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Publication Date

2019-08-01

DOI

10.1016/j.biortech.2019.121365

Peer reviewed

**Conversion of depolymerized sugars and aromatics from engineered
feedstocks by two oleaginous red yeasts**

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Abstract

One of the requirements for efficient biological conversion of lignocellulose to bioproducts is the compatibility of biological catalysts with the processes employed to solubilize and depolymerize the lignocellulosic components. The red yeasts *Rhodospiridium toruloides* and *Rhodotorula mucilaginosa* were evaluated for their ability to assimilate sugars and aromatic compounds extracted from two engineered lines of *Arabidopsis thaliana* with modified lignin or the wild-type using ionic liquid, acid or alkaline pretreatments. Differential amounts of monomeric sugars, organic acids and, in the case of the engineered lines, either 4-hydroxybenzoic or protocatechuic acid were additionally released from the biomass and found to be tolerated and consumed by both microorganisms. Genetically-engineered strains of the two red yeasts successfully converted the depolymerized products into the biofuel precursor bisabolene when cultivated on hydrolysates or synthetic media containing specific sugars, acids and aromatics found in the hydrolysates.

Keywords: *biomass pretreatment, aromatic compounds, bisabolene, red yeasts, biofuels*

1. Introduction

Within a lignocellulosic biorefinery, biomass pretreatment is essential to allow for efficient deconstruction of lignocellulosic feedstocks into substrates that can be biologically converted into biofuels and bioproducts (Jönsson and Martín, 2016). A variety of pretreatment processes have demonstrated to be effective at reducing lignocellulose recalcitrance and releasing fermentable sugars, by promoting chemical hydrolysis reactions and increasing the accessibility of enzymes for degradation of specific components (Bhutto et al., 2017; Hu and Ragauskas, 2012). Popular approaches include the implementation of ionic liquid (IL), acid, or alkaline pretreatments, usually coupled with enzymatic saccharification (Behera et al., 2014; Menon and Rao, 2012; Rabemanolontsoa and Saka, 2016), as well as the use of organic or deep eutectic solvents, among many other strategies (Mamilla et al., 2019; Sindhu et al., 2016; K. Zhang et al., 2016). However, the extent of solubility and depolymerization of the cellulosic, hemicellulosic and lignin components for a given process is highly dependent on the biomass source and reaction conditions. Moreover, the successful coupling of pretreated and deconstructed hydrolysates to a biological fermentation step requires organisms that tolerate potential toxic compounds in the media derived from the pretreatment process or the lignocellulose itself, and are capable of growing rapidly on the generated substrates (Jönsson and Martín, 2016; Klinke et al., 2004).

Until recently, lignin has played a secondary role in processes centered on the biological conversion of lignocellulose. The main reasons are that this polymer features a high degree of heterogeneity, hydrophobicity, resistance to chemical or enzymatic depolymerization, and the fact that sugars are preferred carbon and energy sources for most microorganisms over lignin-

derived compounds (Nieves et al., 2015). Nevertheless, lignin is an important constituent of biomass (10-30 % wt.) and its valorization has been deemed essential to improve biorefinery economics (Ragauskas et al., 2014; Rinaldi et al., 2016). Several proposed biological lignin valorization schemes consider the use of microorganisms as cell factories to funnel the different types of molecules present in pretreated streams into common intracellular metabolic intermediates, which can then be converted to valuable products (Beckham et al., 2016; Bugg and Rahmanpour, 2015; Linger et al., 2014; Palazzolo and Kurina-Sanz, 2016; Xu et al., 2019). A drawback of this approach is that the yields of lignin-derived monomeric compounds generated during pretreatment that can be biologically assimilated are often very low. Different strategies have been applied to increase lignin monomer yields, often requiring severe reaction conditions such as high temperature, pressure or catalyst concentrations (Abdelaziz et al., 2016; Schutyser et al., 2018). However, compounds commonly found in liquors generated under harsh conditions are known to cause growth inhibition to microorganisms even at relatively low concentrations (Jönsson et al., 2013). Even if this toxicity can be partially overcome, a single organism may only be able to metabolize a fraction of these compounds and, if a previous separation step is not included, thermochemical degradation of sugars or other undesired effects may also occur (Ko et al., 2015). One alternative to circumvent this problem and obtain an integrated bioprocess could be the generation of feedstocks that favor the release of known metabolizable compounds with low toxicity, coupled to the use of organisms with natural tolerance and the capability to assimilate these types of compounds.

