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Demonstrating a separation-free process coupling ionic liquid pretreatment, saccharification, and fermentation with *Rhodosporidium toruloides* to produce advanced biofuels†

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Achieving low cost and high efficiency lignocellulose deconstruction is a critical step towards widespread adoption of lignocellulosic biofuels. Certain ionic liquid (IL)-based pretreatment processes effectively reduce recalcitrance of lignocellulose to enzymatic degradation but require either costly separations following pretreatment or novel IL compatible processes to mitigate downstream toxicity. Here we demonstrate at benchtop and pilot bioreactor scales a separation-free, intensified process for IL pretreatment, saccharification, and fermentation of sorghum biomass to produce the sesquiterpene bisabolene, a precursor to the renewable diesel and jet fuel bisabolane. The deconstruction process employs the IL cholinium lysinate ([Ch][Lys]), followed by enzymatic saccharification with the commercial enzyme cocktails Cellic CTec2 and HTec2. Glucose yields above 80% and xylose yields above 60% are observed at all scales tested. Unfiltered hydrolysate is fermented directly by *Rhodosporidium toruloides* – with glucose, xylose, acetate and lactate fully consumed during fermentation at all scales tested. Bisabolene titers improved with scale from 1.3 g L^{-1} in 30 mL shake flasks to 2.2 g L^{-1} in 20 L fermentation. The combined process enables conversion of saccharified IL-pretreated biomass directly to advanced biofuels with no separations or washing, minimal additions to facilitate fermentation, no loss of performance due to IL toxicity, and simplified fuel recovery *via* phase separation. This study is the first to demonstrate a separation-free IL based process for conversion of biomass to an advanced biofuel and is the first to demonstrate full consumption of glucose, xylose, acetate, and lactic acid in the presence of [Ch][Lys].

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Introduction

Lower cost and higher efficiency biomass deconstruction remains a critical hurdle towards large-scale deployment of affordable lignocellulosic biofuels. Certain ionic liquids (ILs) have unique solvent properties that enable more efficient and uniform deconstruction of a wide array of lignocellulosic biomass types, including disruption of lignin and decrystallization of cellulose.[1] These advantages are counterbalanced by process development challenges created by the toxicity of many ILs towards hydrolytic enzyme mixtures and biofuel production strains.[2] This toxicity can be mitigated by extensive water washing to remove residual ILs prior to saccharification or fermentation,[3–5] by use of a new class of lower toxicity ILs,[4,6] by development of IL tolerant enzyme mixtures and biofuel production strains,[7,8] or by a combination of these techniques.

Due to the high cost of solid–liquid separation and the associated loss of biomass and sugars during washing and separation steps, consolidation of deconstruction, saccharification, and fermentation unit operations is critical to reducing the operating and capital costs for biomass conversion.[9] Direct fermentation of whole slurry following enzymatic saccharification has been demonstrated successfully in combination with a number of pretreatment strategies, including both dilute acid and sulfite pretreatment.[10,11] This whole slurry approach reduces process complexity and completely eliminates sugar losses during separation. Because fermentation inhibitors are retained throughout the process, biocompatible pretreatment

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and robust biocatalysts are required for efficient conversion. For ionic liquid-based processes, combining IL pretreatment and saccharification with no intermediate separation leads to significant improvement in process economics due to improved yields and elimination of extensive water washing for IL removal between unit operations.[12] Consolidation of pretreatment and saccharification into a one pot process was recently demonstrated with both IL tolerant enzyme cocktails[13] and more recently with bio-derived ILs and commercial enzymes.[14] Further benefits may be obtained by consolidating pretreatment, saccharification and fermentation, eliminating the need to separate ILs from hydrolysates prior to fermentation. These unit operations can be combined either sequentially or in a simultaneous saccharification–fermentation (SSF) process. For example, a fully consolidated high gravity process combining IL pretreatment and SSF was recently demonstrated for ethanol production in *Saccharomyces cerevisiae*.[15] Technoeconomic analysis of the fully consolidated process indicates a 40% cost reduction when compared to an equivalent process with IL removal and recovery prior to fermentation. This promising result demonstrates the value of a separation-free process, in which IL pretreated biomass is converted into biofuels or bioproducts with no intermediate separation steps between conversion unit operations (Fig. 1). While ethanol production has been demonstrated in the presence of concentrated ionic liquids, no process has yet demonstrated production of advanced biofuels under such conditions, including molecules suitable as diesel and jet fuel, and no fermentation process has demonstrated conversion of both pentose and hexose sugars in the presence of concentrated ionic liquids.

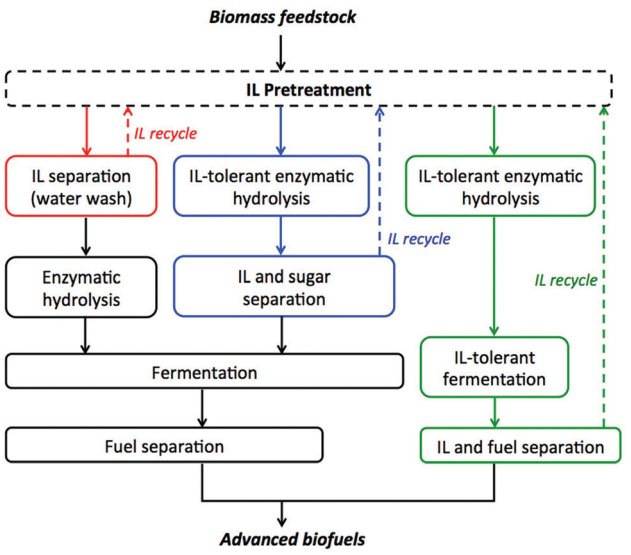

Fig. 1 Three process configurations for conversion of IL pretreated biomass to advanced biofuels: fully separated unit operations (red), one pot deconstruction–saccharification (blue), and the fully consolidated process (green). The fully consolidated process eliminates the requirement for IL separation prior to saccharification and fermentation, but requires IL tolerant enzyme cocktails and an IL tolerant host organism. Separation of both fuel molecules and residual ionic liquids can then be consolidated into a single step following fermentation.

