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Authors
Okonkwo, Onyinye
Dou, Chang
Oksen, Ethan
et al.

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Corn stover variability drives differences in bisabolene production by engineered *Rhodotorula toruloides*

Onyinye Okonkwo[1], Chang Dou[1], Ethan Oksen[1], Akash Narani[1], Wilian Marcondes[1], Xiaowen Chen[2], Joonhoon Kim[3,4,5], Yuqian Gao[4,6], Meagan C. Burnet[4,6], Bobbie-Jo M. Webb-Robertson[4,6], Brenton C. Poirier[4,6], Deepti Tanjore[1], Jon K. Magnuson[3,4,5], Nathalie Munoz Munoz[4,6], James Gardner[1]

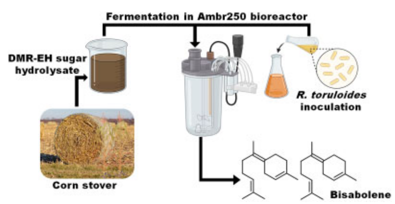
[1]Advanced Biofuels and Bioproducts Process Development Unit, Lawrence Berkeley National Lab, Berkeley, CA 94608, USA
[2]National Biology Center, National Renewable Energy Lab, 1617 Cole Blvd., Golden, CO 80401, USA
[3]Energy and Environment Directorate, Pacific Northwest National Laboratory, Richland, WA 99352, USA
[4]US Department of Energy Agile BioFoundry, Emeryville, CA 94608, USA
[5]US Department of Energy Joint BioEnergy Institute, Emeryville, CA 94608, USA
[6]Earth and Biological Sciences Directorate, Pacific Northwest National Laboratory, Richland, WA 99354, USA
Correspondence should be addressed to: Onyinye Okonkwo, E-mail: ojokonkwo@lbl.gov; James Gardner, E-mail: jgardener@lbl.gov

Abstract: Microbial conversion of lignocellulosic biomass represents an alternative route for production of biofuels and bioproducts. While researchers have mostly focused on engineering strains such as *Rhodotorula toruloides* for better bisabolene production as a sustainable aviation fuel, less is known about the impact of the feedstock heterogeneity on bisabolene production. Critical material attributes like feedstock composition, nutritional content, and inhibitory compounds can all influence bioconversion. Further, the given feedstocks can have a marked influence on selection of suitable pretreatment and hydrolysis technologies, optimizing the fermentation conditions, and possibly even modifying the microorganism's metabolic pathways, to better utilize the available feedstock. This work aimed to examine and understand how variations in corn stover batches, anatomical fractions, and storage conditions impact the efficiency of bisabolene production by *R. toruloides*. All of these represent different facets of feedstock heterogeneity. Deacetylation, mechanical refining, and enzymatic hydrolysis of these variable feedstocks served as the basis of this research. The resulting hydrolysates were converted to bisabolene via fermentation, a sustainable aviation fuel precursor, using an engineered *R. toruloides* strain. This study showed that different sources of feedstock heterogeneity can influence microbial growth and product titer in counterintuitive ways, as revealed through global analysis of protein expression. The maximum bisabolene produced by *R. toruloides* was on the stalk fraction of corn stover hydrolysate (8.89 $\pm$ 0.47 g/L). Further, proteomics analysis comparing the protein expression between the anatomic fractions showed that proteins relating to carbohydrate metabolism, energy production, and conversion as well as inorganic ion transport metabolism were either significantly upregulated or downregulated. Specifically, downregulation of proteins related to the iron–sulfur cluster in stalk fraction suggests a coordinated response by *R. toruloides* to maintain overall metabolic balance, and this was corroborated by the concentration of iron in the feedstocks.

One-Sentence Summary: This study elucidates the effects of different sources of corn stover on bisabolene production by engineered *Rhodotorula toruloides*, highlighting the importance of understanding feedstock variability to enhance bioprocess efficiency and economic outcomes.

Keywords: Corn stover variability, Anatomic fractions, Self-heating, Bisabolene, *R. toruloides*, Fermentation

Graphical abstract



Introduction

A global shift to more sustainable resources and technologies has offered a great opportunity for biomanufacturing, particularly microbial fermentation. Given the technological advances in renewable energy generation, the global community is attempting to establish a more circular economy that includes driving the shift of the chemical industry and biorefineries towards using renewable feedstocks, to minimize net carbon dioxide (CO_2) emission per unit of generated chemical or fuel (Blank et al., 2020; Prather, 2020; Tagliapietra & Wolff, 2021). Under this circumstance, it is crucial to develop production processes based on the wastes generated

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by other sources of economic activity. Such wastes include lignocellulosic biomass, plastics, and CO_2. An emerging solution is the transformation of these wastes into simple molecules, which can be subsequently used as feedstock for microbial fermentation. In the recent White House report (The White House Office of Science and Technology Policy, 2023), the Department of Energy identified areas where biotechnology and biomanufacturing can play a critical role in reducing greenhouse gas (GHG) emissions and removing carbon from the atmosphere. According to the report, in 20 years, the United States hopes to collect and process on an annual basis, 1.2 billion metric tons of conversion-ready, purpose-grown plants and waste-derived feedstocks suitable for conversion to fuels and products, while minimizing emissions, water use, habitat loss, and other sustainability challenges. Furthermore, the United States anticipates the production of 3 billion gallons of sustainable aviation fuels (SAF) in 7 years, with at least 50% reduction in GHG lifecycle emissions relative to conventional aviation fuels, with production rising to 35 billion gallons in 2050 (The White House Office of Science and Technology Policy, 2023).

It is evident that the United States has a great potential for expanding the use of renewable feedstocks due to the ability to grow, harvest, store, and transport agricultural products on an enormous scale. These feedstocks will be critical for achieving transportation fuel production goals as well as providing sustainable feedstock for decarbonization efforts across other sectors. Large quantities of biomass are necessary to supply emerging bioenergy conversion facilities, on the scale of 2,100 tons per day for a 61-million gal per year biorefinery (Humbird et al., 2011). However, it is challenging to provide a consistent supply of biomass throughout the year, since most agricultural residues are seasonally harvested and will require extended on-farm storage. Furthermore, there are significant challenges to reliable and efficient biomass conversion to fuels and products as a result of the different sources of biomass variability. Corn stover is one such residue that has gained considerable attention as a bioenergy feedstock due to its current availability and collection infrastructure. Current U.S. estimates suggest that up to 256 million tons per year of corn stover could be sustainably harvested ("US Billion Ton Update: Biomass supply for a bioenergy and bioproducts industry (executive summary)," 2011). However, several factors contribute to the compositional variability of corn stover. For example, feedstock ash and moisture content vary considerably in corn stover harvested for bioconversion and this well translates to inherent variability in the chemical composition (Shah et al., 2011; Shinners et al., 2007).

Ash accelerates the rate of wear of processing equipment, which can vary depending on the composition of the ash, the type of biomass being processed, and the equipment used. Researchers reported that corn stover with high ash content due to contamination from soil consumed higher power during grinding, resulting in reductions of processing rates and producing a larger fraction of fine particles in the feedstock that were lost to dust mitigation systems (Sievers et al., 2020). Corn stover, which consists of stalks, leaves, husks, and cobs left behind after harvest, contains varying levels of ash depending on factors like soil conditions, fertilization, and harvesting practices. Therefore, a comprehensive approach that considers material selection, equipment design, and maintenance practices is essential to minimize the effects of ash-related wear in biomass processing (Li et al., 2020; Yan et al., 2020). Furthermore, researchers have observed that corn stover feedstock from fields with a high residual moisture content results in bale degradation due to self-heating. In turn, self-heating caused a more pronounced drop in preprocessing throughput due to grinder overloads and process upsets, leading to equipment downtime (Ray et al., 2020). Despite the challenges in corn stover preprocessing, Sievers et al. (2020) reported that conversion yield to sugars was not affected. The implications of biological degradation potentially impact the overall efficiency, conversion, and yields of integrated biorefineries. One significant challenge associated with production of biofuels from lignocellulose is its recalcitrance with respect to bioconversion (Himmel et al., 2007). However, there is no study to show the impact of corn stover variability on fermentation performance of the corn stover sugar streams.