In Nature, fungi contribute to biomass degradation by producing and secreting enzymes that break down lignocellulosic components into metabolizable substrates (Bomble et al., 2017). The use of several species of fungi with the natural ability to assimilate lignocellulose-derived

components has recently gained interest because they can attain high cell densities under controlled conditions and are tolerant to inhibitory compounds (Poontawee et al., 2017). In particular, some oleaginous red yeasts from the phylum Basidiomycota are known to consume hexoses, pentoses, organic acids and aromatic monomers, while efficiently transforming them into cell biomass that is rich in lipids and carotenoids (Patel et al., 2016; Shi and Zhao, 2017; Yu et al., 2011). It has previously been shown by our group and others that two red yeast species, *Rhodosporidium toruloides* (also called *Rhodotorula toruloides*) and *Rhodotorula mucilaginosa*, are good potential candidates for valorization of biomass hydrolysates. In particular, *R. toruloides* is known to exhibit fast growth in media with high sugar concentrations and has arisen as a promising host for conversion of sugars and aromatics into terpene biofuels and bioproducts, such as bisabolene (Sundstrom et al., 2018; Yaegashi et al., 2017). Bisabolene can then be hydrogenated to produce bisabolane, which is considered an advanced biofuel with diesel and jet fuel applications (Peralta-Yahya et al., 2011). On the other hand, a related *R. mucilaginosa* strain isolated from soil has recently shown the ability to thrive in lignin-rich liquors with high concentrations of salt and aromatic monomers (Rodriguez et al., 2017). A direct comparison of the performance of these species as conversion hosts in different types of hydrolysates would allow for a better understanding of their capabilities and limitations, and could help improve process design.

In this work, we assessed the potential of these red yeasts to convert lignocellulosic hydrolysates into biofuels by employing three common lignocellulosic pretreatments, and evaluating the ability of engineered strains to grow in them and produce the biofuel precursor bisabolene. This comparison was extended by using two engineered lines of *Arabidopsis thaliana* (*Arabidopsis*) that express bacterial genes coding for either a 3-dehydroshikimate dehydratase (QsuB) or a

hydroxycinnamoyl-CoA hydratase/lyase (HCHL) enzymes, resulting in the accumulation of specific aromatics derived from lignin biosynthetic pathways and modification of the lignin content and structure (Eudes et al., 2015, 2012).

2. Materials and methods

2.1. Strains, plasmids and plant lines

The strains and plasmid sequences used in this work are deposited in the Joint BioEnergy Institute public registry and can be accessed at <https://public-registry.jbei.org/login> with the following ID numbers (in parentheses): *Rhodospiridium toruloides* BIS18 (JBx_086882), *Rhodotorula mucilaginosa* J31 (JBx_061726), *Rhodotorula mucilaginosa* BIS11 (JBx_100577) and *Agrobacterium tumefaciens* EHA 105 (ABF_003056). The employed *Arabidopsis* lines (C4H::qsuB-1.2, pIRX5::HCHL-2 and their respective wild-type controls, ecotype Columbia Col-0) have previously been described (Eudes et al., 2015, 2012). *R. mucilaginosa* J31 was transformed with plasmid 2-GPD1-BIS-Tnos (JBx_065214). This plasmid contains a bisabolene synthase (BIS) gene that is codon-optimized for *R. toruloides* IFO0880 (JBx_086520) and controlled by the GAPDH promoter from the same species. The same plasmid was used to generate *R. toruloides* BIS18 and other bisabolene-producing strains from *R. toruloides* IFO0880 in a previous report (Yaegashi et al., 2017). *R. mucilaginosa* J31 and *R. toruloides* IFO0880 were the wild-type yeast strains used in this work.

2.2. Generation and selection of bisabolene producers

127 *Agrobacterium tumefaciens* mediated transformation (ATMT) was the technique of choice to
128 generate the *R. mucilaginosa* recombinant strains, using plasmid 2-GPD1-BIS-Tnos and
129 following a previously reported protocol (S. Zhang et al., 2016). Transformants carrying
130 genomic integrations of the bisabolene synthase gene were selected on agar plates with 300
131 µg/mL carbenicillin, 300 µg/mL cefotaxime, and 50 µg/mL nourseothricin for 5 days at 30 °C
132 and verified by PCR using the following primers (5'-3'): BisFw, atggccggcgctcggccgtct and
133 BisRv, tcagagagggaggggctcgatg. For bisabolene production screening, 40 positive colonies were
134 cultured at 30 °C in test tubes with 5 mL of BD Difco YPD broth (ThermoFisher, USA)
135 supplemented with additional glucose (100 g/L final concentration). A 40% (v/v) dodecane
136 overlay was used to capture bisabolene. After 5 days of incubation, samples were collected from
137 the dodecane layer and diluted (1:1) in ethyl acetate containing 5 mg/L of caryophyllene as
138 internal standard, prior to GC-MS analysis. From this screening, *R. mucilaginosa* strain BIS11
139 was identified as the highest bisabolene producer obtained for this species. *R. toruloides* BIS18
140 was selected from other *R. toruloides* bisabolene producing strains in our archive generated with
141 the same procedure, because of its similar production levels and BIS gene copy number,
142 compared to *R. mucilaginosa* BIS11. The relative copy number comparison was performed by
143 qPCR using genomic DNA from wild-type and recombinant strains as template, extracted with a
144 Maxwell RSC Instrument and Maxwell RSC Plant DNA Kit (Promega, USA) from overnight
145 cultures in YPD broth. Templates were diluted to a final concentration of 0.5 ng/µL and mixed
146 with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, USA) and primers at a final
147 concentration of 0.25 µM, with the following sequences (5'-3'): Bis5'Fw, atcaagaacggcatcacct;
148 Bis5'Rv, gagttgagctccgtgaggac. A primer pair that anneals to the actin gene was used as a
149 reference, with sequences (5'-3'): actinFw, aagctctgctacgtcgcgct and actinRv,