To achieve high fermentation performance in the presence of ionic liquids, we employ the robust and metabolically flexible host *Rhodosporidium toruloides*. This oleaginous yeast is a native xylose utilizer and readily consumes glucose, xylose, and aromatic lignin decomposition products when cultivated on cellulosic hydrolysates, including those generated from [Ch][Lys] pretreated biomass.[16] Cell densities exceeding 150 g L^{-1} dry cell weight have been achieved with *R. toruloides* in high gravity fermentation, with endogenous lipid concentrations of nearly 70% as a fraction of dry biomass.[17] Uninhibited growth with over 100 g L^{-1} glucose in solution during high-gravity fermentation reveals a high tolerance for osmotic stress. Due to its high lipid productivity, *R. toruloides* has been explored extensively for production of triglyceride-based biodiesel, including production of cellulosic fuels.[18] Numerous genetic tools exist for engineering *R. toruloides*,[19,20] and strains have now been engineered for production of both monoterpene and sesquiterpene biofuel precursors[13] as well as C16–C18 fatty alcohols.[21]

To demonstrate the potential of *R. toruloides* as a robust host for conversion of IL-rich hydrolysates into advanced biofuels, this work targets production of the sesquiterpene bisabolene. Bisabolene is a chemical precursor to bisabolane, a potential renewable diesel and jet fuel that phase separates readily when released to the fermentation broth, enabling efficient recovery *via* two-phase extractive fermentation with an organic overlay.[22,23] In this work, we demonstrate bisabolene production from sorghum hydrolysate in a scalable one-pot process comprising IL pretreatment, saccharification, and fermentation. High titers of bisabolene were achieved relative to those previously obtained using a multi-step process,[13] and conversion efficiency improved with scale, demonstrating the feasibility of integrating all biomass conversion unit operations within the biorefinery.

Experimental

Biomass source

Dried sorghum supplied by Idaho National Laboratory was milled and passed through a 2 mm screen. The initial biomass had a moisture content of 10% and contained 28 ± 1.4% glucan, 17 ± 1.8% xylan, and 18.8 ± 0.2% lignin as a fraction of dry weight.

Pretreatment

Three reactors were used for one-pot pretreatment and saccharification – a 1 L 4520 Parr bench top reactor (Parr Instrument Company, Moline, IL, USA) equipped with three-arm, self centering anchor with PTFE wiper blades, a 10 L Parr vessel equipped with anchor impellers with scraper blades, and a 210 L vessel equipped with a helical impeller (Andritz AG, Graz, Austria). Biomass pretreatment parameters were adapted from the optimized conditions identified by Xu

et al.[14] Initial biomass loading was 70 g in the 1 L vessel, 700 g in the 10 L vessel, and 5.25 kg in the 210 L vessel. The pretreatment vessels were loaded with 30% w/w biomass and 70% w/w aqueous fraction, with the aqueous fraction consisting of 90% DI water and 10% cholinium lysinate [ChLys]. During pretreatment, the reaction vessels were heated to a reaction temperature of 140 °C for one hour under completely mixed conditions.

Enzymatic saccharification

Following pretreatment, the reactors were cooled and adjusted to pH 5 with 50% v/v H_2SO_4. Additional DI water was added to reach 20% w/w solids as measured by initial solids loading. An enzyme mixture of Cellic® CTec2 and HTec2 (Novozymes, Franklington, NC, USA) at ratio of 9 : 1 v/v, respectively, was used at a total enzyme loading of 10 mg g^{-1} biomass dosing 3.19 mL of the (hemi) cellulolytic enzymatic cocktails to each reactor (*i.e.* 53 mL kg^{-1} biomass). The reaction vessels were completely mixed and heated to 50 °C for 72 hours with periodic sampling.

Fermentation strain

The BIS3 strain of *Rhodosporidium toruloides* was used for all fermentation in this study (JBEI registry ID JBx_065244). This bisabolene-producing strain was engineered from wild-type *R. toruloides* strain IFO0880 obtained from the NBRC culture collection. Construction and initial characterization of this strain was described by Yaegashi *et al.*[16]

Seed cultures

Seed cultures were inoculated from single colonies of *R. toruloides* strain BIS3 grown on YPD agar plates. YPD medium containing 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, and 20 g L^{-1} glucose was used for all seed cultures. 50 mL seed cultures were grown for 24 hours in 250 mL baffled flasks at 30 °C with 250 rpm agitation. For the 20 L scale-up fermentation, four 2.5 L baffled flasks containing 500 mL seed medium were inoculated with 25 mL exponential-phase seed culture and incubated for an additional 24 hours prior to inoculation of the main fermentation. All bioreactor fermentations were inoculated at 10% inoculum size from seed cultures with a final OD_{600} of ~20. This inoculum size was chosen to ensure rapid and robust growth and to minimize any potential risk from contaminating organisms.