The variability caused by storage and handling conditions would likely "trickle down" to variability in the processed biomass and ultimately the production titer, yield, and productivity. Industrial processes can be financially viable with some variability, but certain processes are more robust than others. Most notably, processes with low-value products will have lower profit margins, making them more sensitive to variability. Researchers have used elements of the quality by design approach such as critical material attributes, critical process parameters, and critical quality attributes to understand and mitigate impacts of feedstock variability on bioconversion processes for potential application at an industrial scale (Leal et al., 2020). *Rhodotorula toruloides* has also been the subject of intense research for bioconversion, due in part to its native ability to efficiently convert glucose and xylose from hydrolyzed lignocellulose. Its growth and productivity were reportedly enhanced in lignocellulosic hydrolysates relative to purified substrates (Yaegashi et al., 2017). *Rhodotorula toruloides* was engineered to produce bisabolene, a potential jet fuel precursor that can be transformed into its saturated form (bisabolane) through hydrogenation to ensure compatibility with jet engines (Kirby et al., 2021). Due to the physicochemical and structural variability that exists within corn stover biomass, remarkable efforts have been made to de-risk the variability and material attributes that affect the biomass value chain (Ray et al., 2020). However, there is still a lack of comprehensive information that addresses how lignocellulosic biomass variability affects microbial fermentation.

The goal of this study is to understand how different sources of variability in corn stover affect conversion to bisabolene by *R. toruloides* after deacetylation, mechanical refining, and enzymatic hydrolysis (DMR-EH) pretreatment. Understanding how variability of various DMR-EH-treated corn stover affects conversion to bisabolene by *R. toruloides* would provide a clearer picture of the breadth of influence that feedstock variability imparts. By examining factors such as the sugar and elemental composition of different corn stover sources, variations of different anatomic fractions, and the effects of biological degradation during storage, this study contributes to the understanding of bioproduction variability, supporting the development of more efficient and robust bioprocesses. This body of work would provide valuable insights into optimizing the process, addressing challenges, and potentially uncovering new avenues for improving bisabolene production from corn stover. This work is part of an ongoing collaboration between multiple national laboratories to investigate the effects of feedstock properties, as illustrated in Fig. 1. Investigators at Sandia National Laboratories (SNL), National Renewable Energy Laboratory (NREL), Lawrence Berkeley National Laboratory (LBNL), and Pacific Northwest National Laboratory (PNNL) contributed to this research. Each of the collaborating research teams offered key contributions in the study of biochemical conversion of sourced corn stover to bisabolene via *R. toruloides*: NREL researchers chose several batches of corn stover as representatives for different ash and moisture compositions, storage conditions, and anatomical fractions, and applied the DMR-EH process for use of resulting

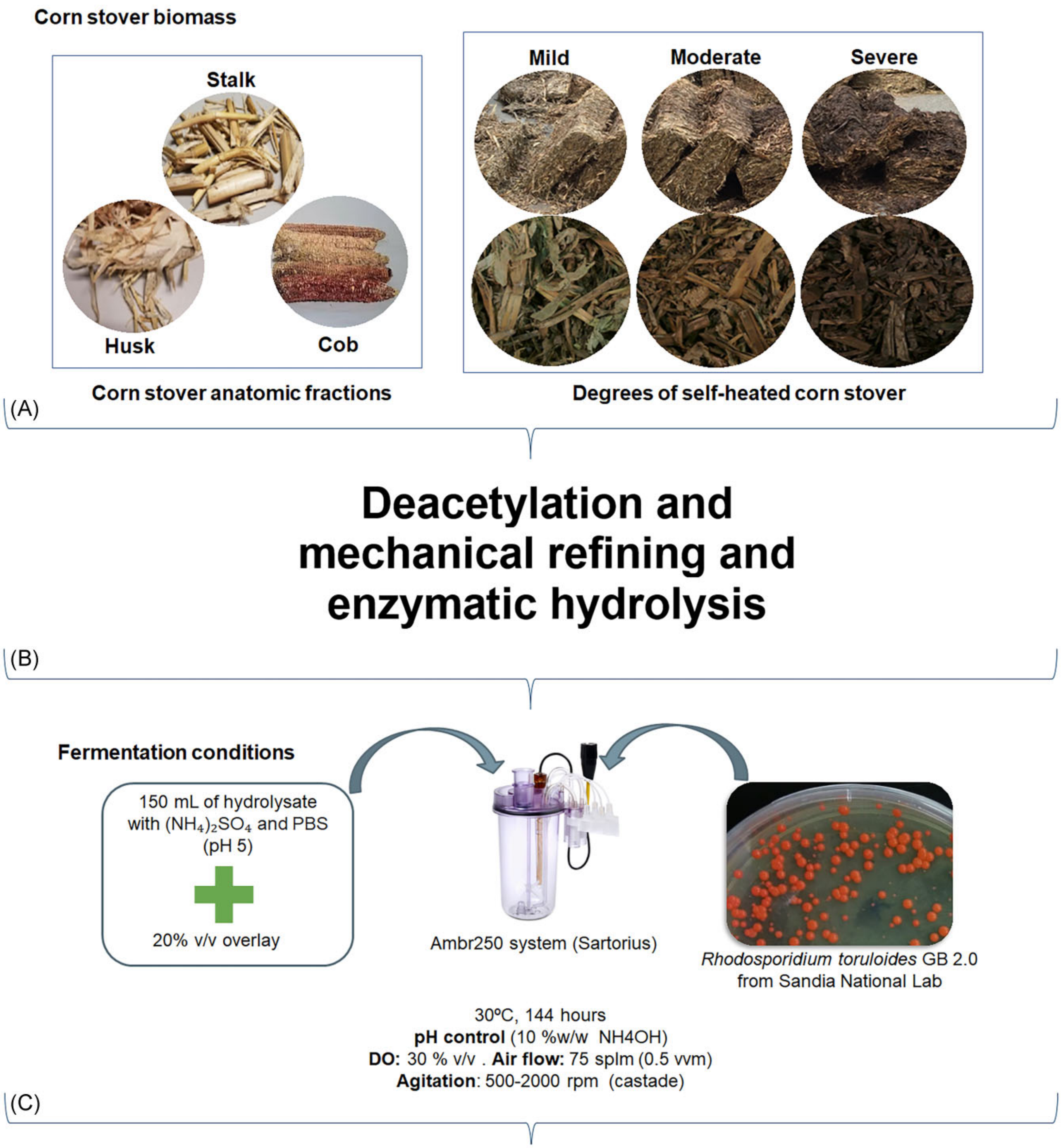

Fig. 1. Biomass conversion processes. (A) Characterized biomass. Figure modified from previously published images by Bose et al. (2020). (B) National Renewable Energy Laboratory's deacetylation and mechanical refining method was used to treat the different corn stover batches (Chen et al., 2016). (C) Fermentation by *R. toruloides* was performed using AMBR250 bioreactor (this study).

hydrolysates in fermentation; LBNL researchers conducted the fermentation experiments described in this study, using an engineered strain of *R. toruloides* provided by SNL. PNNL experts in global proteomics conducted these experiments and supported subsequent analysis.

Materials and Methods

Feedstock Preparation

The National Renewable Energy Laboratory provided the corn stover for these experiments. The different corn stover samples showed a broad range of characteristics in ash, metal, organic acids, and carbohydrate content (manuscript in review). Initially, the ash and moisture contents were identified as critical material attributes impacting surface energy, wettability, and cohesion of corn stover and the corn stover samples were categorized as high ash–low moisture, high ash–high moisture, low ash–low moisture, and low ash–high moisture sample sets (Leal et al., 2020). However, the moisture and ash content of the raw materials underwent changes during subsequent handling, storage, and pretreatment. As such, the heterogeneity of the different lignocellulosic sugar streams generated was then based on the composition of the hydrolysate sugar stream generated from NREL's DMR-EH process.

Table 1 shows the heterogeneity of the feedstock types based on the metal composition established in this study, and these served as the bases for testing feedstock heterogeneity and how it influences low temperature conversion for *R. toruloides*.

