 ± 0.3	12.4 ± 1.1	7.1 ± 0.3
Glucan (%)	38.5 ± 0.3	26.3 ± 0.7	29.8 ± 0.6
Xylan (%)	16.0 ± 0.1	9.9 ± 0.3	13.5 ± 0.3
Klason Lignin (%)	21.6 ± 0.1	17.6 ± 0.9	24.7 ± 0.1
Ash (%)	8.1 ± 1.2	19.2 ± 2.3	0.0 ± 0.1

Yeast strain and inoculum preparation: The xylose-utilizing strain, *Saccharomyces cerevisiae* NS 22273, used for this study was engineered by Novozymes and maintained in a 25 % (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 YPDX media (10 $g.L^{-1}$ yeast extract, 20 $g.L^{-1}$ peptone, 20 $g.L^{-1}$ glucose and 10 $g.L^{-1}$ xylose) using defrosted cell suspension from glycerol stock. Cells grown in the YPDX media were then used to inoculate seed 2 in a 50:50 mixture of YPDX and filtered hydrolysate using a 10 % (v/v) inoculum size. In both steps, the cells were incubated at 30 °C or 35 °C at 220 rpm for 24 h. YPDX media and hydrolysate for seed cultivation was filtered sterilized with 0.2 µm pore filters. 100,000 U/L Penicillin and 100 mg/L Streptomycin were added prior inoculation. Microbial growth was measured by optical density at 600 nm via spectrophotometer (Thermo Scientific™ GENESYS™ 10S UV-Vis Spectrophotometer.

Scale-up of one-pot ionic liquid pretreatment and enzymatic hydrolysis: To address process scalability and optimize pretreatment and fermentation parameters, the one-pot ionic liquid pretreatment and saccharification process developed by Das et al.[19] was scaled-up to 10 L and 210 L sequentially. A summary of the pretreatment and saccharification conditions used by Das et al.[19] are shown in Table 2. Biomass pretreatment was carried out using cholinium lysinate [Ch][Lys] (Proionic, Grambach, Styria) ionic liquid as the catalyst. The 10 L scale experiments were carried out in a 10 L Hastelloy C276 Parr vessel (Parr Instrument Company, model: 4555-58, Moline, IL, USA). Two biomass solid loading conditions were evaluated (15 % and 25 % solid loading) with a 3 kg final working weight. A mixture of pine, almond and walnut wood (1:2:2) was used as substrate. Pretreatment conditions for both experiments were: 10 % wt. [Ch][Lys], 160 °C, 50 rpm for 3 h. After pretreatment, the reaction was cooled to room temperature and adjusted to pH 5 using 50 % (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, which corresponds to 40 FPU/g biomass and 95 U/g biomass.

Table 2. Pretreatment and saccharification conditions at scale-up process

Pretreatment		**Saccharification**	
Temperature	160 °C	*Temperature*	50 °C
Reaction time	3 h	*pH*	5
Biomass ratio	20:40:40 (Pine:Almond:Walnut)	*Reaction time*	72 h
[Ch][Lys] loading	10 % wt.	*Enzyme loading*	30 mg/g biomass

The one-pot pretreatment and enzymatic saccharification at 210 L scale was conducted in an Andritz 210 L Hastelloy C276 pressure reactor (AG, Graz, Austria) with a helical impeller. Three solids loadings (19 %, 22 % and 25 %) and two working volumes (75 kg and 90 kg) were evaluated. Pine, almond and walnut (1:2:2) were premixed with 10 % wt. [Ch][Lys] and water and heated to 160 °C for 3 h at 45 rpm. After IL pretreatment, the contents were cooled to 50 °C and adjusted to pH 5.0 with 50 % (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, 50,000 U/L Penicillin and 50 mg/L Streptomycin were added after harvest and the hydrolysate was stored at 4 °C.

Optimization of fermentation process parameters: Small-batch fermentations were carried out in 100 mL sealed glass bottles with 80 mL working volume in duplicate. 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 4000g for 20 min to remove the solids and sterilizing through a 0.2 µm filter prior to use.

Scale-up of fermentation process: Following process optimization, 5 L scale fermentations were performed in a Sartorius fermenter (Sartorius

BIOSTAT® B, Germany). No solids separation was performed, and the unfiltered hydrolysate was pasteurized at 70 °C for 30 min prior to fermentation. 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 ° or 35 °C; pH 5 or pH 5.5, 300 rpm, 5 days fermentation time.

A 1500 L scale fermentation was demonstrated in an ABEC fermenter equipped with a 17'' Rushton impeller (ABEC, Springfield, MO) with a final working volume of 680 L and a 10 % (v/v) inoculum size. The seed train for this process was completed in two stages. First, 2 L shake flasks containing 1 L YPDX 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. Next, 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 YPDX and filtered hydrolysate. YPDX 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. After 10 h, 68 L of acclimated cells were transferred to the fermentation tank containing 615 L of unfiltered hydrolysate, totaling 680 L.

Prior to inoculation, unfiltered hydrolysate was transferred 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.

Distillation process: Following fermentation, distillation was conducted in a 21-plate column with a 120 L bottom reboiler (Solar Spirits LLC, WA). During each batch, ≈100 kg final fermentation medium was pumped into the reboiler. The primary condenser cooling water was turned on to a flow rate of 30 L/h, and the secondary condenser to 7 L/min. 5 psig steam was supplied to the reboiler heating coil; when the temperature of the top plate began increasing, the steam was turned down to 2.5 psi and the column was given 30 min to establish vapor-liquid equilibrium on each plate. The cooling water flow to the primary condenser was then reduced to 15 L/h, and the distilled liquid was collected in the first collector (hearts). When the hydrometer

reading of the distilled liquid dropped below 75 % v/v, the liquid was collected in a second collector (tails). After the hydrometer reading reached approximately 5 % v/v, the distillation was stopped. All liquid collected in tails was subsequently redistilled to increase ethanol recovery.