Fermentation with variable sugars and organic acids

Growth and bisabolene production was quantified in shake flask fermentation for four carbon sources evaluated alone and in combination: glucose, xylose, acetic acid, and lactic acid. 500 mL shake flasks were inoculated with 10 mL seed culture, 80 mL of carbon substrate, 20 mL dodecane overlay, and 10 mL of 10× YEP medium to achieve an initial concentration of 10 g L^{-1} yeast extract and 20 g L^{-1} peptone. Initial sugar and organic acid concentrations in these experiments were 22 g L^{-1} glucose, 14 g L^{-1} xylose, 8 g L^{-1} acetic acid, and 21 g L^{-1} lactic acid. All conditions were tested at a starting pH of 7 with 10 N NaOH used to adjust pH to the initial set point. Shake flasks were incubated at 30 °C with 250 rpm agitation. Each fermentation condition was tested in triplicate.

Fermentation with variable pH and nitrogen sources

To test the effect of variable pH and nitrogen sources, 100 mL shake flasks were inoculated with 2 mL seed culture, 2 mL 10× nitrogen source, 16 mL unfiltered hydrolysate, and 4 mL dodecane overlay. Two nitrogen sources were tested – YEP medium and 5 g L^{-1} ammonium sulfate. Two initial pH values were tested, pH 5 and pH 7, with 50% NaOH added to adjust pH to the initial set point. Each of the four conditions was tested in triplicate. Unfiltered hydrolysate was pasteurized for 1 hour at 80 °C prior to inoculation, nitrogen sources were autoclaved, and the dodecane overlay was filter sterilized. Shake flasks were incubated at 30 °C with 250 rpm agitation.

2 L bioreactor fermentations

Four 2 L Biostat B fermentors (Sartorius Stedim, Göttingen, Germany), each agitated with two Rushton impellers, were batched with 900 mL unfiltered hydrolysate, 90 mL nitrogen source, 220 mL dodecane overlay, and 110 mL seed culture for an initial volume of 1.32 L. The dodecane overlay contained 0.13% v/v pentadecane as an internal standard to control against dodecane evaporation during fermentation. Nitrogen was supplied as either 5 g L^{-1} ammonium sulfate or 10 g L^{-1} yeast extract and 20 g L^{-1} peptone (YEP medium) in the aqueous phase. Two antibiotics were added to the batch medium to inhibit contamination – 1 mL 30% w/v cefotaxime and 1 mL 10% w/v nourseothricin. Unfiltered hydrolysate was pasteurized at 80 °C for 1 hour, all other media components were filter sterilized with 0.2 μm filters. All bioreactor fermentations were inoculated at pH 7 with 2 N NaOH added on a pH trigger to control pH above the set point of pH 5. No upper set point was used for pH. 10% v/v PPG-PEG-PPG antifoam (Sigma Aldrich) was used as needed to control foaming. Dissolved oxygen was controlled at 20% saturation by varying agitation from 400–800 rpm, then varying air flow from 0.5–1.5 LPM. Fermentation temperature was held constant at 30 °C.

20 L fermentation

Scale-up fermentations were conducted in a 50 L working volume fermentor (ABEC, Bethlehem, PA, USA) sterilized by steaming in place and agitated with three Rushton impellers, two of which were submerged at the volume tested. The fermentor was batched with 17.2 L unfiltered hydrolysate, 1.7 L concentrated ammonium sulfate (sufficient to achieve 5 g L^{-1} AS in the aqueous phase), 4.2 L dodecane overlay, and 2.1 L seed culture for an initial volume of 25.2 L. The dodecane overlay contained 0.13% v/v pentadecane as an internal standard. As with 2 L fermentations, antibiotics were added to mitigate contamination risk: 21 mL 30% w/v cefotaxime and 21 mL 10% w/v nourseothricin. Antifoam, DO control, and pH control were unchanged from 2 L fermentations. Unfiltered hydrolysate was pasteurized in the fermentation vessel at 70 °C for 15 minutes, ammonium sulfate and antifoam solutions

were autoclaved and added to the vessel following pasteurization, and dodecane and antibiotic solutions were sterilized with 0.2 μm vacuum filters and added to the vessel following pasteurization.

Sugar and organic acid analysis

Sugars and organic acids were quantified by high performance liquid chromatography (Thermo Fisher Scientific, Ultimate 3000, Waltham, MA, USA) using an Aminex HPX-87H column (Bio-Rad Laboratories, USA) and a refractive index detector. The mobile phase was 5 mM sulfuric acid with a flow rate at 0.6 ml min^{-1} and a column oven temperature of 65 °C. Prior to analysis, samples were filtered using 0.45 μm centrifuge filters and diluted with 5 mM sulfuric acid prior to injection.

Nutrient analysis

Ammonium and phosphate concentrations in fermentation supernatant were analyzed *via* colorimetric assays with a Gallery automated photometric analyzer (Thermo Fisher Scientific, Waltham, MA, USA).

Bisabolene analysis

To quantify bisabolene, 10 μL samples of the dodecane overlay were diluted with 390 μL ethyl acetate and quantified by the gas chromatography-mass spectroscopy (GC-MS) method described by Özaydın *et al.*[24] Final bisabolene titers in bioreactor fermentations were adjusted based on a pentadecane internal standard to control for dodecane evaporation during the course of fermentation. All bisabolene titers are normalized to the aqueous volume of the fermentation.

Analysis of water-soluble lignin and sugars by size-exclusion/gel-permeation chromatography (SEC/GPC)

For analysis of water-soluble hydrolysate constituents, a defined volume of supernatant was taken from each sample, diluted 10-fold with water and filtered using 0.45 μm nylon filters. 20 μl of each filtered sample was injected into an Agilent 1200 series HPLC, equipped with a PL aquagel-OH 20 column (Agilent Technologies, Santa Clara, CA, USA) maintained at 40 °C. Water was used as mobile phase at a rate of 1 mL min^{-1} and detection was performed with both RID and DAD. The retention time was converted to molecular weight by calibration against polyethylene glycol standards.