gtacgtcgtctcgtggatgc. The following amplification protocol was used for all templates and primer pairs: 95 °C for 2 min, 40 cycles of 95 °C for 15s and 95 °C for 1 min, followed by a dissociation protocol going from 60 °C to 95 °C, increasing 0.2 °C every 10 s.

2.3. Plant growth conditions

Arabidopsis seeds were germinated directly on soil and plants grown individually using the Arasystem (Betatech, Belgium). Growing conditions were 14 h light/day at 100 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$, 22 °C and 55% humidity for the IRX5::HCHL-2 line, and 10 h light/day at 150 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$, 22 °C and 60% humidity for the C4H::qsuB-1.2 line. For each line, wild-type control plants were grown in the same tray and under the same conditions. Stems from dried mature senesced plants were harvested without leaves, siliques and seeds, completely dried in an oven at 50 °C overnight, and ball-milled to fine powder using a Mixer Mill MM 400 (Retsch Inc., USA) and stainless steel balls for 2 min at 30 s^{-1} .

2.4. Biomass pretreatments and generation of hydrolysates

Ball-milled biomass was mixed with either 10% (w/w) cholinium lysinate (ChLys), 1.2% H_2SO_4 (w/v) or 2% (w/v) NaOH in water. For ionic liquid (IL) pretreatment, 40 mg of biomass was mixed with 220 μL of 10% ChLys (solids loading 18.2% (w/v)). For acid and base pretreatment, 40 mg of biomass was mixed with 300 μL of dilute H_2SO_4 or NaOH, respectively (solids loading 13.3% (w/v)). These pretreatment experiments were performed in duplicate. Samples were set up to react in an autoclave at 121 °C for 1 h. IL-pretreated samples were then diluted with 780 μL of deionized water, and the acid and base samples with 700 μL of water to reach a final volume of 1

mL. Solids were then separated by centrifugation. The concentrations presented in all experimental graphs represent the final concentrations after water addition. A separate scale-up reaction using acid treatment was prepared in order to increase the final hydrolysate volume and concentration for bisabolene production experiments. To do this, proportionally higher amounts of biomass (424 mg) and dilute H₂SO₄ (3180 µL) were mixed in 20 mL glass pressure tubes and reacted in an autoclave at 121 °C for 1 h. Then, water was added (2120 µL) to reach a final volume (5300 µL) that resulted in hydrolysates with 2-fold higher concentration relative to the small scale. The resultant liquid fractions were separated by centrifugation and used for bisabolene production experiments, after addition of 10-fold concentrated SD media, adjusting the pH to a value of 7.4 and filtering the final media with 0.45 µm cellulose acetate syringe filters (Thermo Fisher Scientific, USA).

2.5. Analysis of monomeric aromatic compounds

To quantify aromatics, sample supernatants were collected and filtered through 0.45 µm centrifuge filters before HPLC analysis. The separation of compounds was performed using an Agilent Technologies 1260 infinity series HPLC system equipped with an Eclipse Plus Phenyl-hexyl column (250 mm length, 4.6 mm diameter, 5 µm particle size; Agilent Technologies, USA) that was maintained at 50 °C, using 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), 80 % B (12 min; 0.5 mL/min), 100 % B (12.1 min; 0.5 mL/min), 100 % B (12.6 min; 1 mL/min), 30 % B (12.8 min; 1 mL/min), 30 % B (15.6 min; 1 mL/min). Diode array

detectors set at 254, 280 and 310 nm wavelengths were used for detection. Concentrations were determined using calibration curves made with peak areas obtained from pure standards.

2.6. Analysis of sugars, organic acids and furfurals

The concentrations of glucose, xylose, arabinose, acetic acid, formic acid, 5-hydroxymethylfurfural and furfural were quantified using an Agilent Technologies 1260 infinity series HPLC system equipped with an Aminex HPX-87H column (Bio-Rad, USA). The mobile phase employed was 4 mM H₂SO₄ (pH = 2) with a flow rate of 0.6 mL/min and a column temperature of 65 °C. Sample injection volumes of 5 µL were used. Prior to analysis, samples were filtered through 0.45 µm centrifuge filters. Compounds were monitored by a refractive index detector (kept at 35 °C and configured for a 4 s response time) and their concentrations were calculated by integration of peak areas and comparison to a calibration curve made from pure standards.