Analytical methods and compositional analysis: Extractives were determined by solvent extraction as described by Mansfield[23], and structural carbohydrates and lignin content were quantified following the National Renewable Energy Laboratory (NREL) Laboratory Analytical Procedure (LAPs) standard protocol.[24] All experiments were performed in triplicate. Sugars (glucose, xylose, cellobiose and arabinose), ethanol, acetic acid and lactic acid were quantified by High Performance Liquid Chromatography (Thermo Fisher Scientific, Ultimate 3000, Waltham, MA, USA) with an Aminex HPX-87H column (Bio-Rad Laboratories, Inc) equipped with a refractive index detector. The HPLC was operated using 4 mM H_2SO_4 mobile phase at 0.3 $mL.min^{-1}$ and column oven temperature of 65 °C. The samples were filtered through 0.22 µm filter membranes and diluted with 4 mM H_2SO_4 for injection.

Commercial-scale process model development and analysis: To evaluate process economics, a biorefinery model was developed and sized to process 2000 bone-dry-metric ton (bdt) of woody biomass residues per day. The woody biomass residues include almond, walnut, and pine residues in the ratio of 2:2:1. The process model for biomass feedstock supply was developed following the methods and assumptions reported by Idaho National Laboratory.[25,26] Based on feedback from the supplier, the delivered cost of biomass feedstock blend was calculated using a zero farm-gate price (assuming the woody residue has no competing value-added application) and an estimated biomass trucking distance of 160 km (100 miles). Other assumptions and the methods used to calculate the delivered cost of woody residues and associated GHG emissions are documented in the SI Table S1, including ranges used for the IL cost ($1-2/kg), enzyme cost ($4-5/kg protein), and IL recovery rate for a commercial-scale facility (95-98%). The process model for deconstruction and bioconversion was developed in SuperPro Designer using the one-pot pretreatment and enzymatic hydrolysis configuration, measured feedstock composition, reactor-operating conditions, and biomass-to-ethanol conversion rates based on the findings of this study (Tables 1-3, and Figure 7). Results are based on a commercial-scale facility sized to process 2000 bdt biomass/day. Where appropriate, the model's methods and assumptions, including ethanol recovery, combustion

of residual lignin following distillation, wastewater treatment, and onsite energy generation stages, are consistent with those reported in similar past studies developed at JBEI[27] and NREL.[28] Other capital costs, operating costs, and discounted cash flow analysis to determine the minimum selling price of ethanol are consistent with prior NREL analyses.[28] Life-cycle GHG emissions per unit of ethanol produced were calculated based on material and energy balances for feedstock logistics and the biorefinery; the detailed input data and methods are documented by Neupane et al.[29] The carbon footprint credit of onsite electricity was determined assuming that it offsets the average U.S. electricity mix. Because the feedstock is considered a waste product, we do not include any emissions associated with collection of the biomass prior to transportation and pre-processing.

RESULTS AND DISCUSSION

Scale-up of one-pot ionic liquid pretreatment and enzymatic saccharification: In previous work, Das et al.[19] demonstrate proof of concept for one-pot conversion of woody biomass to ethanol, utilizing a 2:2:1 blend of almond, walnut, and pine wood, coupled to [Ch][Lys] pretreatment. Blending hardwood (almond and walnut) with softwood pine synergistically increased the conversion efficiency of the mixed feedstock. This effect is attributed to lower lignin content and higher methoxy content in the hardwoods, likely resulting in reduced condensation and an overall reduction in recalcitrance to ionic liquid pretreatment.[30] To develop this promising result via co-optimization of pretreatment conditions, fermentation conditions, and mixing parameters, we initially tested scale-up performance of the ionic liquid pretreatment in a 210 L pressure vessel. Three solids loading conditions and two working weights were evaluated in batch operation: 19 %, 22 %, 25 %, and 75 kg and 90 kg, respectively. Temperature, reaction time, and IL percent were kept constant during pretreatment. Saccharification was conducted at 50 °C, pH 5 for 72 h with 30 mg/g biomass enzyme loading.

Figure 2 depicts the concentration of glucose and xylose released during the 210 L scale enzymatic saccharification over a period of 72 h. As expected, sugar concentrations increased when solid loading increased from 19 % to 25 %. Final glucose concentrations at 19 %, 22 % and 25 % were 46.4, 53.1 and 59.3 g/L, with hydrolysis conversion efficiencies of 76.6 %, 75.5 % and 74.3 % respectively. For each condition, xylan conversion was nearly complete after 24 h of saccharification and glucan conversion improved

marginally from hours 24 to 72, suggesting that the reaction time for the saccharification step could potentially be reduced by 1/3 with minimal loss of efficiency. Despite the small difference in conversion efficiency at 72 h of saccharification, sugar concentrations after harvest decreased, resulting in a lower overall conversion efficiency of 77.7 % for the 19 % solids loading, 73.6 % for the 22 % solid loading, and 71.8 % for the 25 % solid loading batches. This decline in saccharification efficiency is likely attributable to a mass and heat transfer limitation in the reactor vessel. The increase in water-insoluble solids reduced the overall mixing efficiency and resulted in a mixing "dead-zone" at the base of the vessel, thereby reducing the aggregate sugar concentration of the fully mixed hydrolysate after harvest as compared to sugar concentrations at the fully mixed sample port. Xylan conversion was near complete after 24 h of saccharification, exceeding 95 % in all three conditions.

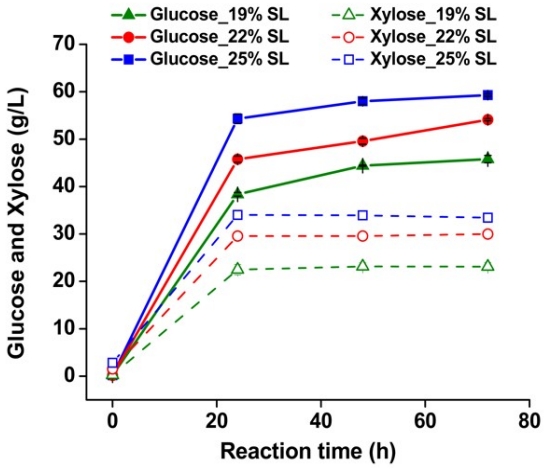


Figure 2. Glucose and xylose concentrations during enzymatic hydrolysis in 210 L scale at 19 %, 22 % and 25 % solid loading. Working weight: 75 kg, enzyme loading: 30 mg/g biomass, temp.: 50 °C, reaction time: 72 h, pH 5. Error bars: standard deviations of experimental replicate

Biomass conversion efficiency declined when working weight was increased from 75 kg to 90 kg at 19 % solid loading due to reductions in mixing efficiency at higher pretreatment volumes (Table 3). Glucan conversion dropped from 77.7 % to 73 % while xylan conversion decreased from 95.5 % to 87.2 %. Overall, the 19 % solids loading and 75 kg working weight conditions resulted in the highest overall pretreatment efficiency, while simultaneously reducing fermentation toxicity as compared to the 25 % solids loading hydrolysate (further discussion below). These parameters were

therefore selected for the remainder of the scale-up work. Overall combined glucan and xylan conversion efficiency at 19 % solids loading and 75 kg was 83 %.