Quantification of monomeric aromatic compounds

To quantify lignin-derived monomeric compounds, sample supernatants were collected and filtered through Nanosep 3 K centrifuge filters (Pall Laboratories, Port Washington, NY, USA) prior to analysis. Separation of metabolites was performed with an Eclipse Plus Phenyl-hexyl column (250 mm length, 4.6 mm diameter, 5 μm particle size; Agilent Technologies, Santa Clara, CA, USA) kept at 50 °C, with an injection volume of 5 μl. The mobile phase was composed of 10 mM ammonium acetate in water (solvent A) and 10 mM ammonium acetate in acetonitrile 90% (solvent B), prepared from a stock solution of 100 mM ammonium acetate and 0.7% formic acid in water. The following mobile phase gradient profile was used: 30% B (0 min; 0.5 mL min^{-1}), 80% B (12 min; 0.5 mL min^{-1}), 100% B (12.1 min; 0.5 mL min^{-1}), 100% B (12.6 min; 1 mL min^{-1}), 30% B (12.8 min; 1 mL min^{-1}), 30% B (15.6 min; 1 mL min^{-1}). Metabolites were quantified using calibration curves made with authentic standard compounds.

Results and discussion

Pretreatment and saccharification

To examine the scalability of the consolidated IL pretreatment and saccharification process at high solids loadings, a comparison of three scales (70 g, 700 g, and 5.25 kg of sorghum) was made in 1 L, 10 L, and 210 L reaction vessels (Table 1). Sorghum was loaded at 30% (w/w) during biomass pretreatment and then diluted to 20% (w/w) for enzymatic saccharification. This level of biomass loading requires high solids mixing capabilities due to minimal free water, and the efficiency of the process therefore depends heavily on the reactor design. A comparison of saccharification rates shows improvement with scale, with over 87% of final monomeric glucose and 80% of xylose released during the first 24 hours of saccharification in the 210 L reactor, compared to an average of 69% of glucose and 68% of xylose in the 10 L Parr reactors (Fig. 2). While the wall scraping impellers in the 1 L and 10 L Parr vessels provide effective mixing in the horizontal axis, the helical impeller in the 210 L Andritz vessel appears to improve reaction kinetics by providing additional mixing on the vertical axis. Improvements in process kinetics at scale are encoura-

Table 1 Reactor size, biomass loading, and sugar yields over four deconstruction campaigns. Lower glucose during run 4 is attributed to contamination by thermophilic lactic acid-producing bacteria, resulting in conversion of glucose to lactic acid during saccharification. Glucose yields in run 4 are comparable to smaller-scale campaigns after accounting for loss of sugars to lactic acid production. Error estimates represent the standard deviation of three analytical replicates

Run	Reactor volume (L)	Initial dry biomass (g)	Total mass pretreated (g)	Glucose (g L^{-1})	Xylose (g L^{-1})	Glucose yield (%)	Xylose yield (%)	Glucose + lactic acid yield (%)
1	1	70	233	50 ± 0.6	22.0 ± 2	75 ± 1.2	57 ± 3	n/a
2	10	700	2330	54.4 ± 0.7	22.2 ± 0.3	88 ± 1.1	57 ± 0.8	n/a
3	10	700	2330	52.5 ± 0.2	24.3 ± 0.1	85 ± 0.3	62 ± 0.3	n/a
4	210	5250	17 500	42.1 ± 0.3	24.1 ± 0.1	68 ± 0.5	63 ± 0.3	83 ± 1.0

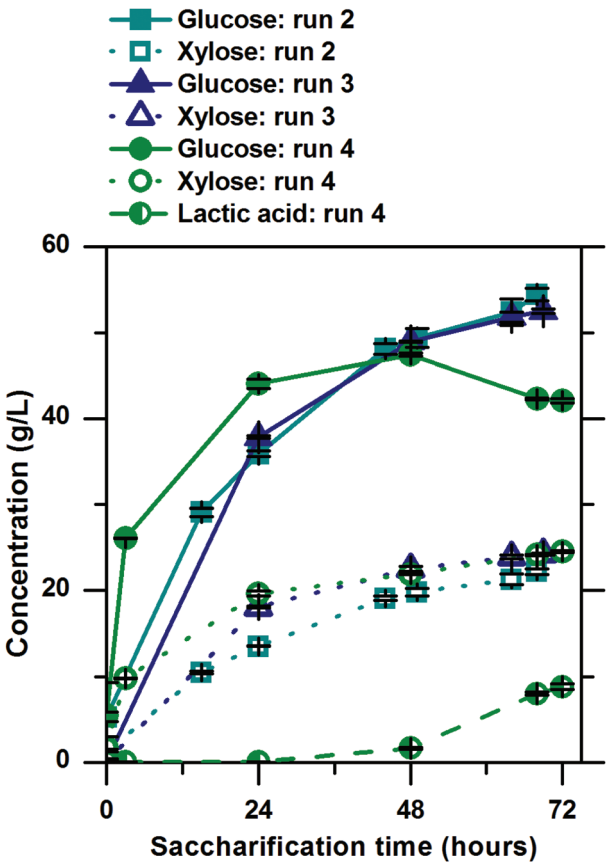

Fig. 2 Release of glucose and xylose during three 72-hour saccharification campaigns. Run 2 and run 3 were completed in a 10 L vessel; run 4 was completed in a 210 L vessel. Production of lactic acid was observed only in the 210 L campaign. Error estimates represent the standard deviation of three analytical replicates. Operating conditions and final glucose and xylose yields are detailed in Table 1.

ging and indicate potential compatibility with fed-batch or continuous process configurations.