The feedstock was divided into three categories: whole stover (Feedstocks 1, 2, 3, and 4), manually separated anatomical fractions (cob, husk, and stalk), and corn stover subjected to three different self-heating conditions (mild, moderate, and severe). All samples were pretreated and hydrolyzed using NREL's DMR-EH approach. Severe self-heated corn stover was also subjected to a two-stage (labeled as *Severe two-stage self-heated* in Table 1) process where sodium carbonate was used before sodium hydroxide to deacetylate the stover prior to enzyme hydrolysis, so as to increase fermentable sugar yield. The hydrolyzed biomass was then used for fermentation using *R. toruloides* as a biocatalyst for bisabolene production.

NREL researchers subjected the corn stover to DMR-EH. The first step was a dilute alkali extraction of the biomass, which decouples the acetyl groups from the hemicellulose and partially removes the lignin component. The biomass then passed through a disk refiner and Szego mill to reduce the particle size and increase the surface area. Hydrolytic enzymes HTEC-3 and CTEC-3 liberated the hemicellulose and cellulose, respectively, from the solids before use (Chen et al., 2016). Researchers at LBNL then assessed the potential of these feedstock categories for conversion to bisabolene via *R. toruloides*.

Fermentation

Sandia National Laboratory provided a modified strain of *R. toruloides* for the production of bisabolene. The construction of this strain is described by Kirby et al. (2021). *Inoculum*: Seed culture of *R. toruloides* was prepared by adding 1 ml of *R. toruloides* GB 2.0 glycerol stock in 100 ml of steam-sterilized Sigma YPD media in a 500-ml glass baffled flask. Cultures grew at 30 °C with shaking at 200 rpm to maintain optimal growth conditions for yeast.

The fermentations were conducted using Ambr® 250 high-throughput instrumentation and vessels (Sartorius Stedim Biotech) and software (Ambr® 250 runtime_R15.4.0). Each vessel contained 120 ml of its respective DMR-EH supplemented with 5-g/L ammonium sulfate, 10.34-g/L potassium phosphate monobasic, and 4.18-g/L potassium phosphate dibasic. All media underwent mixing and filter sterilization by a 0.2-µm bottle top filter in a biosafety cabinet. Thirty milliliters of dodecane or oleyl alcohol was also added to each vessel to trap the bisabolene from the aqueous phase, as high concentrations of bisabolene can be toxic to *R. toruloides*. The inoculum was added at 5% vessel working volume for 4 optical density (OD) of seed, giving an initial main fermentation OD of 0.2.

The initial mixing was 500 rpm and ramped up to the upper control limit of 2,000 rpm, to control the dissolved oxygen at a setpoint of 30%. Gassing was kept constant in all vessels at 0.5 vvm (75 slpm). The pH was initially set to pH 6 by adjusting the media with sodium hydroxide before starting the experiment. The control setpoints for pH and temperature were 5 and 30 °C, respectively, which is optimal for yeast growth. The fermentations lasted for a period of 144 or 240 hr. By considering these fermentation durations, researchers were able to gain a comprehensive understanding of how fermentation time influences the growth, sugar consumption, and end product to identify the optimal conditions for achieving desired outcomes. The programmed robotic liquid handler was carried out by the sampling at intervals of 24 hr. The first sample was taken shortly after inoculation, and

Table 1. Metal Composition in Variable Corn Stover Feedstock

	Whole stover				Anatomical fractions				Self-heated feedstock			
Metal composition	**Feedstock 1**	**Feedstock 2**	**Feedstock 3**	**Feedstock 4**	**Cob**	**Husk**	**Stalk**	**Whole stover**	**Mild self-heated**	**Moderate self-heated**	**Severe one-stage self-heated**	**Severe two-stage self-heated**
Aluminum (mM)	0.11	0.08	0.08	0.04	0.05	0.03	0.06	0.05	0.10	0.12	0.11	0.06
Calcium (mM)	10.39	11.01	11.22	5.48	4.29	6.65	9.92	6.18	8.55	7.09	7.42	7.70
Iron (mM)	2.10	1.89	1.98	0.31	0.19	0.44	0.82	0.57	2.00	1.59	1.11	0.76
Potassium (mM)	1.11	0.94	1.11	0.69	1.92	0.61	1.95	0.87	0.91	0.78	0.94	0.76
Magnesium (mM)	11.58	10.76	7.01	2.56	3.13	4.09	7.33	4.36	6.33	4.43	3.93	2.95
Sodium (mM)	15.03	6.98	6.06	4.57	17.71	4.57	10.87	5.12	3.82	2.81	3.73	3.35
Phosphorus (mM)	0.85	0.64	0.66	0.48	0.52	0.52	1.70	0.81	0.57	0.45	0.51	0.64
Sulfur (mM)	13.26	0.79	0.89	1.24	8.66	0.45	1.70	0.77	1.78	0.56	0.59	0.81
Silicon (mM)	3.79	4.00	4.94	3.30	1.54	3.37	3.56	3.09	2.83	2.35	2.85	2.21
Zinc (mM)	0.03	0.04	0.02	n.d.	n.d.	n.d.	0.03	0.00	0.02	0.01	0.01	n.d.

Note. The initial impact of ash and moisture in the raw material diminished across different sample processing levels. The results reported in the table were obtained after pretreatment and enzymatic hydrolysis. n.d. = not detected.

one was taken each day after until the end of fermentation. The autosampler dispensed 2 ml of well-mixed broth from each vessel into a centrifuge tube chilled to 4 °C. The samples were mixed well enough that the 2 ml in the tube had representative fractions of organic overlay and aqueous phase. Additional 5-ml samples were taken in the log phase at 36- and 60-hr or 24- and 48-hr time points for investigating the dynamics in protein expression across various samples. The reason for selecting different sampling points was to capture the full scope of the protein expression, that is, the long-term trends and early expression dynamics.

Elemental Composition Analysis Using ICP-OES

To prepare corn stover hydrolysate samples for elemental composition analysis, each sample was digested in 2% nitric acid and a total volume of 10 ml. The samples were placed in the incubator for 24 hr. Samples were weighed prior to incubation and after incubation and 2% nitric acid was added to each sample to make up for the loss due to evaporation and centrifuged to remove the solids formed during digestion. Digested samples were then analyzed using a Varian 720-ES inductively coupled plasma optical emission spectroscopy (ICP-OES) system.

Bioreactor Sampling

High-performance liquid chromatography (HPLC) and gas chromatography mass spectroscopy (GC-MS) served as the basis for compositional analysis. These tests included sugar content analysis by HPLC, bisabolene quantification by GC-MS, optical density testing on a ultraviolet–visible spectroscopy (UV-VIS) spectrophotometer, and proteomics testing performed by PNNL. The samples taken were spun in a centrifuge to separate out the aqueous and overlay fractions. The oleyl alcohol or dodecane layer was removed from the top with a pipette and stored for GC analysis. The aqueous fraction was filtered in a 0.2-µm centrifugal filter for HPLC analysis. The pellet was then resuspended in water, and brought to the level of the original sample. This portion was then used for OD, with distilled water used as a reference blank. Spectrophotometry (Genesys 10S UV-VIS, Thermo Fisher Scientific) was used to quantify the optical density of each sample at 600-nm wavelength. Samples for OD measurements were diluted in water to meet the linear range requirements. The linear range for this equipment was between 0.1 and 0.4 OD units. All other samples were stored in −20 °C freezers until they were tested.

Quantification of Sugar monomer DMR-EH-Treated Corn Stover

Sugars were quantified as previously described (Sundstrom et al., 2018). The concentration of sugars of the fermentation samples was quantified using an HPLC system (Ultimate 3000, Thermo Fisher Scientific) equipped with a refractive index detector and an Aminex HPX-87H column (Bio-Rad, 300 × 7.8 mm). Four millimolar of H_2SO_4 was used as the mobile phase at a flow rate of 0.6 ml/min. The column oven temperature was set at 65 °C. Standards were prepared to encompass the same range of concentrations as the samples. Prior to analysis, samples were filtered using 0.2-μm centrifuge filters.