2.7. Media and cultivation conditions

Seed cultures were obtained by inoculating the yeast strains in 5 mL of YPD broth and incubated overnight at 30 °C and 200 rpm. Overnight cultures were diluted 10 times with fresh media and grown until exponential phase prior to transferring to the cultivation media. Synthetic defined (SD) media was prepared by mixing BD Difco yeast nitrogen base without amino acids (Thermo Fisher Scientific, USA) and complete supplemental mixture (CSM) (MP Biomedicals, USA), following manufacturer's instructions, with the exception that 1/10th of the recommended amount of CSM was used to avoid supplementing high concentrations of amino acids. SD media was added as a 10x stock to experiments with hydrolysates or single substrates.

For growth experiments, the initial pH of the media was adjusted to 7.4 using concentrated NaOH or H₂SO₄, filtered through 0.45 µm nylon centrifuge filters (VWR, USA), and transferred to lidded 96-well plates (Falcon clear plates, Corning, USA), where 140 µL of media and 10 µL of cells per well were used. The cultures were incubated at 30 °C in a DTX 880 Multimode Detector (Beckman Coulter, USA) and optical density (OD) at 595 or 620 nm was recorded every 5 minutes for 48 hours. At the end of the experiments with hydrolysates, the supernatants were separated by centrifugation and filtration, and kept frozen until analysis. In all cases, growth rates were calculated by obtaining the slope of growth curves during the exponential phase, after plotting the natural logarithm of OD values versus time. For bisabolene production experiments, transparent 48-well FlowerPlates (m2p labs, Germany) were used for cultivation, employing 784 µL of media, 16 µL of cells and 200 µL of a dodecane overlay, and covered with sterile AeraSeal films (Excel Scientific, USA). The plates were incubated for 7 days in a humidity-controlled incubator with orbital shaking at 800 rpm. The entire contents of each well were collected in eppendorf tubes, where the dodecane layer, supernatant, and cells were separated by centrifugation. After the removal of the liquids, the cell pellets were resuspended in 800 µL of water, diluted four-fold with water, and 100 µL were transferred to 96-well plates to measure final optical density at 600 nm using a SpectraMax Plus 384 reader (Molecular Devices, USA). All cultivations were performed in duplicate.

2.8. Bisabolene quantitation

Bisabolene producing strains were cultured with 20 % (v/v) dodecane overlay to capture bisabolene. Samples were taken from the dodecane layer and dissolved in ethyl acetate, containing 5 mg/L of caryophyllene as internal standard. The amount of bisabolene in the

dodecane layer was quantified by GC-MS using an Agilent Technologies 6890N system, equipped with a 5973 mass selective detector and a DB-5ms column (30 m x 250 μ m x 0.25 μ m, Agilent Technologies, USA). Splitless 1 μ L injections were used on a GC oven program consisting on 100 °C for 0.75 min, followed by a ramp of 40 °C per min until 300 °C, and held 1 min at 300 °C. Injector and MS quadrupole detector temperatures were 250 °C and 150 °C, respectively. Bisabolene concentrations were calculated by integration of the peak area values obtained in selective ion monitoring mode, compared to a calibration curve made with pure bisabolene, and normalized with the areas obtained from the spiked caryophyllene, as internal standard.

3. Results and discussion

3.1. Generation and characterization of hydrolysates

The first objective of this work was to evaluate the influence of the pretreatment and type of biomass on the composition of hydrolysates, focusing on polysaccharide and lignin-derived monomers. Three common pretreatment conditions, IL (10 % cholinium lysinate), acid (1.2 % H₂SO₄) or base (2 % NaOH), were applied to dried milled *Arabidopsis* biomass from engineered lines C4H::qsuB-1.2 (QsuB), IRX5::HCHL-2 (HCHL), and their respective wild-type controls, called Q and H (Fig. 1). The concentrations of monomeric sugars, aromatics, organic acids and furfurals in the liquid fraction of the resulting hydrolysates were quantified and compared (Fig. 2). Tracked compounds include glucose, xylose, arabinose, protocatechuic acid (PCH), 4-hydroxybenzoic acid (4-HBA), acetic acid (AA), formic acid (FA), furfural, and 5-hydroxymethylfurfural (HMF).

264 The analysis revealed differences in the type and concentrations of monomers released among all
265 pretreatment conditions and biomass types (Fig. 2). Regarding the aromatic compounds, 4-HBA
266 and PCH were found in considerable amounts only in biomass from HCHL and QsuB plant lines,
267 respectively. These observations are consistent with previous metabolite measurements
268 performed on these lines and are inherent to the engineering strategy employed on them (Eudes
269 et al., 2015, 2012). Acid pretreatment was the only condition that released PCH in QsuB biomass
270 samples (328 mg/L), presumably due to its occurrence mainly as phenolic glucosides. In
271 contrast, all pretreatments released 4-HBA from HCHL biomass with the following yields: 142
272 mg/L for IL, 219 mg/L for acid and 280 mg/L for base treatments. This result is in agreement
273 with previous observations showing that most 4-HBA occurs in both its phenolic glucoside and
274 glucose ester forms in HCHL plants (Eudes et al., 2012). Other hydroxycinnamic acids that are
275 typically found associated to the cell walls in grasses such as ferulic and *p*-coumaric acids
276 (Ralph, 2010) were detected in very low amounts or not detected at all.