Final glucose yields were relatively stable across scale, improving slightly from 75% at 1 L reactor scale to 88% in the second 10 L reactor campaign, up to a maximum of 54 g L^{-1} (Table 1). Glucose yields in the 210 L reactor decreased slightly relative to the 10 L reactor scale but remained above 80% after accounting for the glucose converted to lactic acid (see explanation below). Xylose yields improved at each stage of scale-up, from 57% at 1 L scale to a maximum of 63% in the 210 L pretreatment vessel, providing further indication of efficiency gains due to improved mass transfer in the larger-scale reactor. Overall, all major indicators of deconstruction rate and efficiency indicate equal or improved performance with scale.

In addition to the lignocellulosic sugars, two organic acids were generated during pretreatment and saccharification: acetic and lactic acid. Approximately 9 g L^{-1} of acetic acid was produced during pretreatment across all biomass deconstruction campaigns. This observation is not surprising as acetic acid is associated with hemicellulose and is often released from biomass during pretreatment.[25] Lactic acid appeared only during the final 24 hours of enzymatic saccharification in the 210 L reactor, ultimately reaching a concentration of 9 g L^{-1}. The appearance of lactic acid was linked to a decrease in glucose, indicating the presence of contaminating lactic acid bacteria (LAB). LAB contamination is a known risk during enzymatic saccharification of lignocellulose, and is generally considered detrimental to downstream fermentations using conventional conversion hosts.[26,27] However, as described below, the process deployed in this study is not negatively affected by the presence of lactic acid. While contamination is not a desirable outcome, it provided an opportunity to further assess the robustness of an end-to-end process using *Rhodosporidium toruloides*.

Fermentation with *Rhodosporidium toruloides*

Both acetic and lactic acids generated in during pretreatment and saccharification can have negative impacts on growth and productivity during fermentation of lignocellulosic hydrolysates.[28–30] To understand how these acids affect *R. toruloides*, they were tested alone, in combination, and in combination with glucose and xylose to determine their ability to sustain cell growth and bisabolene production (Fig. 3). *R. toruloides* grew on acetic acid and lactic acid at concentrations similar to those found in the hydrolysates (~8 g L^{-1} and ~20 g L^{-1} respectively), both individually and in combination, and produced bisabolene titers from 50–200 mg L^{-1} with OD_{600} ranging from 3–10. This result confirms that both organic acids are metabolized for biomass and product for-

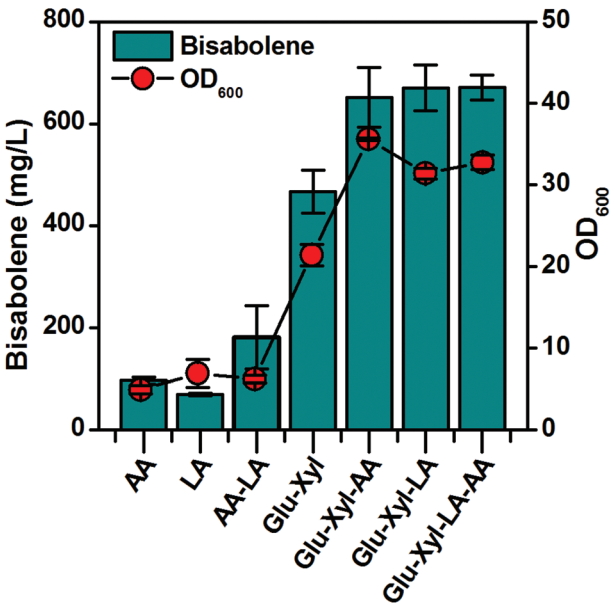

Fig. 3 Impact of acetic and lactic acid on peak OD_{600} after 5 days fermentation and peak bisabolene production after 11 days fermentation. Four carbon sources were evaluated alone and in combination: 22 g L^{-1} glucose (Glu), 14 g L^{-1} xylose (Xyl), 8 g L^{-1} acetic acid (AA), and 21 g L^{-1} lactic acid (LA). Error bars represent the standard deviation of three biological replicates.

mation. Both acetic and lactic acid also improved final bisabolene titers from ~500 mg L^{-1} to ~700 mg L^{-1} when added to a mock hydrolysate medium containing glucose and xylose, indicating that these organic acids act as a carbon supplement to the pentose and hexose sugars and do not have a negative impact on their conversion. This metabolic flexibility and tolerance to relatively high concentrations of organic acids further underscores the advantages of *R. toruloides* as a platform host for conversion of complex lignocellulosic hydrolysates.

Once it was established that acetic and lactic acids are compatible with the bioconversion of lignocellulosic sugars using *R. toruloides*, hydrolysate from 10 L-scale deconstruction run 3 was used to examine the impact of pH and nitrogen source on growth and bisabolene production. As the primary goal of this study is to consolidate unit operations and avoid intermediate separation processes, residual solids remaining after saccharification were retained in all of the following shake flask and bioreactor fermentations. Performance in shake flasks was evaluated at an initial pH of 5 or 7 and with two nitrogen sources: yeast extract-peptone (YEP) or 5 g L^{-1} ammonium sulfate (AS). A previous study indicated that an initial pH of 7.5 is optimal for bisabolene production in shake flasks,[16] but an initial pH of 5 would be advantageous, as it would eliminate the need for pH adjustment following enzymatic saccharification. While an initial pH of 5 resulted in a significant lag phase relative to the pH 7 conditions, particularly in the AS hydrolysate, the final bisabolene titer was higher with a starting pH of 5, up to a maximum titer of 1.3 g L^{-1} in the AS hydrolysate after 12 days of fermentation (Fig. 4). Addition of complex media components proved unnecessary, as hydrolysates containing AS outperformed those containing YEP at both pH 5 and pH 7.