Bisabolene Quantification

To measure the amount of bisabolene produced in the cultivations, the oleyl alcohol overlay was collected and diluted in ethyl acetate spiked with 500 mg/L of caryophyllene as an internal standard (Peralta-Yahya et al., 2011). The samples were then analyzed by GC-MS using an Agilent Technologies 6890N system, equipped with a 5973 mass selective detector and a DB-5ms column (30 m × 250 µm × 0.25 µm, Agilent Technologies, USA). Splitless injections (1 µl vol.) were used on a GC oven program consisting of 100 °C for 0.75 min, followed by a ramp of 20 °C per min until 300 °C, and held 1 min at 300 °C. Injector and MS quadrupole detector temperatures were 250 and 150 °C, respectively. Concentrations were calculated by integration of the peak area values obtained in the selective ion monitoring mode and compared to the areas obtained from a calibration curve made with pure bisabolene. The bisabolene concentrations measured in the oleyl alcohol layer were adjusted to represent the concentrations in the aqueous volume.

Proteomic Evaluation

Samples were processed immediately after they were taken from the vessels. Five milliliters was removed for each sample and placed in a falcon tube. The tubes were centrifuged to separate out the pellet. All aqueous and organic layers were decanted from the top, and then the pellet was resuspended in 2-ml ice-cold phosphate buffered saline. These samples were then placed in liquid nitrogen for 1 min. All samples were then stored in a −80 °C freezer until shipped to PNNL for testing.

Cell pellets were processed using a metabolite, protein, lipid extraction (MPLEx) method (PMID: 27822525, PMID: 29876813). Briefly, in solvent-resistant tubes (Sorenson), the cell pellets were resuspended in water and a solvent mixture of four volumes of cold 2:1 chloroform:methanol mix was added. Samples were vigorously vortexed for 30 s, placed on ice for 5 min, vortexed again for 30 s, and centrifuged at 15,000× *g* for 5 min at 4 °C. After centrifugation, the denatured protein interphase was washed in 1 ml of cold 100% methanol, vortexed, and centrifuged at 15,000× *g* for 5 min at 4 °C to pellet the protein. The methanol was removed, and samples were dried in a fume hood.

The protein pellet was resuspended in 100 mM ammonium bicarbonate (NH_4HCO_3) containing 8 M urea, and protein concentration was measured by a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). Disulfide bonds were reduced by adding dithiothreitol to a final concentration of 5 mM and incubating at 60 °C for 30 min with constant shaking at 850 rpm. Samples were alkylated with a final concentration of 40 mM iodoacetamide for 1 hr at 37 °C at 850 rpm. The reaction was then diluted 10-fold with 100 mM NH_4HCO_3 followed by the addition of calcium chloride to 1 mM final concentration. Sequencing-grade trypsin (Promega) was added to all protein samples at a 1:50 (*w*/*w*) trypsin-to-protein ratio and incubated for 3 hr at 37 °C. Digested samples were desalted with 1 ml of Discovery C18 SPE columns (Supelco, Bellefonte, PA), using the following protocol: 3 ml of methanol was added for conditioning the column followed by 2 ml of 0.1% trifluoroacetic acid (TFA) in water. The samples were then loaded onto each column followed by 4 ml of 95:5 water:acetonitrile, 0.1% TFA. Samples were eluted with 1 ml of 80:20 acetonitrile:water, 0.1% TFA. The samples were concentrated down to ~50 µl using a Speed Vac and a final BCA assay was performed to determine the peptide concentration.

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) was carried out using a Dionex Ultimate 3000 liquid chromatography system (Thermo Fisher Scientific) coupled to Q Exactive HF-X (Thermo Fisher Scientific). Mobile phase A (MPA) is 0.1% formic acid in water while mobile phase B (MPB) is 0.1% formic

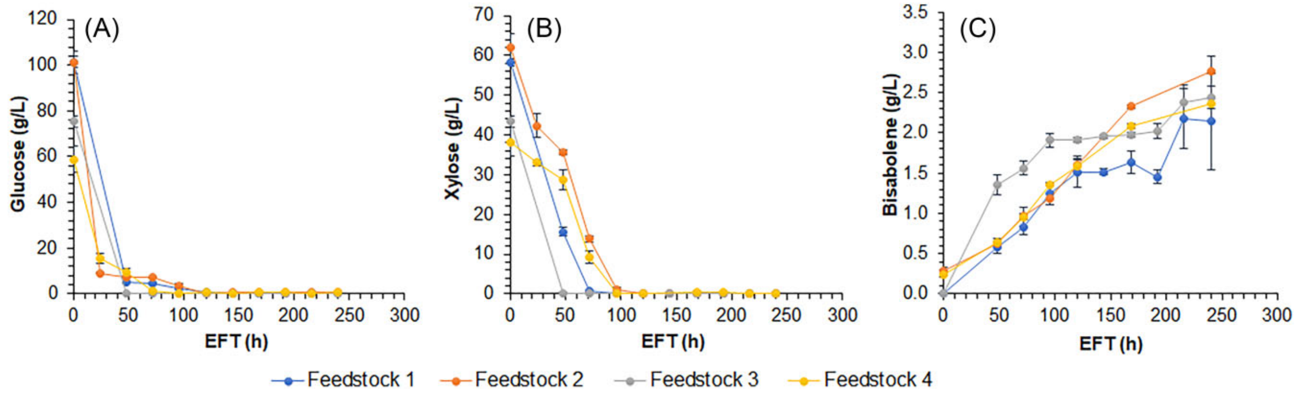

Fig. 2. Fermentation of hydrolysates with different initial sugar concentrations by *R. toruloides*: (A) glucose consumption, (B) xylose consumption, and (C) bisabolene production. Rapid sugar utilization significantly influenced initial rate of production in Feedstock 3 but did not result in higher bisabolene titers, which could indicate a more complex relationship between sugar metabolism and the production of bisabolene. Error bars indicate the standard deviation for each set of triplicate samples of the 250-ml stirred tank fermentations. (Sugars fell to near zero within 2 days.)

acid in acetonitrile. Five hundred-nanogram peptides were first loaded onto a trap column (i.e., 4 cm × 100 μm i.d. column in-house packed with 5-μm Jupiter C18 particles) using 7-μl/min MPA for 4 min and then separated by a reversed-phase capillary column, (i.e., 30 cm long and 75 μm i.d. in-house packed with 1.7-µm Waters BEH C18 particles). The elution was carried out at 200 nl/min with the following gradient: 0 min 1% MPB; 2.6 min 1% MPB; 12.6 min 8% MPB; 107.6 min 25% MPB; 117.6 min 35% MPB; 122.6 min 75% MPB; 125.6 min 95% MPB; 131.6 min 95% MPB; 132.6 min 50% MPB; 134.6 min 95% MPB; 140.6 min 95% MPB; 142.6 min 1% MPB; and 190 min 1% MPB. MS analysis was carried out in data-dependent mode for a duration of 120 min. MS settings were as follows: full MS (AGC, 3×10^6; resolution, 60 ,000; m/z range, 300–1,800; maximum ion time, 20 ms); MS/MS (AGC, 1×10^5; resolution, 45,000; m/z range, 200–2,000; maximum ion time, 100 ms; minimum signal threshold, 5×10^4; minimum AGC target, 5×10^3; isolation width, 0.7 m/z; TopN, 12; dynamic exclusion time setting, 45 s; collision energy, NCE 30).

LC-MS/MS data were searched against proteomic database of *Rhodosporidium toruloides* IFO 0880 v4.0 along with heterologous genes using MSGF+ (PMID: 25358478) and MASIC software (PMID: 18440872). The results were filtered by 1% false discovery rate and 5-ppm mass error. The resulting peptide results were then log2 transformed and subjected to an initial peptide filter to remove peptides that do not have enough data for either a quantitative test (two samples with data in at least two conditions) or a qualitative test (three samples with data within at least one condition). This reduced the initial set of 62,685 identified peptides to 40,970. Outlier sample analysis was then performed with a statistically robust Mahalanobis distance (PMID: 21852304). Visual confirmation of the results is observed via Pearson correlation, boxplots, and principal component analysis (PCA). The log2 transformed data were normalized to median sample abundance. The peptides mapped to 4,218 proteins. Protein quantification was performed with standard reference-based quantification (PMID: 23019139). Statistical analysis was performed on both the peptide and protein-level data using a standard two-sample *t*-test to compare either between anatomical fractions or time points from a quantitative perspective and a g-test to compare the strains from a qualitative perspective. Output is visualized via Volcano plots. Unsupervised clustering by strain at the protein level is observed via PCA.