277 On the other hand, IL and base pretreatments resulted in similar sugar yields compared to each
278 other, although much lower than the ones obtained with acid (Fig. 2a and 2c). On this regard, it is
279 known that dilute acid conditions can dissolve and hydrolyze hemicellulosic components,
280 particularly xylan, resulting in the release of monomeric sugars (Hendriks and Zeeman, 2009).
281 Xylose was the most abundant sugar monomer in all cases, followed by glucose and minor
282 amounts of arabinose. Although these pretreatment methods can be followed by an enzymatic
283 hydrolysis step for efficient release of additional monosaccharides from biomass, it should be
284 emphasized that the scope of this work was not to find optimal conditions for the release of
285 sugars, but rather to test the applicability of different types of hydrolysates as cultivation media
286 towards their biological valorization. Moreover, the low sugar yields obtained in some conditions

does not imply that the employed methods are not efficient to solubilize and depolymerize the constituents of lignocellulosic biomass when an enzymatic hydrolysis step is included, as it has been demonstrated before (Liu et al., 2017). Enzymatic hydrolysis was intentionally not employed in these experiments to make it easier to monitor consumption and conversion of the other components, which have comparatively lower concentrations and are less well studied compared to glucose and xylose. Despite not using enzymes, some of these sugars were still released into the hydrolysates, enabling them to be monitored as well.

The combined concentrations of formic and acetic acids reached several grams per liter with all conditions, although base pretreatment resulted in higher amounts in most cases (Fig. 2b and 2d). Furfural and HMF were detected only after acid pretreatment, with combined concentrations nearing 200 mg/L. It was also observed that the absolute monomer concentration values obtained for the two batches of wild-type plants were different but featured a similar profile (Fig. 2a and 2c). Even if acid pretreatment stands out as the most effective approach to release sugars and aromatics in the conditions evaluated here (and without considering enzyme addition), it is important to consider that potential growth inhibitors such as acetic acid, formic acid, and furfurals were also generated.

3.2. Substrate consumption profiles of R. toruloides and R. mucilaginosa

After identification of the main monomeric compounds present in the hydrolysates, it was important to identify which ones the red yeasts can assimilate. Considering that there is limited information in the literature about consumption of aromatics by these yeast species, other common lignin-derived aromatic acids were also included for comparison, namely vanillic acid, ferulic acid, *p*-coumaric acid and benzoic acid. These experiments were performed using initial

compound concentrations of 1 g/L. This low concentration was selected to minimize potential toxic effects, and the resultant cell biomass values were compared to the ones obtained with only the base SD media. As shown in Figure 3, both yeasts are able to grow on each of the main three sugars, acetic acid, and the two aromatics found in hydrolysates of HCHL and QsuB biomass (i.e., 4-HBA and PCH). However, modest differences in growth rates and maximum cell biomass were observed for the different aromatics at the tested concentration. Glucose was the carbon source that resulted in the fastest growth, which was about 3 times higher than xylose, 4 times higher than arabinose and 2 times higher than acetic acid (Fig. 3). Interestingly, some growth rates on aromatics were higher than the ones obtained on xylose or acetic acid, which shows that compounds like 4-HBA can be rapidly assimilated if present at a sub-toxic concentration in the media. In general, *R. mucilaginosa* accumulated more cell biomass and, except for media with ferulic or benzoic acids, did it faster than *R. toruloides*. For example, while biomass yields were very similar for both yeasts grown on glucose or PCH, the growth rates of *R. mucilaginosa* on these substrates were higher. No growth from any organism was observed on vanillin, formic acid, HMF, and 4-hydroxybenzaldehyde. These results are in agreement with previous observations on molecule toxicity in other microorganisms, where some phenolic or furan aldehydes obtained during pretreatment (e.g. vanillin, HMF and 4-hydroxybenzaldehyde) appear to be especially toxic (Nieves et al., 2015; Zaldivar et al., 1999). The similar substrate consumption profiles are an indicator of the close phylogenetic relationship among these yeast species and suggest that they may harbor similar catabolic pathways.