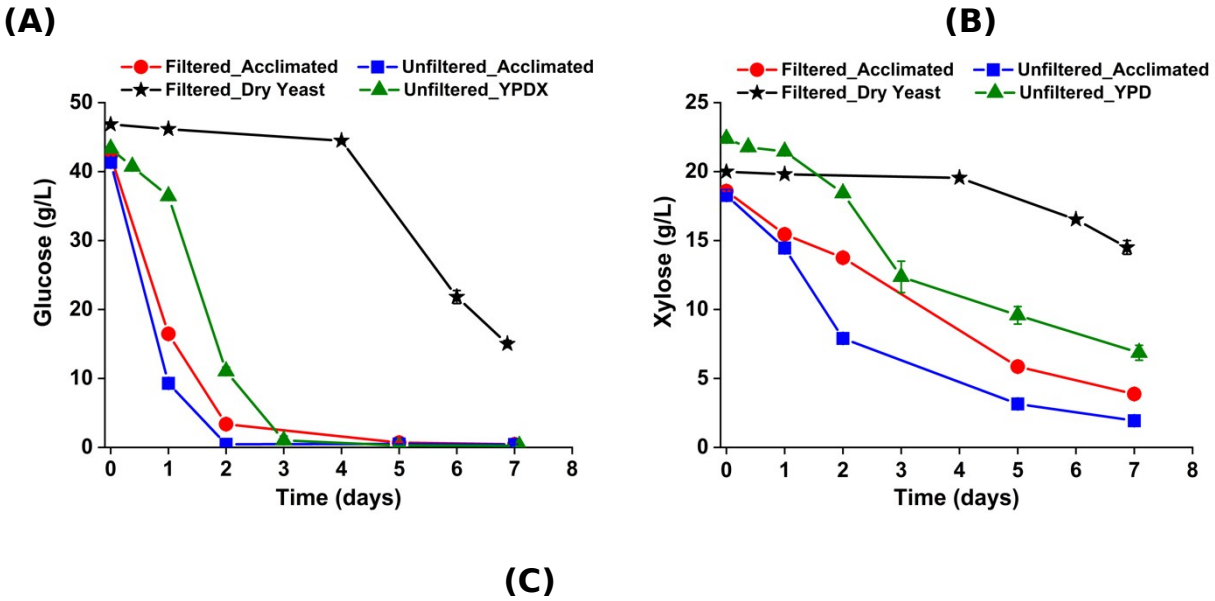
Table 3. Pretreatment and saccharification conditions and efficiencies after harvest across three solids loadings and two working weights at 210 L scale. The 19 % solids loading condition represents the average of three runs – this condition was used as the basis for fermentation process optimization in shake flasks, as well as for the 680 L fermentation campaign

Pretreatment condition	19 % dry solids	19 % dry solids	22 % dry solids	25 % dry solids
Working Weight (kg)	**75**	90	75	75
Final glucose (g/L)	**47.0 ± 0.0**	44.1 ± 0.1	51.4 ± 0.6	57.2 ± 0.5
Final xylose (g/L)	**23.7 ± 1.0**	21.7 ± 0.1	28.4 ±0.6	31.9 ± 0.5
Total glucose + xylose (g/L)	**70.7**	65.8	79.8	89.1
Glucan conversion (%)	**77.7 ± 0.1**	73.0 ± 0.1	73.6 ± 0.8	71.8 ± 0.6
Xylan conversion (%)	**95.5 ± 4.0**	87.2 ± 0.2	98.0 ± 2.4	97.6 ± 1.6
Combined glucan + xylan conversion (%)	**83.0**	77.1	80.7	79.3

Optimization of fermentation process parameters at bench-scale: Media optimization and fermentation conditions play a critical role in the enhancement of product yield. Understanding the factors that limit ethanol yields is important in identifying opportunities for process improvement. To assess critical parameters prior to scale-up, bench-scale experiments were conducted to evaluate the effect of solids removal, nutrient supplementation, initial sugar concentrations, pH, temperature, culture acclimatization, and inhibitors on the fermentation media. These factors were assessed using hydrolysate produced at 19 % solid loading as a base condition, except when testing the effects of solids removal and higher concentration hydrolysate

(25 % solid loading). Xylose utilizing strain NS 22273, supplied and engineered by Novozymes Inc., was utilized for fermentations at 30 °C, pH 5, and with an 80 mL working volume, with the exception of the tests to isolate the impact of variable temperature and pH. No solids separation or detoxification occurred in the hydrolysis and fermentation steps.

Lag phase in the initial stages of fermentation is often a consequence of toxicity of the hydrolysate. One attractive option in improving performance and tolerance to microbial inhibitors is adaptation during propagation, otherwise known as acclimatization. A two-stage seed train with yeast propagated initially in YPDX media (Seed 1) and subsequently in a 50 % YPDX/50 % filtered hydrolysate mixture (Seed 2) was used to help overcome this initial lag. To verify the efficacy of acclimatization, a control with Seed 2 culture cultivation in 100 % YPDX and direct dry yeast inoculation was tested. The presence of inhibitors during propagation significantly improved fermentation, mainly in the early stages, reducing the glucose consumption phase of the fermentation by a full day and enhancing overall xylose utilization (Figure 3). In the first 48 h of fermentation, specific ethanol production rate was over 60 % higher for the adapted cells compared to the inoculum from YPDX media. We therefore retained this acclimatization step in subsequent scale-up fermentations. Contrary to the general observation that glucose in the media inhibits utilization of D-xylose in yeast batch cultivations, xylose was simultaneously consumed during this study.[31]