Results and Discussion

Comparative Analysis of DMR-EH-Pretreated Corn Stover From Varied Batches: Impact on Bisabolene Production

Generally speaking, one of the primary measures of lignocellulosic hydrolysate efficacy is the ability of the microbial strains of interest to utilize the carbon sources that are present (usually sugars). In the corn stover hydrolysates used in this study, the main sugars of interest were glucose and xylose. As is typically the case for lignocellulosic feedstocks (Mosier et al., 2005; Saha, 2003), other sugars were present but in much smaller quantities, thus likely not meaningful values to track for the purposes of this study. While *R. toruloides* is capable of co-consuming multiple sugars, it is evident that glucose is consumed before xylose (Fig. 2A and B) suggesting carbon catabolite repression, a common regulatory mechanism observed in many yeast species including *Yarrowia lipolytica* (Niehus et al., 2018).

DMR-EH-pretreated whole corn stover Feedstocks 1, 2, 3, and 4 had an initial glucose concentration of 101.4 ± 2.3 g/L, 101.3 ± 5.0 g/L, 75.4 ± 2.34 g/L, and 58.9 ± 5.9 g/L, respectively (Fig. 2A). Differences in the performance of feedstock contributed to the differences observed in conversion performance by *R. toruloides*. Glucose and xylose were rapidly consumed in Feedstocks 1 and 3 compared to Feedstocks 2 and 4, suggesting that certain critical material attributes other than the initial sugar concentrations, not identified in this study, must be responsible for observed differences. The resulting bisabolene titers across the four Feedstock studied were not significantly different (2.14 ± 0.6 g/L, 2.77 ± 0.19 g/L, 2.44 ± 0.3 g/L, and 2.37 ± 0.06 g/L for Feedstocks 1, 2, 3, and 4, respectively). The rate of bisabolene production early on in the fermentation (∼48- to 50-hr time point in plot 2C) is higher for Feedstock 3 compared to Feedstock 1, 2, or 4 (Fig. 2C), likely as a result of higher metabolic activity. Variations in the concentration and availability of key nutrients can significantly affect microbial growth and productivity as observed with the differences in sugar consumption and bisabolene production across the hydrolysates. Understanding the specific reasons for the difference in productivity requires a careful and detailed evaluation of the critical material attributes of different corn stover batches before and after pretreatment. Having this detailed information can further lead to the screening of the dose-dependent effects, possible interactions among various material and process

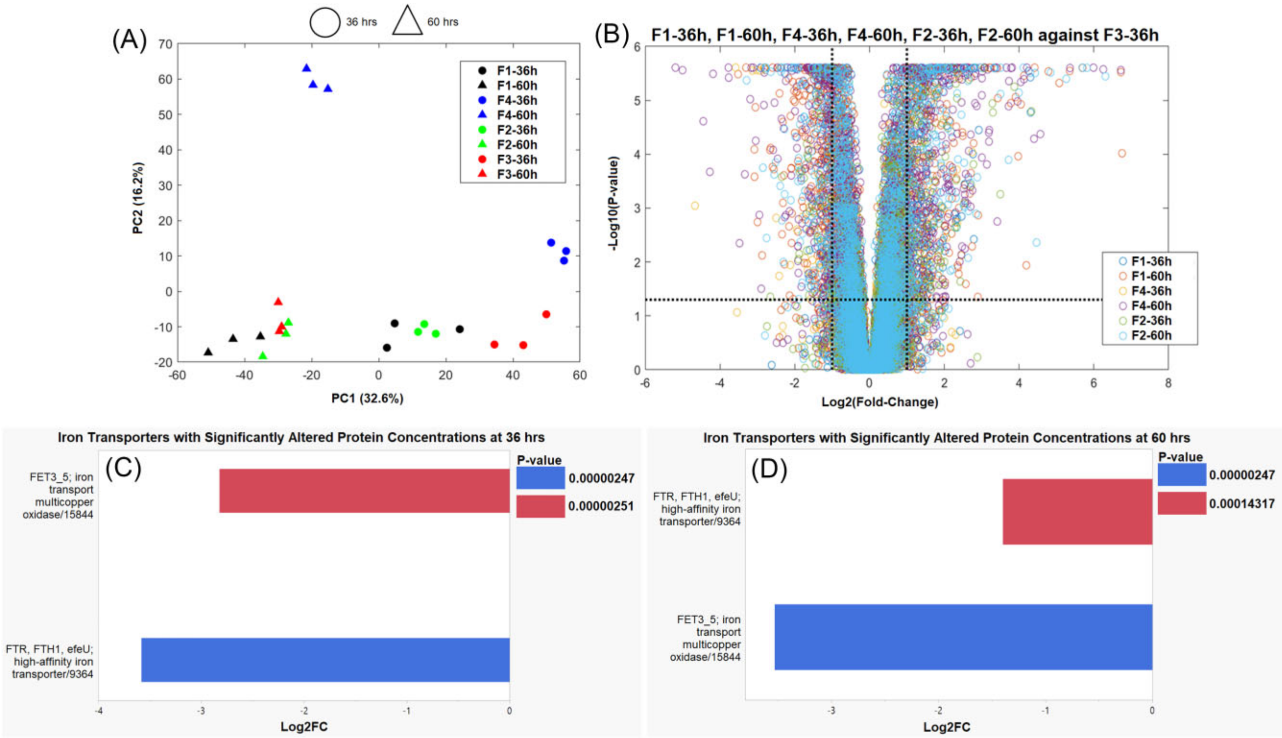

Fig. 3. (A) Principal component analysis of all proteins with complete data (3,688 proteins) showed separation by sampling time along PC1. Both the 36- and 60-h time points for Feedstock 4 separate from other feedstocks along PC2. These data show clear variation in protein expression levels between *R. toruloides* grown with different hydrolysates. (B) Representative volcano plot showing fold changes in protein expression between samples from different hydrolysates, where the vertical dotted lines correspond to −1 and 1 log2(fold change) and the horizontal dotted line is the cutoff between *p* values of <.05. (C) Downregulation of iron transport proteins in Feedstock 4 when compared to Feedstock 3 at 36-hr time point and (D) downregulation of iron transport proteins in Feedstock 4 when compared to Feedstock 3 at 60-hr time point.

attributes, and how these attributes affect the bioconversion process. The critical material attributes of feedstocks can occur as a result of differences in harvesting techniques, environmental factors, genetic variations, growing conditions, among other factors, all of which may have the ability to reduce the utility of a given feedstock or the resulting hydrolysate. Therefore, it is imperative to carefully evaluate the most suitable or reliable pretreatment approach for any given technology. Key variables observed in the composition of these feedstocks were the initial sugar concentrations and differences in the distribution of sulfur, sodium, calcium, magnesium, and silicon, shown in Table 1. While previous studies, as well as this research, support the components described in Table 1 as being influential on hydrolysate performance (Humbird et al., 2010; Palmqvist and Hahn-Hägerdal, 2000), it is quite possible that this influence is confounded by interaction effects among these components as well as the presence or absence of other components. Also notable was the formation of solids in the hydrolysate upon the introduction of potassium phosphate dibasic, which was used as the buffer in the fermentation step. Solid formation occurred in all hydrolysates except for the whole stover hydrolysate, Feedstock 4, suggesting a difference in composition. These solids formed fluffy, light-colored, suspended solids that eventually settled out. Before fermentation, the hydrolysate was sterilized by filtration, thus removing the suspended solids. Accordingly, filtration may have resulted in loss of nutrients or removal of growth inhibitors (or both). In any case, hydrolysate filtration at commercial scales is very likely cost-prohibitive, so a better understanding of such DMR-EH-derived precipitates may be an important consideration.