Following process verification in shake flasks, performance was evaluated at 2 L scale in batch fermentation using the same 10 L scale hydrolysate as the shake flask campaigns (Fig. 5A–D). Both the YEP and the less costly AS were tested as nitrogen sources. To balance the higher bisabolene titer observed in shake flasks in the pH 5 AS hydrolysate with the shorter lag phase observed at pH 7, all of the bioreactor campaigns were initiated at pH 7 and the pH was allowed to drop naturally until reaching a set point of pH 5. In each case, the carbon sources were fully consumed within two days, with immediate co-consumption of glucose and acetate followed by full consumption of xylose in the second day of fermentation (Fig. 5A and C). Both ammonium and phosphate were also depleted within two days (Fig. 5B and D). Ammonium levels rose again at the end of fermentation, likely due to cell lysis. Notably, a large fraction of observed bisabolene production in both reactors occurred after carbon, nitrogen, and phosphorus were fully depleted, with bisabolene production continuing throughout the 14 days of fermentation.

Considering that *R. toruloides* is an oleaginous yeast, we hypothesize that a portion of the sugars consumed during the first 2 days were stored as triglycerides and then slowly remobilized and converted into bisabolene in days 2–14 *via* diversion of acetyl-CoA generated from fatty acid beta-oxidation into the mevalonate pathway. This explanation is consistent with the depletion of both nitrogen and phosphorus, as nutrient depletion is a trigger of lipid production in oleaginous yeasts.[31] Final bisabolene titers reached 2.0 g L^{-1} in the YEP medium and 1.9 g L^{-1} in the ammonium sulfate medium, a 1.5-fold improvement over the equivalent conditions in shake flask fermentation (Fig. 3). This increase in titer is likely caused by a combination of improved mixing, oxygen transfer, DO control, and pH control in the bioreactor.

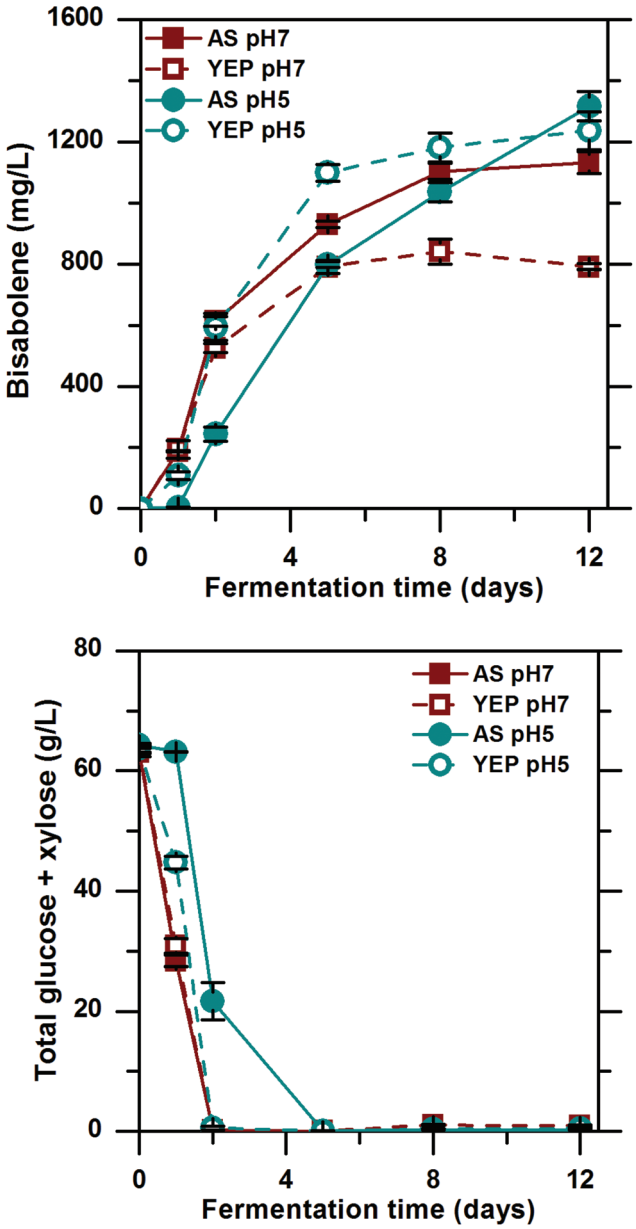

Fig. 4 Effect of variable pH at inoculation and variable nitrogen sources on bisabolene production and sugar consumption in 30 mL shake flask cultivation with unfiltered hydrolysate. Conditions tested include pH 5, pH 7, addition of YEP medium, and addition of 5 g L^{-1} ammonium sulfate (AS). Error bars represent standard deviation of three biological replicates.