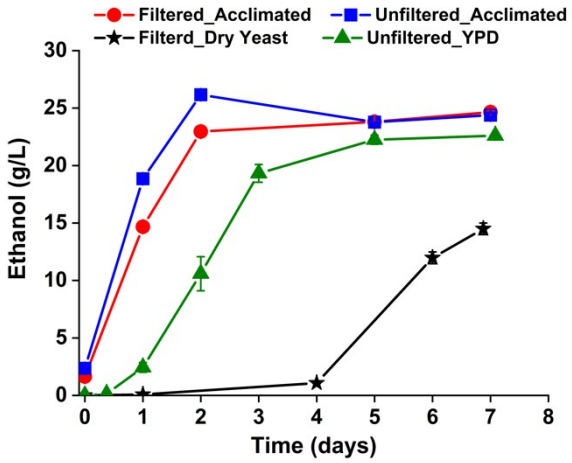


Figure 3. Effect of cells acclimatization on the alcoholic fermentation by *S. cerevisiae* using woody biomass hydrolysate pretreated at 19 % solid loading and 75 kg working weight. **(A)** Glucose consumption, **(B)** Xylose consumption, **(C)** Ethanol production. Filtered and unfiltered hydrolysate were inoculated with either dry yeast, cells cultivated in YPDX media or cells acclimated in 50 % hydrolysate/50% YPDX media. Shake flasks fermentation at 30 °C and pH 5. Error bars: standard deviations of experimental replicate

Separation of residual lignin following saccharification is costly and results in either significant loss of sugar, or significant dilution due to intensive washing of the residual solids. This can be circumvented via whole slurry processing in which residual lignin is retained during fermentation. To evaluate the effect of lignin separation on fermentation, shake flasks studies were performed using filtered and unfiltered hydrolysates as fermentation media. Filtration did not improve fermentation performance, and in fact led to a slight reduction in glucose and xylose consumption (Figure 4A). The specific mechanism for this effect is not known, but it is likely that the fermentation process is indifferent to the lignin, with the decrease in fermentation time resulting from displacement of fermentable sugar with inert lignin. Critical nutrients present in the hydrolysate may also adhere to the insoluble lignin, resulting in nutrient removal during centrifugation and filtration. Due to the significant process advantages of separation-free whole slurry fermentation, we used unfiltered hydrolysate for all subsequent process development and scale-up efforts.

Although high initial biomass loading is desired to produce a higher ethanol titer and thereby enhance process intensity, this study found that biomass concentrations exceeding 19 % negatively impacted both enzymatic

saccharification via impaired mixing and fermentation via increased toxicity. While a high solids process produces a more concentrated hydrolysate, the increase in sugars typically leads to an increase in fermentation inhibitors. Proper management of inhibitors and initial sugar concentration in fermentation can improve ethanol productivity, as xylose fermentation using *Saccharomyces cerevisiae* is particularly sensitive to the inhibitor profile.[32]

To evaluate the optimal solids loading conditions for efficient biomass to ethanol conversion, hydrolysates pretreated at 19 % and 25 % solid loading were evaluated in fermentation studies (Figure 4B). Increased solids loading resulted in longer fermentation times with reduced xylose consumption even after 7 days of fermentation. Higher initial sugars improved ethanol titers, but only after 72 h of fermentation. The toxicity of lignocellulosic hydrolysates has been associated with the presence of various aldehydes, organic acids and phenolic derivatives. Even in low concentrations, these compounds inhibit yeast metabolism by prolonging the lag phase, damaging cellular membranes and leading to cytoplasm acidification.[33] Xylose-rich hydrolysates contain more acetyl groups, cleaved off from hemicelluloses and in the form of acetic acid, causing cell stress and thus reducing the fermentation rate.[34] This suggests that the longer fermentation times are likely the result of increased inhibitor concentrations resulting from higher solids loading. While we opted to limit solids loading to 19 % for further work in this study, a combination of pretreatment optimization, strain adaptation, and adaptive laboratory evolution could ultimately be used to enable high-efficiency fermentation of more concentrated hydrolysates.

Figure 4C isolates the inhibitory effect of the whole slurry hydrolysate on ethanolic fermentation by comparison to a control fermentation media mimicking the glucose and xylose concentrations found in the 19 % solid loading pretreatment hydrolysate. Glucose consumption was slightly faster and the specific xylose consumption rates obtained in the synthetic media were clearly higher than for the hydrolysate fermentation. Glucose and xylose were completely depleted in 48 h in the mimic media, while almost 20 % of the original xylose remained after 7 days of fermentation.