The proteomic dataset obtained from the four feedstock samples evaluated had 62,991 peptides from 4,863 proteins. Peptides without adequate data for a quantitative test or qualitative test were filtered out, giving 55,539 peptides and 4,790 protein families. The peptide data did not indicate outlier behavior based on either a robust Mahalanobis distance squared value associated with the peptide abundance vector, the standard Pearson correlation heat map, and standard boxplot. Significant protein expression differences were seen between all the hydrolysates compared, regardless of time point suggesting influence of different hydrolysate components on the growth of *R. toruloides*. Samples for proteomics were taken at 36 and 60 hr for each condition to allow for meaningful proteome comparisons (Fig. 3).

Comparison of proteins expressed between feedstocks showed that Feedstocks 3 and 4 had greater protein variations. Proteins involved in metal transport were found to be significantly enriched, pointing to potential differences in the concentration of these metals between hydrolysate conditions, as observed in Table 1. When we examined proteins with significantly different expression between feedstocks, we found that proteins annotated as iron transporters were more highly expressed in the Feedstock 3 condition than in Feedstock 4 at both the 36- and 60-hr time points (Fig. 3C and D). This correlates with the iron concentration obtained from ICP-OES analysis where the iron concentration was about four times higher for Feedstock 3 than for Feedstock 4 (1.98 mM and 0.31 mM in Feedstock 3 and Feedstock 4, respectively). A similar effect was observed when Feedstocks 1 and 2 were compared to Feedstock 4, which further suggests that iron plays an important role in the metabolic process. High iron concentration in Feedstocks 1, 2, and 3 compared to Feedstock 4 correlates with protective metal ion antiporter upregulation observed in this study. Copper and zinc transporters were also differentially expressed between Feedstock 4 and Feedstock 3, pointing to influence of metals in conversion of lignocellulosic biomass by *R. toruloides*. The proteomic data obtained from this study directly point to the importance of metal transporter proteins in the conversion of corn stover biomass to bisabolene. Therefore, understanding the complex interactions between metal concentrations and fermentation performance requires a multifaceted approach that might help elucidate the underlying mechanisms and provide clearer insights into the influence of metals in the conversions of lignocellulosic biomass. Measurement of key enzymes involved in dependent processes to see whether their activity aligns with protein expression data would also provide valuable insights.

Previous research has shown that DMR-EH pretreatment approach consistently yields a readily fermentable carbon source (Chen et al., 2016). In terms of sugar consumption by *R. toruloides*, the interbatch differences observed with sugar consumption and productivity may translate into significant impacts on hydrolysate performance at the commercial scale. This echoes a deeper understanding of the hydrolysate and feedstock heterogeneity, and strategies to remove or dampen the negative effects of heterogeneity. For example, DMR-EH pretreatment effectively removes lignin from lignocellulosic biomass, resulting in biomass with lower lignin content. Lignin has been shown in previous studies to interfere with enzymatic hydrolysis and fermentation processes (Li et al., 2009; Yang and Wyman, 2008). The reduced recalcitrance and inhibitor levels promote better microbial growth and fermentation performance. Nonetheless, observed differences in sugar consumption and productivity in the batches within this study suggest the presence of other critical material attributes that influences the metabolic processes.

Varying Conversion Performance of DMR-EH Hydrolysates Correlated With Compositional Differences Found in Corn Stover Anatomical Fractions

It is hypothesized that the individual anatomical fractions of corn stover vary in both composition and recalcitrance, offering biorefineries different options for processing either fractionated or whole biomass. Understanding the techno-economic impacts of variability helps end users evaluate trade-offs in conversion yield and economics for biorefinery feedstock decisions. This requires data on the composition and conversion performance of whole biomass and its anatomic fractions. The degree of conversion from four distinct DMR-EH hydrolysates (derived from husk, cob, stalk, and whole stover) to bisabolene via *R. toruloides* served as a test of this hypothesis. Glucose consumption, xylose consumption, growth rates, and bisabolene productivity and titer provide the basis of comparing performance among the corn stover fractions. Secondarily, a proteomics-based approach served as a means of understanding the underlying differences observed across the different fractions. Generally, *R. toruloides* was reasonably efficient in utilizing all studied fractions for the production of bisabolene. However, the oleaginous yeast did exhibit better performance with stalk than cob or husk, in terms of sugar consumption, growth, and bisabolene titers. With the stalk fraction of this study, the sugars were completely consumed after 48 hr, and for the whole stover, glucose was completely consumed after 72 hr while xylose was completely depleted after 96 hr (Fig. 4A and B).

The glucose in the cob was completely depleted by 144 hr but not xylose. About 71% of xylose in the cob was consumed by *R. toruloides* meanwhile only 81.9% glucose and 58.4% xylose were consumed in the husk hydrolysate. Both glucose and xylose were completely consumed in the whole corn stover and stalk fraction. In contrast, cob and husk performed poorly in comparison. The highest bisabolene titer was observed with stalk (8.89 ± 0.47 g/L) followed by whole stover (5.68 ± 0.51 g/L). Husk and cob produced 1.80 ± 0.09 and 3.61 ± 0.59 g/L, respectively. A consistently high performance was observed in the stalk fraction with respect to the growth of *R. toruloides*, sugar consumption, and bisabolene production (Fig. 4). Bisabolene titer for the whole stover feedstock was greater than the four feedstocks tested in previous section. It was also greater than the self-heated whole stover feedstocks tested in the next section of this report. Given that all other processing conditions were the same, the observed increase with the whole stover feedstock (in this section) compared to the other whole stover feedstocks in other sections can be attributed to specific differences inherent to the whole stover itself. These differences include nutrient content, chemical composition, structural differences, and intrinsic variability in the whole stover's natural composition. These differences are also reflected in Table 1. Consequently, the results observed underscore the importance of addressing critical material attributes to mitigate feedstock variability and ensure the efficiency of integrated biorefinery operations.

To understand the variability in the anatomic fractions at a molecular level, proteomic evaluation was carried out on the samples obtained from 24- and 48-hr fermentation intervals to capture the early protein expression dynamics. The distribution of identified proteins across the anatomical fractions corresponds to the differences observed during fermentation. Additionally, the grouping of the triplicate samples demonstrated the reliability of the generated data. PCA of all proteins showed significant separation by sampling time along principal component 1 (PC1). Samples collected at 48 hr showed clear separation across anatomic fractions along principal component 2 (PC2) (Fig. 5). Protein evaluation of *R. toruloides* grown on different anatomic fractions showed significant differences in protein expression as seen in the PCA plot in Fig. 5. Whole stover and stalk samples collected at the 48-hr interval showed weak correlation on negative PC2 while husk and cob were correlated on positive PC2. Additionally, Fig. 6 shows significantly expressed *R. toruloides* proteins from different anatomic fractions.

Generally, comparing the protein expression between the anatomical fractions showed that proteins relating to lipid transport, and metabolism, energy production and conversion exhibited significant differential were significantly expressed (Fig. 6). Proteins responsible for inorganic ion transport and metabolism and energy production and conversion were specifically downregulated in the following hydrolysates when compared to each other: whole stover/cob, whole stover/husk, stalk/husk, and stalk/cob (Fig. 6). Notable is the fact that contained in these proteins are iron–sulfur (Fe–S) clusters, which are essential cofactors found in a wide variety of proteins and play critical roles in numerous biological processes, including electron transport, enzyme catalysis, and regulation of gene expression. Interestingly, associating the downregulation of inorganic ion transport and metabolism, and energy production and conversion data showed that iron played a vital role in the conversion of lignocellulosic feedstock to bisabolene. However, it is unclear whether the downregulation of proteins responsible for inorganic ion transport and metabolism and energy production and conversion was as