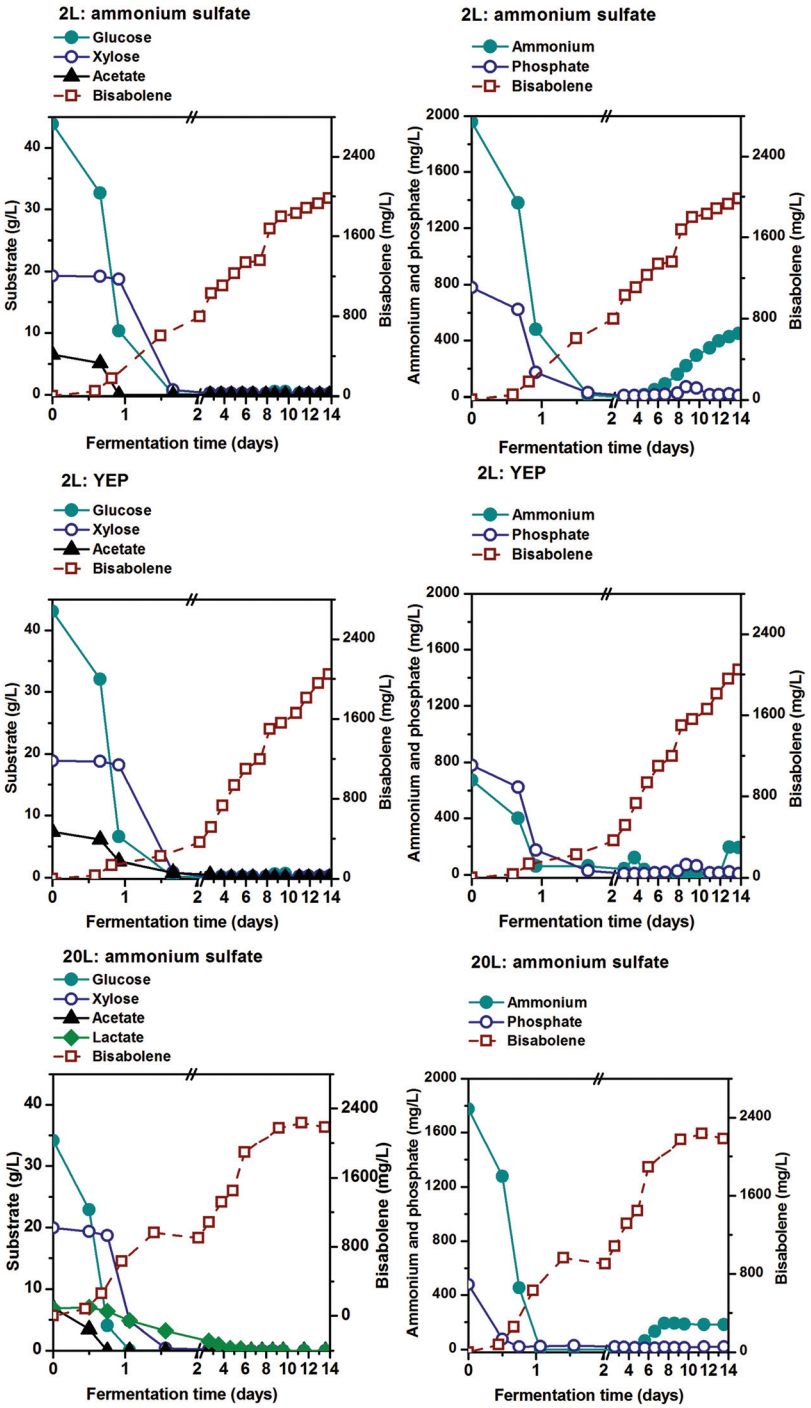

Fig. 5 Carbon consumption, nutrient consumption, and bisabolene production over the course of three scale-up fermentations. Three conditions were tested: 2 L scale with ammonium sulfate medium, 2 L scale with YEP medium, and 20 L scale with ammonium sulfate medium and residual lactic acid from enzymatic saccharification.

To further demonstrate process scalability, unfiltered hydrolysate produced in the 210 L deconstruction vessel was used to test the fermentation process at 20 L scale with AS as the sole media addition. Despite the presence of 9 g L^{-1} lactic acid produced during scale-up of enzymatic saccharification, bisabolene titers reached 2.2 g L^{-1} at the 20 L scale, the highest value recorded to date with *R. toruloides* (Fig. 5E and F). Lactic acid was fully consumed after 4 days of fermentation, with its consumption beginning after glucose, xylose, and acetate were depleted (Fig. 5E). Similar to the 2 L fermentations, the carbon and nutrients in the hydrolysate were depleted quickly, while bisabolene consumption continued over the course of the 14-day fermentation.

Conversion of solubilized lignocellulose beyond monomeric sugars

The heterogeneity of lignocellulosic hydrolysates is a hurdle towards efficient carbon utilization, and numerous components beyond monomeric sugars must be identified and quantitated in order to fully assess their bioconversion. Therefore, in addition to monomeric sugars and organic acids, we attempted to quantify monomeric aromatic compounds and higher molecular weight components, including soluble oligomeric sugars and lignin. Analysis *via* HPLC reveals very low concentrations of aromatic monomers present in the hydrolysate. 4-Hydroxybenzoic acid was the primary monomer detected with a starting concentration of 238 mg L^{-1}, all of which was completely consumed after 25 h of cultivation (ESI Table 1†). The combined yields of other aromatic compounds including benzoic acid, vanillic acid, ferulic acid, vanillin and coumaric acid represented less than 40 mg L^{-1} in total and were not appreciably consumed. Although *R. toruloides* has the capability to tolerate and assimilate several lignin-derived aromatics at a gram per liter scale,[16] it is clear that the [Ch][Lys] pretreatment used in this study does not significantly depolymerize sorghum lignin into known aromatic monomers. However, an examination of higher molecular weight solubilized lignocellulose *via* gel permeation chromatography (GPC) suggests that some degree of lignin depolymerization may be occurring during cultivation (Fig. 6).