(A) **(B)**

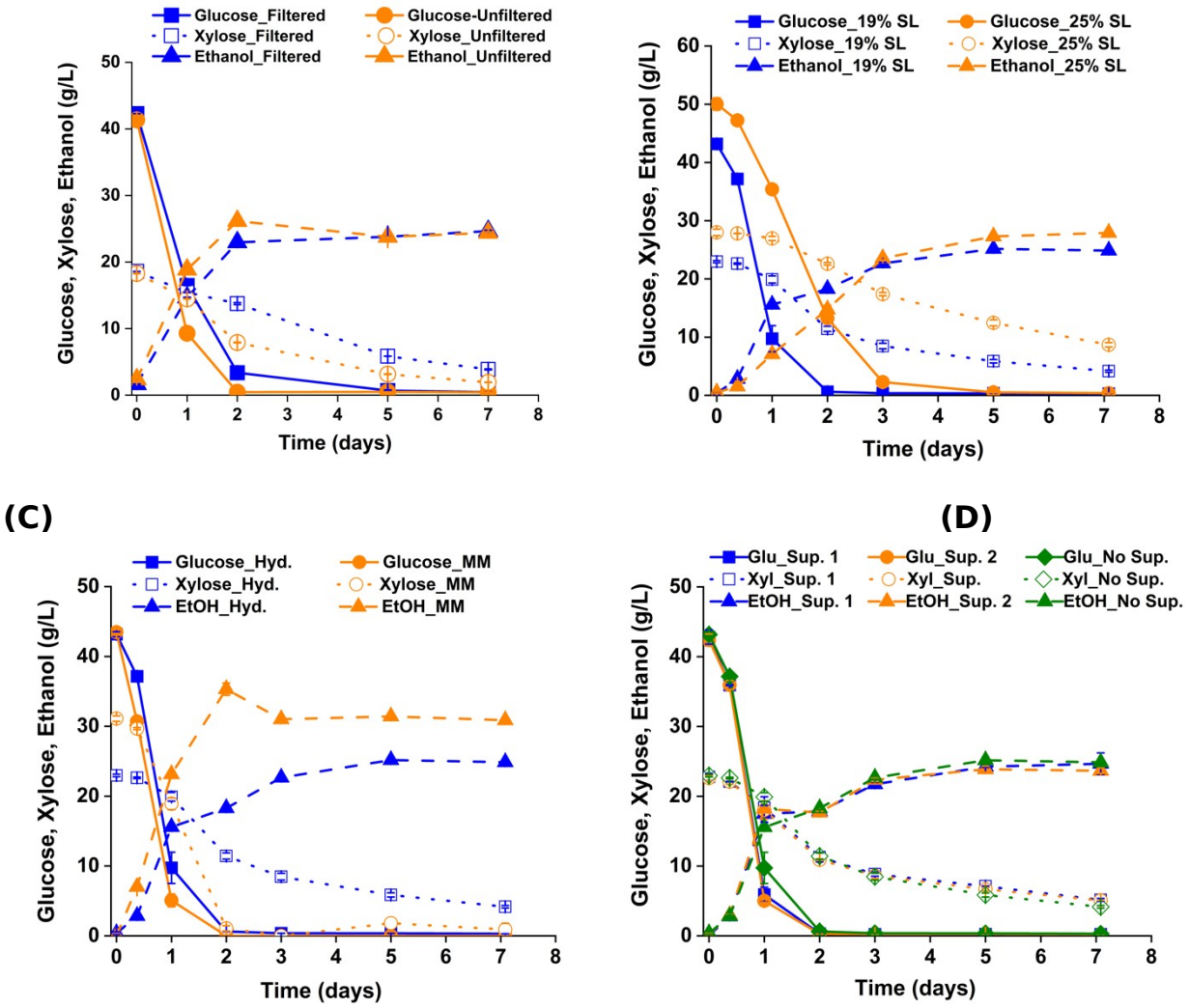


Figure 4. Effect of solids content **(A)**, initial sugar concentration **(B)**, inhibitors **(C)** and nutrient addition **(D)** on the ethanol fermentation process. Supplementation 1: 0.2 g/L KCl, 3.8 g/L $MgSO_4.7H_2O$, 1 % yeast extract and 1 g/L KH_2PO_4; Supplementation 3: Sup. 2 + 0.1 mg/L biotin, 1 mg/L thiamine and trace elements. SL: solid loading; Hyd: woody biomass hydrolysate; MM: mimic media. All fermentations were carried out at 30 °C and pH 5. Error bars: standard deviations of experimental replicate

While ethanol production mainly depends on sugar availability, macronutrients, vitamins, and trace elements are also necessary to maintain the health of the biocatalyst. As these experiments demonstrated a similar sugar consumption profile - rapid glucose uptake followed by gradual xylose consumption, with 4-10 g/L residual xylose at the end of fermentation – a nutrient supplementation study was conducted to determine if fermentation time and xylose consumption were constrained by nutrient deficiency. Two

supplementations were tested; the first comprises yeast extract and mineral salts and the second contains yeast extract, mineral salts, biotin, thiamine and trace elements, which are necessary for most xylose-fermenting yeast.[35] As seen in figure 4D, process performance was largely unaffected by nutrient supplementation, indicating that residual xylose was driven by alternative metabolic factors. We therefore limited future fermentation studies to the unsupplemented hydrolysate.

Ethanol production is greatly influenced by pH and temperature, as it affects the enzymatic activity, membrane turgidity of yeast cells and permeability of essential nutrients.[36,37] To further address residual xylose in the fermentation, two pH values – pH 5 and pH 5.5, and two temperatures – 30 °C and 35 °C were evaluated (Figure 5) using unfiltered hydrolysate. High acetate concentrations in the hydrolysate are known to impact fermentability, and fermentation at higher pH is known to reduce toxicity in similar [Ch][Lys] one pot processes due to the reduction of inhibitory effect of undissociated acids in the hydrolysate pretreated with [Ch][Lys].[38]

Fermentation results revealed pH 5.5 as preferable to pH 5 at both temperatures (Figure 5). Increased temperature was only beneficial at pH 5.5 – at pH 5, increased temperature was detrimental to both xylose consumption and ethanol titer. According to Azhar et al.[39], growth rate and metabolism of yeasts increases as the temperature increases until it reaches an optimum value. Thus, we hypothesize that the reduced toxicity at pH 5.5 enables more rapid metabolism of sugars at higher temperature; however, in the presence of increased toxicity at pH 5 this affect appears to be reversed, with more rapid metabolism exacerbating hydrolysate toxicity. Based on these results, pH 5.5 and 35 °C were chosen as the optimal pH and temperature for further scale-up fermentations.