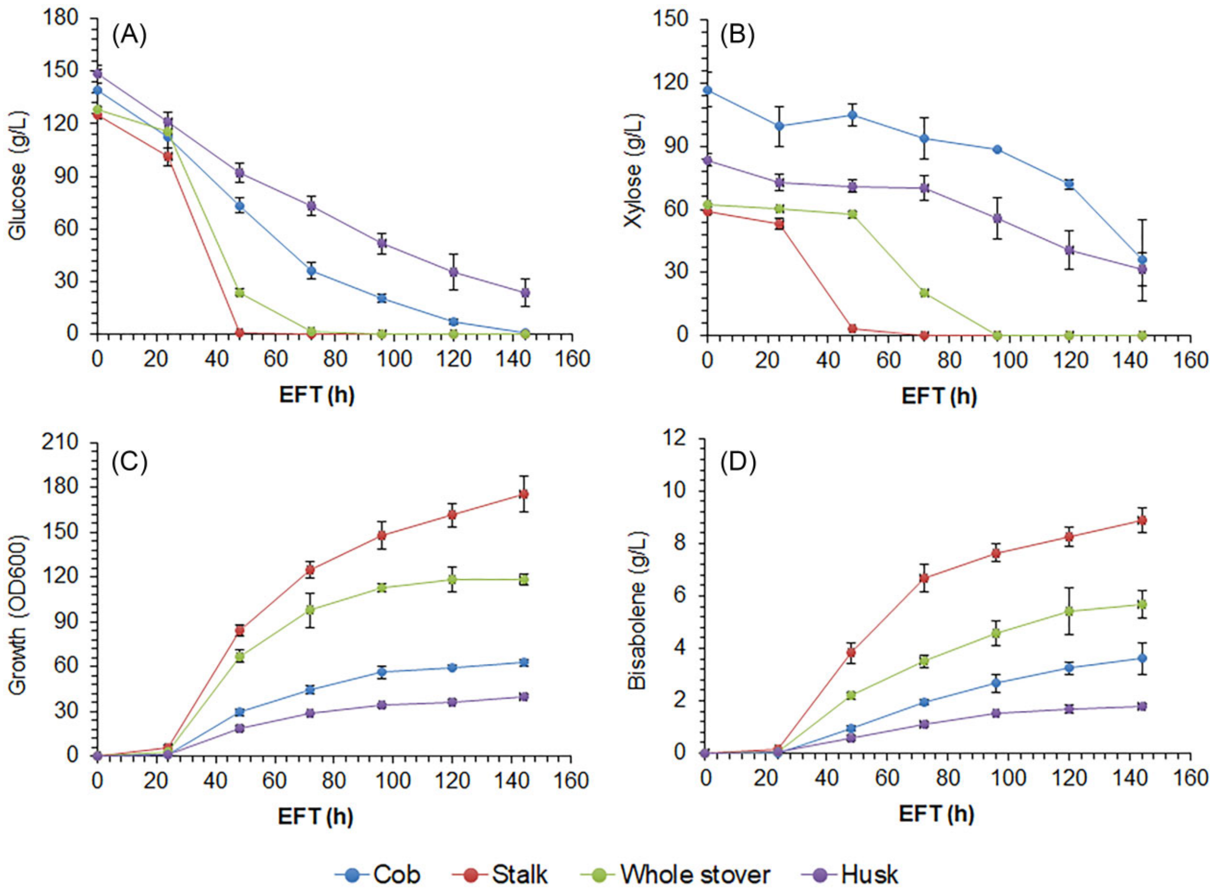

Fig. 4. Fermentation of different corn stover anatomical fraction hydrolysates by *R. toruloides*. (A) Glucose consumption (stalk > whole stover > cob > husk); (B) xylose consumption (stalk > whole stover > cob > husk); (C) growth; and (D) bisabolene production (stalk > whole stover > cob > husk).

a result of iron limitation or possible toxicity. This is subject to further investigation.

In this study, proteins related to lipid metabolism and transport were observed to be differentially expressed. For example, acyl-CoA synthetase which activates fatty acids by converting them into acyl-CoA for their subsequent metabolism and incorporation into lipids. Another proteins related to lipid metabolism and transport is the hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase which facilitates the efficient breakdown of fatty acids into acetyl-CoA through the β-oxidation pathway which is then utilized in the mevalonate pathway to produce bisabolene. These proteins were seen to be significantly upregulated in stalk compared to cob, husk, or whole stover. This means that, for the purposes of this study, *R. toruloides* performed best on corn stalk hydrolysates, compared to other corn stover anatomical fractions. Different levels or interactions of various material attributes of the anatomic fractions certainly contributed to the differences observed in the conversion performance observed. Notable are the levels of magnesium in stalk, which was about two times higher than in cob or husk and phosphorus, which had a 2.2 times higher concentration in stalk than in cob or husk (Table 1). Adequate phosphorus is essential for normal growth and cell metabolism. Phosphorus limitation can lead to reduced growth rates and metabolic activity as observed in conversion performance of cob and husk. However, *R. toruloides* has developed (Wu et al., 2010) mechanisms to adapt to low phosphorus-limited conditions, such as altering its metabolic pathways to optimize phosphorus usage. For example, under phosphorus-limited conditions, *R. toruloides* may exhibit stress responses such as changes in enzyme activity and gene expression. The yeast may upregulate genes associated with phosphorus uptake and storage, allowing it to better survive and grow in nutrient-poor environments (Wang et al., 2022). Additionally, the availability of phosphorus can impact the production of other metabolites, including organic acids and secondary metabolites. Therefore, understanding how phosphorus affects these processes especially in a complex media such as corn stover hydrolysate stream can be crucial for optimizing fermentation processes.

Previous studies of *R. toruloides* often explore its use with whole biomass or generic lignocellulosic materials. For example, general studies have focused on its lipid production capabilities using whole corn stover hydrolysates or other biomass sources (Dai et al., 2019; Huang et al., 2022). This yeast has been studied for its lipid transport and metabolism proteins due to its potential in biotechnological applications, particularly in the production of biofuels and bioproducts. Evaluating the differences in the conversion of the different anatomical fractions stresses the need for advanced modeling of fermentation data combined with omics technologies to provide a comprehensive approach to understanding and optimizing conversion of waste streams. Fermentation processes involve numerous variables and complex interactions between microorganisms, nutrient, and process parameters and as such advanced modeling would help to better understand these interactions in detail. Given the high amount of

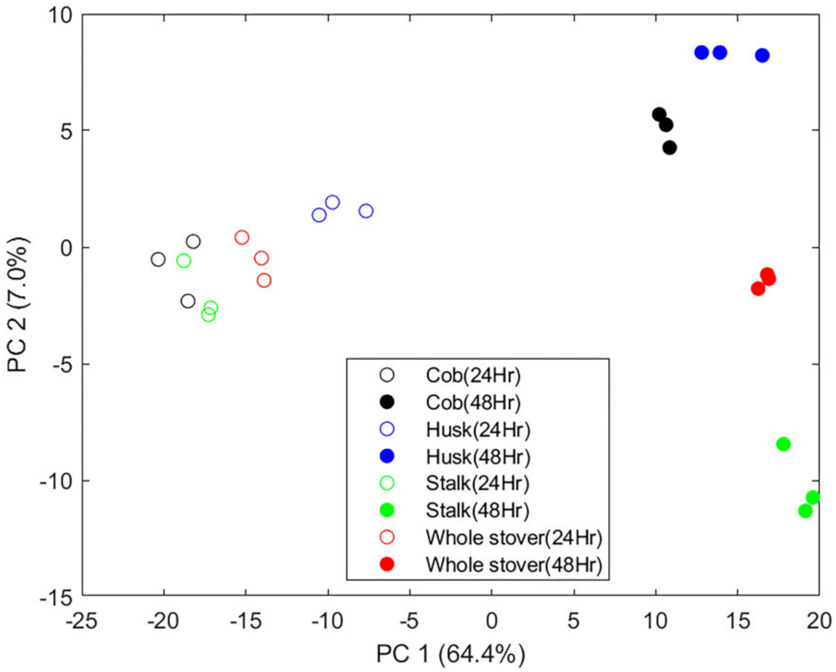

Fig. 5. Analysis of differential protein expression in *R. toruloides* as a function of growth on deacetylation, mechanical refining, and enzymatic hydrolysis hydrolysates of four different anatomical fractions of corn stover. A principal component analysis of proteomic data (principal component analysis plot) of biological replicates, showing their distribution as a function of hydrolysate (stalk, husk, cob, and whole stover) at 24 and 48 hr. There are distinct differences among the groups, as well as clear separation by time on the first principal component.