Residual solids following fermentation contained 28.7 ± 4% glucan, 14 ± 3% xylan, and 32 ± 1.9% lignin, as compared to 28 ± 1.4% glucan, 17 ± 1.8% xylan, and 18.8 ± 0.2% lignin in the initial biomass. This increase in the carbohydrate fraction of the solids, despite efficient liberation of both C6 and C5 sugars during saccharification, is consistent with significant solubilization of lignin and a concurrent reduction in total insolubles. Analysis of the underivatized water-soluble fraction of the hydrolysate indicates that most compounds below a molecular weight of 1000 Da that produce a refraction index (RI) signal are consumed within the first four days of fermentation (including monomeric and dimeric sugars). In addition, molecules in the range of 1000–4000 Da exhibit a modest but marked decrease in molecular weight over time when traced with both RI and UV detectors. These components likely correspond to oligomeric sugars and lignin detected by RI, and water-soluble lignin oligomers detected by UV.[32] Lignin degradation is well documented in basidiomycetes, and evidence provided here suggests that *R. toruloides* may share some of the same lignin-degrading abilities with its relatives, including the white rot fungi *Phanerochaete chrysosporium*.[33] However, further studies are needed to thoroughly assess and verify its potential ability to degrade lignin and sugar oligomers.

Opportunities for process intensification

Adapting this process for widespread commercialization will require process improvements targeting volumetric productivity, carbon efficiency, and downstream processing cost.

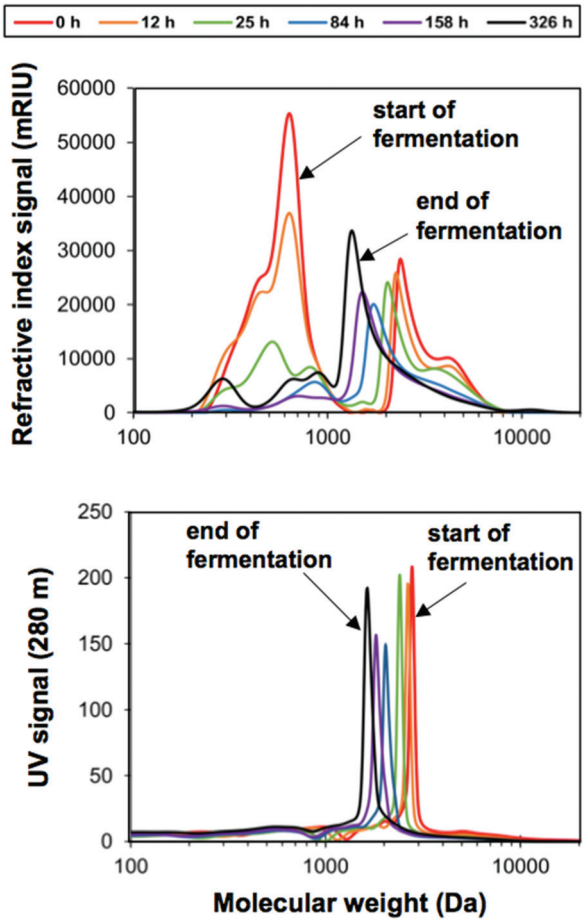

Fig. 6 Gel permeation chromatography (GPC) analysis of water-soluble organics during fermentation at 20 L scale, analyzed on both a refractive index and UV detector. Data is presented from six time points throughout the fermentation campaign.

Given the resilience of *R. toruloides* under osmotic stress, high gravity fed-batch saccharification could be employed to increase sugar concentrations in the hydrolysate. The strain employed in this study is minimally engineered; bisabolene titers, rates, and yields could be improved by routing additional carbon flux to the mevalonate pathway, including re-routing of carbon currently directed towards lipid production. Volumetric process throughput could also be improved by adapting the process for simultaneous saccharification–fermentation. Perhaps most critically, an efficient IL separations and recycle process will be required to cost effectively recover ILs and purify biofuels for downstream use. A number of promising techniques have now been demonstrated for high-yield recovery and reuse of ionic liquids from complex mixtures, including pervaporation, electrodialysis, and three-phase separation *via* addition of kosmotropic salt solutions.[34–36] In the case of pervaporation, up to 99.9% recovery of 1-ethyl-3-methylimidazolium acetate was reported following pretreatment, with pretreatment effectiveness retained over five cycles of pretreatment and recovery. In the fully consolidated process, downstream processing would include

density based solid–liquid–liquid separation to partition the organic phase, aqueous phase, and residual biomass, followed by IL recovery from the aqueous phase and recycling for subsequent rounds of pretreatment.

Conclusions

This study is the first demonstration of a fully consolidated process combining IL pretreatment, enzymatic saccharification, and biocatalysis for production of the advanced biofuel precursor bisabolene using an engineered host. This is also the first such demonstration with *R. toruloides*, or with any host capable of co-consuming alternative substrates including xylose, lactic acid, and aromatic lignin decomposition products. The process proved to be robust under high-intensity conditions at bench and larger scales, with both the deconstruction and bioconversion platforms improving performance at scale despite the presence of organic acids produced during biomass deconstruction. No separations were required prior to saccharification or fermentation, and minimal media additions are required to facilitate bioconversion, resulting in an efficient and streamlined process. Bisabolene titers improved when transitioning from mock hydrolysate to unfiltered IL hydrolysate and full consumption of all measured carbon sources was observed within 48 hours of fermentation, demonstrating the promise of *R. toruloides* as a metabolically flexible platform organism for conversion of IL pretreated biomass at industrial scale. Given recent advances in synthetic biology, use of a non-canonical platform organism with a highly flexible metabolism, high IL tolerance, and high osmotic tolerance is an increasingly viable alternative to engineering such properties into a canonical host. With further intensification and optimization, this process is a promising new approach towards commercial production of low-cost and low-impact lignocellulosic biofuels.

Conflicts of interest

There are no conflicts of interest to declare.

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