3.3. Evaluation of hydrolysates as cultivation media

332 To test the capabilities of the recombinant *R. toruloides* and *R. mucilaginosa* strains to grow on
333 and convert the depolymerized components produced by the different pretreatments, the pH of
334 the hydrolysates was first adjusted to 7.4 and the liquid fraction was separated and supplemented
335 with phosphate and nitrogen salts, before incubation with the cells. The results show that both
336 species are able to grow on media obtained from all pretreatment conditions, although some
337 differences in maximum cell biomass (ranging from 0.47 to 1.09 OD₅₉₅) and growth rates (from
338 0.11 to 0.26 h⁻¹) were observed (Fig. 4a and 4b). Based on these parameters, it was found that *R.*
339 *mucilaginosa* BIS11 performed as well or better than *R. toruloides* BIS18 in all types of
340 hydrolysates. However, when comparing the effects of using wild-type or transgenic feedstocks,
341 virtually no differences in growth performance between hydrolysates obtained from the same
342 pretreatment could be observed. This is probably a consequence of the similar total amounts of
343 monomers released between various feedstocks, with only slightly higher values obtained for the
344 engineered types, particularly under acid conditions (Fig. 2). Moreover, this suggests that sugars
345 and acetic acid, but not the comparatively less abundant aromatics, are the main determinant of
346 cell growth under these conditions.

347 The concentrations of the main compounds known to be growth substrates were measured after
348 48 hours of cultivation to monitor their consumption (Table 1). Both yeasts in all cases achieved
349 70-100% consumption of glucose, although the starting concentrations were very low in some
350 cases and incomplete conversion occurred with base treatments. Interestingly, strong xylose
351 consumption was only obtained in acid-derived hydrolysates. Rather than poor biocompatibility
352 of IL or base treatments (which do not seem to affect the overall growth), it is possible that the
353 low xylose concentrations observed under these conditions (as shown in Fig. 2) have a negative
354 impact on the transport rate. This is supported by previous reports regarding the existence of at

least two different transport systems for glucose and xylose with different substrate affinities in related strains (Barnett, 2008). A similar trend was observed with arabinose, being preferentially consumed in acid-derived hydrolysates, where it is found at higher concentrations compared to the other pretreatments. Since the amount of glucose and arabinose present in samples treated with IL or base is lower than the amount of acetic acid and even aromatics in some cases, a different metabolic state could be active in the yeasts, inducing the consumption of those types of compounds. Concurrently, both organisms efficiently consumed acetic acid, 4-HBA and PCH in the hydrolysates, suggesting that no significant toxicity was present at these concentrations. The fact that both yeasts tolerated the presence of organic acids and furfurals, while consuming the main sugars and aromatics, reinforces their potential for valorization of biomass hydrolysate streams generated by different pretreatments.

3.4. Bisabolene production with recombinant yeast strains

The next step comprised the assessment of bisabolene production by *R. toruloides* and *R. mucilaginosa* recombinant strains grown on pretreated biomass hydrolysates. Acid treatment of QsuB and HCHL feedstocks were selected for these experiments because they resulted in the highest combined substrate yields, including the aromatic monomers. To further increase substrate concentrations and final hydrolysate volume, scale-up reactions were performed with each feedstock (as described in section 2.4), resulting in hydrolysates with a 5-fold larger volume and 2-fold higher concentration, relative to the small scale experiments. In parallel, a set of simulated hydrolysates containing a mixture of glucose, xylose, acetic acid and either 4-HBA or PCH were also prepared according to the concentrations obtained with acid treatment in previous experiments (Fig. 2), as well as simulated hydrolysates containing 2, 5 or 10-fold higher amounts

of each components. One of the reasons to aim for higher concentrated media is to increase bisabolene titers from higher total substrate consumption, especially because the product/substrate yields obtained for a similar *R. toruloides* bisabolene producing strain are reported to still be far from the theoretical maximum (Yaegashi et al., 2017). Furthermore, it would be interesting to determine if a higher concentration of aromatics can be released from the modified feedstocks without resulting in hydrolysates that have strong toxic effects on the yeasts. The concentrations of compounds measured in the larger scale acid-treated hydrolysates after addition of SD media and pH adjustment are shown in Table 2. The concentrations considered for preparing the simulated HCHL or QsuB hydrolysates (and taken as reference for preparing the 2, 5 and 10-fold higher concentrations) are also shown in Table 2.

It can be observed in Figure 5 that both strains are able to produce bisabolene from all different media after a 7-day incubation. However, clear differences in cell biomass and product yields were found. *R. toruloides* BIS18 reached higher bisabolene titers in most conditions (most notably when grown on real hydrolysates) and *R. mucilaginosa* BIS11 produced more cell biomass in general, in agreement with previous experiments. One exception was the 10x QsuB condition, where both strains produced similar and their highest cell biomass values, and in the case of *R. mucilaginosa* BIS11, this media condition also correlated with a higher bisabolene titer (Fig. 5). It appears that the more concentrated substrates in the simulated hydrolysates gradually resulted in the production of more cells and bisabolene, but changes in media substrate composition must be large to result in observable differences in product and biomass yields. The differences in concentration of substrates between real hydrolysates from wild-type vs. the QsuB or HCHL lines are seemingly not large enough to result in clear differences in bisabolene production. Nevertheless, the fact that both organisms can tolerate much higher concentrations of

sugars, acids and aromatics and convert them into cell biomass and bisabolene is encouraging to continue the design of combined pretreatment, yeast engineering and plant engineering strategies.