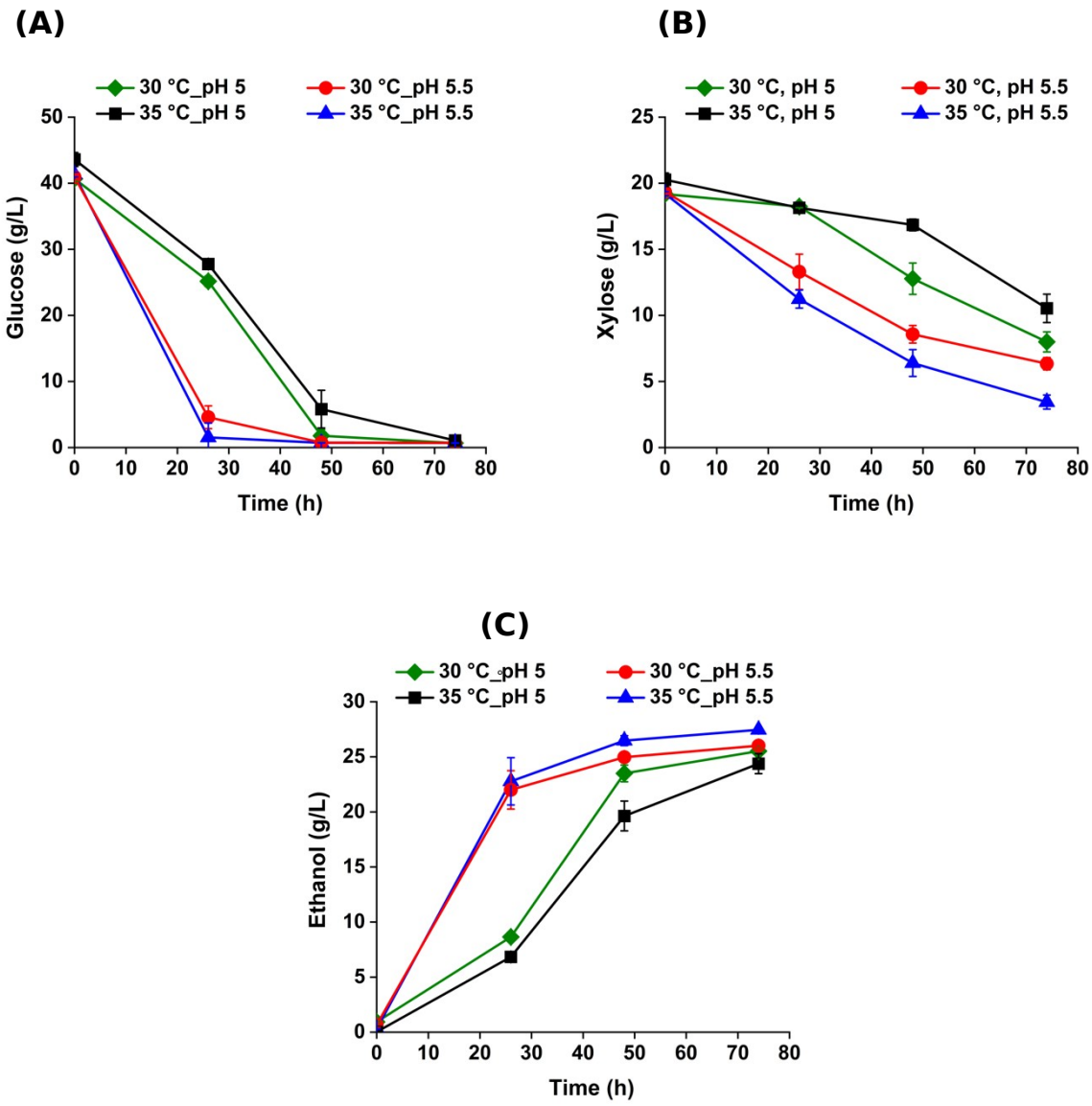


Figure 5. Effect of pH and temperature on ethanol fermentation by *S. cerevisiae* with woody biomass hydrolysate pretreated at 19 % solid loading. **(A)** Glucose consumption, **(B)** Xylose consumption, **(C)** Ethanol production. Error bars: standard deviations of experimental replicate

Pilot scale validation and distillation: Following fermentation optimization in shake flasks, the optimized process was replicated in a 5 L scale bioreactor before proceeding to pilot scale (Figure 6A). Both the original condition (pH 5, 30 °C) and the optimized condition (pH 5.5, 35 °C) performances were compared in bioreactor studies. Glucose was depleted after two days, with 60-70 % of the xylose consumed after 5 days of fermentation for both conditions. At this scale, ethanol titer was 28.6 g/L for

the optimized condition, corresponding to 81.5 % fermentation efficiency. Troubleshooting after the 5 L campaign revealed two potential inhibitory factors – harsh pasteurization conditions for the slurry hydrolysate at 5 L scale resulted in the formation of toxic byproducts, and faster growth of the seed 2 culture in the 5 L experiments resulted in inoculation with stationary phase yeast. For the subsequent pilot scale fermentation pasteurization, pasteurization hold time was reduced to 5 minutes, and the seed 2 culture was intensively monitored to ensure inoculation in late-log phase growth.

(A) **(B)**

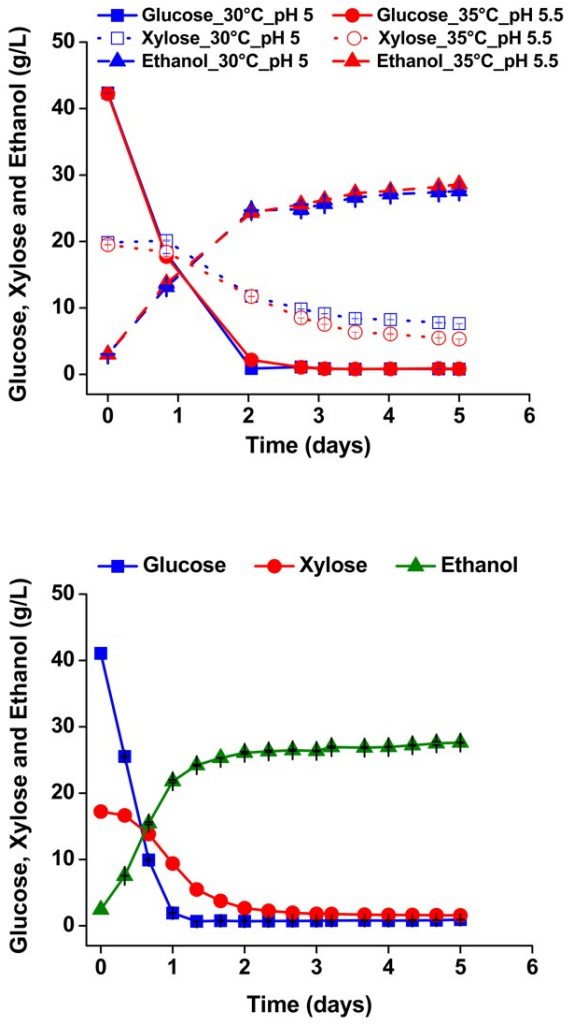


Figure 6. Scale-up fermentation process using woody biomass hydrolysate pretreated with [Ch][Lys]. **(A)** 5 L scale at 30 °C/pH 5 and 35 °C/pH 5.5, **(B)** 1500 L scale at 35 °C and pH 5.5. Error bars: standard deviations of analysis replicate

After successful demonstration of the process in 5 L bioreactor fermentation, the process was tested at pilot scale in a 1500 L ABEC fermenter using a composite batch of 680 L unfiltered hydrolysate produced over multiple deconstruction campaigns in the 210 L Andritz pretreatment vessel. With the seed train and pasteurization improvements following the 5 L fermentation procedure, the pilot scale campaign achieved near complete glucose utilization after 24 h, with >90 % xylose utilization after 72 h (Figure 6B). The fermentation achieved a final titer of 27.6 g/L after 120 h, with an overall conversion efficiency of 93.3 % as a function of initial glucose and xylose. When coupled with the deconstruction efficiency of 83.0 %, this result yielded an aggregate deconstruction and fermentation efficiency of 77.4 % (Figure 7). The results obtained at this scale corresponded well with the ethanol titers obtained in shake flasks and 5 L fermentation scales - 27.5 g/L and 28.6 g/L, respectively. In addition to toxicity generated during pasteurization, challenges encountered during fermentation scale-up included difficulty pumping and mixing the viscous whole slurry hydrolysate and accumulation of highly abrasive silica particles in pumps and agitator assemblies. While manual solutions were sufficient to ensure the success of a single campaign, these materials handling considerations must be addressed during facility design to achieve robust, continuous operation in a demonstration or commercial plant.