Since biomass hydrolysates are typically generated at high solids loading for biological valorization, and to obtain a better idea of how high the aromatic compound concentration can be in hydrolysates before becoming toxic to microbial strains, tolerance assays of the yeasts to high concentrations of the common aromatics 4-hydroxybenzoic acid, protocatechuic acid and *p*-coumaric acid were performed. The results showed that both organisms are naturally resistant to these types of compounds and were able to grow in media containing up to 40 g/L of 4-hydroxybenzoic acid (Fig. 6). In addition, *R. toruloides* BIS18 can tolerate at least 20 g/L of protocatechuic acid and 10 g/L of *p*-coumaric acid. *R. mucilaginosa* BIS11 displayed a higher tolerance, being able to grow on at least 40 g/L of protocatechuic acid and 20 g/L of *p*-coumaric acid. Evidently, these closely related yeast species possess physiological traits that can be advantageous for a biomass valorization process. While *R. toruloides* BIS18 can produce higher bisabolene titers, *R. mucilaginosa* BIS11 can grow faster and is more resistant to high concentrations of aromatics and other compounds present in hydrolysates.

Conclusions

This study illustrates the feasibility of using two red yeast species to convert not only sugars but also aromatics accumulated *in planta*, which were easily released with common pretreatment conditions. By demonstrating tolerance to the main compounds in authentic or simulated hydrolysates and the production of bisabolene from all tested conditions, this work encourages a

combined optimization of the feedstocks, pretreatment and yeasts to obtain more efficient and integrated processes for the biological valorization of biomass that include both plant polysaccharides and lignin.

E-supplementary data of this work can be found in online version of the paper.

Acknowledgements

This work was part of the DOE Joint BioEnergy Institute (<http://www.jbei.org>) supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research, through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the U. S. Department of Energy. The United States Government retains and the publisher, by accepting the article for publication, acknowledges that the United States Government retains a non-exclusive, paid-up, irrevocable, worldwide license to publish or reproduce the published form of this manuscript, or allow others to do so, for United States Government purposes. The Department of Energy will provide public access to these results of federally sponsored research in accordance with the DOE Public Access Plan (<http://energy.gov/downloads/doe-public-access-plan>).

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Figure captions

Figure 1. Workflow employed to generate and characterize lignocellulosic hydrolysates for the production of bisabolene with recombinant red yeasts. Monomeric sugars, organic acids and aromatics in the liquid fraction of hydrolysates were obtained after pretreatment of milled *Arabidopsis* biomass and served as carbon sources in the media after nutrient supplementation and pH adjustment.

Figure 2. Concentrations of monomeric compounds measured in the hydrolysates obtained from ionic liquid (IL), acid or base pretreatment applied to *Arabidopsis* biomass. a) and c) glucose, xylose, arabinose, and 4-hydroxybenzoic acid (4-HBA) or protocatechuic acid (PCH); b) and d) formic acid, acetic acid, hydroxymethylfurfural (HMF) and furfural. Hydrolysates were generated using biomass from plants expressing either a bacterial hydroxycinnamoyl-CoA hydratase/lyase (HCHL) or a 3-dehydroshikimate dehydratase (QsuB) enzyme, and from their corresponding wild-type controls (wild-type H and wild-type Q, respectively). Bars represent the averages from two experiments and the error bars indicate the absolute error. Values on top of the bars indicate the combined concentration of compounds in mg/L measured for each condition.

Figure 3. Cell growth profiles of wild-type *R. toruloides* (blue) and *R. mucilaginosa* (orange) after 48 h incubation on SD media with individual substrates at an initial concentration of 1 g/L. Bars indicate the average maximum cell biomass obtained from biological duplicates after subtracting the initial cell biomass, the error bars indicate the absolute error, and the numbers on top indicate the corresponding average growth rates in h⁻¹. Cell biomass values obtained when no specific substrate was added to the SD media are shown for comparison.

Figure 4. *R. toruloides* BIS11 (blue) and *R. mucilaginosa* BIS18 (orange) growth after 48 h cultivations on hydrolysates obtained with ionic liquid (IL), acid, or base pretreatment. a) *Arabidopsis* HCHL and corresponding wild-type lines; b) *Arabidopsis* QsuB and corresponding wild-type lines. Bars indicate the average maximum cell biomass obtained in each case from

biological duplicates, the error bars represent the absolute error, and the numbers on top indicate the corresponding average growth rates in h^{-1} .

Figure 5. Bisabolene titers (bars) and final cell biomass (dots) obtained by cultivating a) *R. toruloides* BIS18 and b) *R. mucilaginosa* BIS11 strains on authentic or simulated HCHL and QsuB hydrolysates for 7 days. Values in graphs are the average from biological duplicates and the error bars indicate the absolute error. WTQ acid, scaled up acid-pretreated wild-type Q biomass; QsuB acid, scaled up acid-pretreated QsuB biomass; WTH acid, scaled up acid-pretreated wild-type H biomass; HCHL acid, scaled up acid-pretreated HCHL biomass. Media with a mixture of glucose, xylose, acetic acid and 4-HBA or PCH were generated to test the effect of proportionally increasing 2, 5 or 10-fold the amount of carbon sources in QsuB or HCHL hydrolysates, using the concentrations reported in Fig. 2 and Table 2. These simulated hydrolysates are indicated in the graphs as 10x, 5x, 2x or 1x, QsuB or HCHL.

Figure 6. Tolerance of the red yeasts to higher concentrations of 4-hydroxybenzoic acid, protocatechuic acid and *p*-coumaric acid. The compounds were added to SD media at 10 g/L (gray bars), 20 g/L (yellow bars) or 40 g/L (purple bars) final concentrations and used to grow *R. toruloides* BIS18 (left) or *R. mucilaginosa* BIS11 (right) for 48 hours. Bars indicate the average values obtained for cell biomass at different time points from biological duplicates and the error bars indicate the absolute error.

Tables

Table 1. Percentile utilization of substrates by *R. toruloides* BIS18 (top) and *R. mucilaginosa* BIS11 (bottom). For each compound, values represent the average difference between concentrations at the beginning and end of cultivations from biological duplicates, with the absolute error indicated in parentheses. Instances where the initial concentration of a compound was lower than 50 mg/L were not considered for this calculation and are indicated with a dash.

<i>R. toruloides</i> BIS18 % substrate utilization		glucose	xylose	arabinose	acetic	PCH	4-HBA
wild-type H	IL	93 (14)	53 (27)	-	100 (0)	-	-
	acid	100 (0)	94 (0)	95 (2)	100 (0)	-	-
	base	95 (8)	0	19 (5)	100 (0)	-	-
HCHL	IL	100 (13)	19 (45)	-	100 (0)	-	100 (0)
	acid	100 (0)	92 (6)	91 (3)	100 (0)	-	100 (0)
	base	86 (0)	0	17 (1)	99 (0)	-	100 (0)
wild-type Q	IL	86 (0)	15 (3)	-	100 (0)	-	-
	acid	100 (0)	79 (0)	97 (6)	100 (0)	-	-
	base	74 (1)	0	91 (6)	100 (0)	-	-
QsuB	IL	94 (5)	34 (22)	-	100 (0)	-	-
	acid	100 (25)	81 (7)	97 (1)	98 (3)	97 (0)	-
	base	70 (54)	33 (76)	63 (35)	100 (1)	-	-
<i>R. mucilaginosa</i> BIS11 % substrate utilization		glucose	xylose	arabinose	acetic	PCH	4-HBA
wild-type H	IL	100 (0)	55 (19)	-	100 (0)	-	-
	acid	100 (0)	97 (5)	92 (16)	100 (0)	-	-
	base	88 (4)	0	2 (4)	100 (0)	-	-
HCHL	IL	100 (0)	25 (37)	-	100 (0)	-	99 (0)
	acid	100 (0)	93 (5)	85 (5)	100 (0)	-	100 (0)
	base	82 (3)	0	0	100 (0)	-	100 (0)
wild-type Q	IL	100 (0)	38 (0)	-	100 (0)	-	-
	acid	100 (0)	83 (0)	99 (0)	100 (0)	-	-
	base	88 (2)	0	83 (1)	100 (0)	-	-
QsuB	IL	99 (3)	43 (18)	-	100 (0)	-	-
	acid	100 (0)	86 (4)	95 (0)	100 (0)	97 (0)	-
	base	94 (1)	58 (29)	58 (46)	100 (0)	-	-

Table 2. Monomer concentrations in mg/L measured in authentic (WTQ acid, QsuB acid, WTH acid and HCHL acid) or used for the simulated (1x QsuB, 1x HCHL) hydrolysates, employed in bisabolene production experiments. Values for the authentic hydrolysates represent the averages of technical duplicates, with the absolute error indicated in parentheses. The name abbreviations are described in the legend of Fig. 5.

	glucose (mg/L)	xylose (mg/L)	arabinose (mg/L)	formic (mg/L)	acetic (mg/L)	PCH (mg/L)	4-HBA (mg/L)
WTQ acid	1837 (188)	6326 (645)	497 (50)	2276 (234)	1065 (277)	-	-
QsuB acid	2394 (5)	5723 (37)	646 (7)	2660 (116)	1211 (142)	579 (2)	-
WTH acid	776 (9)	6279 (48)	372 (48)	516 (79)	1537 (1)	-	-
HCHL acid	1776 (6)	6664 (66)	331 (66)	438 (9)	1658 (4)	-	519 (3)
1x QsuB	1100	3700	-	-	1000	330	-
1x HCHL	700	3600	-	-	880	-	220

Fig. 1

a)

b)

c)

d)

Fig. 2

Fig. 3

a)

b)

Fig. 4

a)

b)

Fig. 5

Fig. 6