Following pilot scale fermentation, the resulting whole slurry was distilled to recover high-purity cellulosic ethanol. A total of 518 kg of hydrolysate was distilled across five batches, resulting in recovery of 11 kg ethanol of 90.9 % v/v ethanol, for an overall ethanol recovery rate of 81 %. This result validates the feasibility of direct ethanol distillation from whole slurry containing ionic liquids, residual lignin, residual yeast, and byproducts of deconstruction and fermentation. At 81 %, recovery efficiency was significantly lower than for traditional corn ethanol distillation. Distillation efficiency could be improved by optimizing for process intensity and improved ethanol titer, and by transitioning to a more industrially representative continuous distillation process.

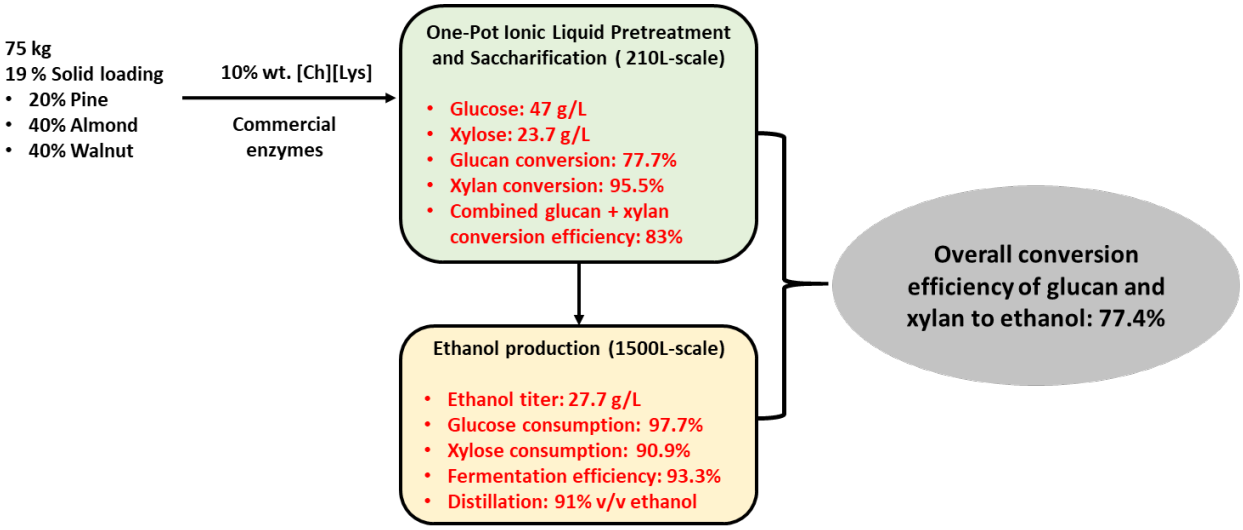


Figure 7. Final deconstruction and fermentation efficiencies for the 19 % solids loading condition used for fermentation process development and scale up, and final fermentation efficiencies for the 680 L pilot-scale fermentation

Selling price and carbon footprint of ethanol: After applying a commercial scale process model to the documented pilot-scale titers and conversion efficiencies, we find a predicted ethanol selling price and carbon footprint of \$8.8/gge and 66.1 g CO_{2e}/MJ, respectively, assuming 19 % solids loading in pretreatment (Figure 8). Based on current process data, increasing the solids loading to 25 % increases the minimum selling price to \$9.4/gge due to reduced saccharification and fermentation efficiencies (Figure 8A), while the net carbon footprint is reduced to 65.3 gCO_{2e}/MJ (Figure 8B). Lower biomass-to-ethanol conversion efficiency increases fuel availability to the boiler, resulting in higher electricity credits while increasing capital cost due to increase in the sizes of boiler and turbine. However, reduced overall operating cost, and the cost credits from additional electricity are not sufficient to offset the revenue generated from increased ethanol production efficiency at 19 % solids loading. Improvements in both saccharification and fermentation efficiency at high solids loading are therefore required to realize a net benefit from process intensification during pretreatment.

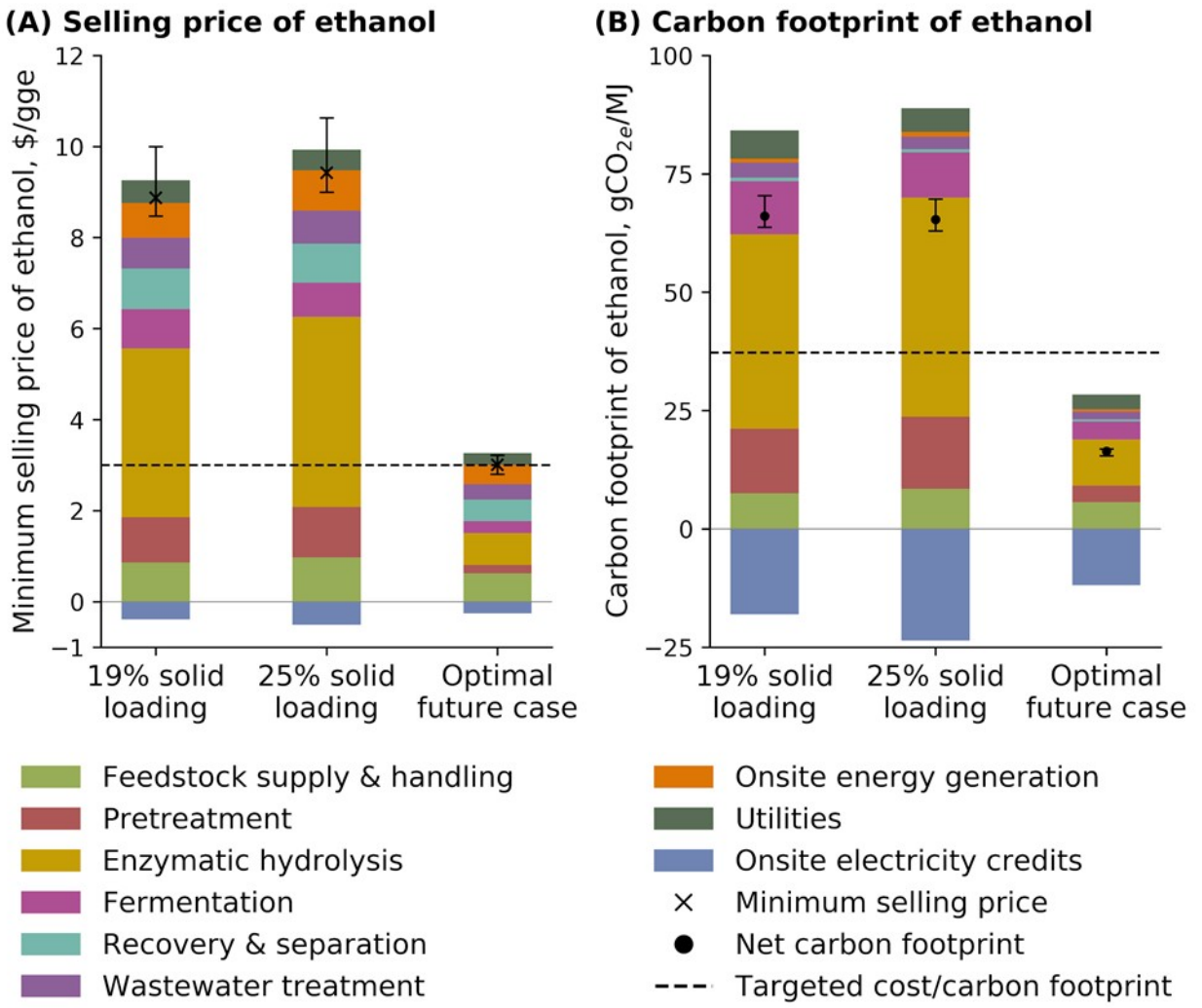


Figure 8. Selling price and carbon footprint of ethanol at the current-state-of-the-art of the technology and for the optimal future case. The horizontal dashed lines represent **(A)** targeted selling price of ethanol of $3/gge, and **(B)** carbon footprint of 37.2 gCO_{2e}/MJ (60% reduction relative to gasoline)

Enzyme cost is the single largest contributor to the predicted ethanol production cost and carbon footprint in the current system, accounting for 40 % - 49 % of total costs, primarily due to a high enzyme-loading rate of 30 mg protein/g biomass[40] (Figure 8). This study targeted overall conversion efficiency during saccharification, with a relatively high enzyme loading employed throughout. Our experimental results do show room for significant improvement in enzyme-loading rate, as the vast majority of the initial glucan and xylan was hydrolyzed within 24 h (Figure 2). For the optimal future case, we model a reduction in enzyme loading from 30 to 10 mg

protein/g biomass, increase in combined glucan and xylan conversion efficiency from 83 % to 95 %, and increase in combined C5- and C6-sugar utilization in ethanol fermenters from 93 % to 95 %. These process improvements could reduce the ethanol selling price and carbon footprint to $3/gge with GHG emissions of 16.4 gCO_{2e}/MJ, 56 % below the GHG emissions reduction mandate for cellulosic biofuel under the Renewable Fuel Standard.

To achieve reduced enzyme loading, increased solids loading, and high conversion efficiencies, additional strategies could be exploited to boost final ethanol titer and productivity. These strategies include hydrolysate toxicity reduction via manipulation of pretreatment parameters, optimization of enzyme cocktails specifically for woody biomass, and improvement of strain resilience via tolerance engineering. To further boost the solids content and efficiency of saccharification, a simultaneous saccharification and fermentation process (SSF) could be employed. This strategy reduces enzyme inhibition from by-products as well as osmotic stress from high concentration of saccharides, as fermentable sugars are continuously released and consumed throughout the process.[41] Xu et al.[16] demonstrate a similar approach, producing 41 g/L ethanol at 74.8 % fermentation efficiency by employing a fed-batch SSF strategy with cholinium aspartate ($[Ch]_2[Asp]$) pretreated corn stover.

CONCLUSIONS

This study is the first demonstration integrating ionic liquid pretreatment and fermentation at pilot scale. When compared to previous studies integrating woody biomass deconstruction with ethanol fermentation, the results presented here demonstrate the highest conversion efficiency from woody biomass to ethanol of any study to date. While most pretreatment technologies require washing of biomass, sugar concentration, solvent removal, or detoxification, the integrated [Ch][Lys] process achieves high efficiency biomass conversion with no intermediate unit operations.

By achieving efficient conversion of recalcitrant woody biomass, this study demonstrates the feasibility of woody biomass residues as a promising alternative to herbaceous feedstocks. We have successfully demonstrated scale-up of a one-pot process with a combined pretreatment and saccharification conversion of 83 %, 93.3 % fermentation efficiency for C5 and C6 sugars, and 81% ethanol recovery direct from the whole slurry hydrolysate in batch distillation. No solids separation or nutrient addition was

necessary to achieve fermentation efficiency comparable to industrial scale ethanol production using first-generation feedstocks.[39] Current operating conditions for biomass deconstruction and biomass-to-ethanol conversion rate result in ethanol selling price of $8.8/gge and carbon footprint of 66.1 gCO_{2e}/MJ. Further improvements in overall yield and titer are therefore required to make this technology commercially attractive – particularly focusing on solids loading, pretreatment efficiency, and enzyme loading. Stacking moderate improvements in each of these areas into an optimal design case results in a projected ethanol-selling price of $3/gge and carbon footprint of 16.4 gCO_{2e}/MJ, an 82% reduction relative to gasoline.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION: Assumptions and methods used to calculate the delivered cost of woody residues and associated GHG emissions.

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SYNOPSIS

This study demonstrates an efficient end-to-end conversion process at pilot scale, converting raw woody biomass to purified ethanol with ionic liquid pretreatment and no intermediate separations.

ABSTRACT GRAPHIC