unidentified proteins that were either significantly upregulated or downregulated, integrating these data with modeling would help in deciphering the role of unidentified proteins and their impact on the conversion process. Other researchers have used advanced techniques such as fluorescence lifetime imaging, analytical pyrolysis coupled with GC-MS, and machine learning to analyze corn stover biomass properties. Such studies have yielded insights into biomass heterogeneity, including effects of storage on the biological degradation of the cell wall (Lacey et al., 2016; Leal et al., 2022; Li et al., 2018). Certain fractions of corn stover can produce compounds during the processing (e.g., furfural, hydroxymethylfurfural (HMF)), which negatively affect microbial fermentation. By selectively processing parts with lower inhibitor generation potential, biorefinery operators may be able to increase the overall process efficiency (Humbird et al., 2011). However, modeling of fermentation data combined with different omics layers such as transcriptomics, proteomics, and metabolomics can provide a holistic view of the fermentation process and thus provide a powerful approach for generating large datasets that would allow for a more informed decision-making regarding process adjustments, troubleshooting, and scale-up, exploring different scenarios such as biomass blending, help with designing more robust and efficient conversion processes as well as uncovering the roles of unidentified proteins, and overall, drive innovation in biotechnology applications.

Assessing the Variability in R. *toruloides* Conversion as a Function of Corn Stover Bale Self-heating During Storage

Previous studies on biological self-heating of anatomical corn stover fractions revealed significant variations in the surface attributes due to self-heating and degradation during storage. Researchers observed that degradation leads to structural and molecular changes and reported that biologically induced degradation affects surface properties such as texture, surface area, and porosity of biomass (Leal et al., 2022). Furthermore, self-heating results in changes to cell structures that lead to increase in properties such as brittleness (Chu et al., 2016). Researchers also showed that self-heating and microbial degradation can impact corn stover's physical and chemical properties, creating variability that could impact downstream processes such as fermentation (Bose et al., 2020; Leal et al., 2022; Li et al., 2020). Darku et al. showed that self-degraded biomass stored for a period of 220 days accelerated pretreatment for bioconversion (Darku et al., 2010). These findings suggest that the inherent heterogeneity of lignocellulosic feedstocks and differences across distinct plant fractions could complicate standard bioprocessing approaches.

This part of the study focused on conversion performance of *R. toruloides* using DMR-EH hydrolysate from biological heating and degradation during bale storage of corn stover. The degree of self-heating was evaluated based on visual identification and was categorized as mild, moderate, or severe self-heated biomass as Bose et al. (2020) described. It is notable that the sugar concentration in mild self-heated hydrolysate was significantly higher (136.6 g/L of glucose and 74.5 g/L of xylose) than in moderate (79.1 g/L of glucose and 34.37 g/L of xylose) or severe self-heated (96.5 g/L of glucose and 34.5 g/L of xylose) hydrolysates (Fig. 7A and B). Comparing the sugar consumption by *R. toruloides* on the different hydrolysate showed variability in sugar consumption profile across the different degrees of self-heated samples. For example, glucose and xylose in severe self-heated hydrolysate were completely consumed by 48 and 72 hr, respectively (Fig. 7A and B). Glucose in moderate self-heated hydrolysate was completely depleted by 144 hr but xylose was not completely consumed. For mild self-heated hydrolysate, neither glucose nor xylose was completely consumed after 144-hr fermentation time (Fig. 7). *Rhodotorula toruloides* grew better on severe self-heated hydrolysate than on

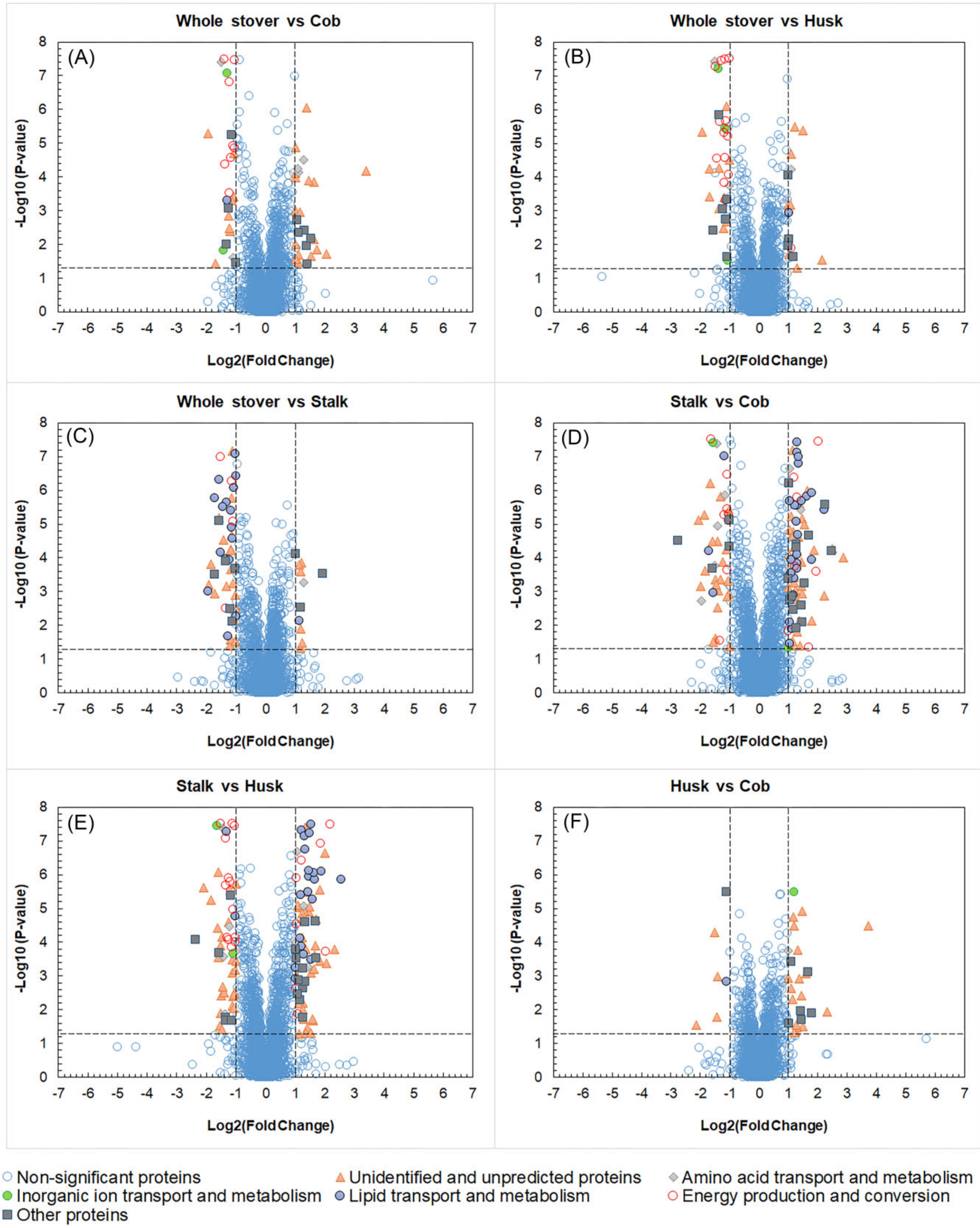

Fig. 6. A plot of logarithmic fold change versus significance (i.e., volcano plot) is a pairwise comparison of protein expression of *R. toruloides* after 48 hr of growth on hydrolysates from the four different anatomical fractions. The horizontal line corresponds to a significance value of $p < .05$. The dotted vertical lines bound the minimal fold change for the most differentially expressed genes. To right and left: functional categories for differentially expressed proteins (significantly upregulated and downregulated protein). Distribution of significantly upregulated and downregulated differentially expressed proteins, with an emphasis on proteins related to energy production and conversion, inorganic ion transport metabolism, and lipid transport metabolism, for their possible influence on bisabolene production.

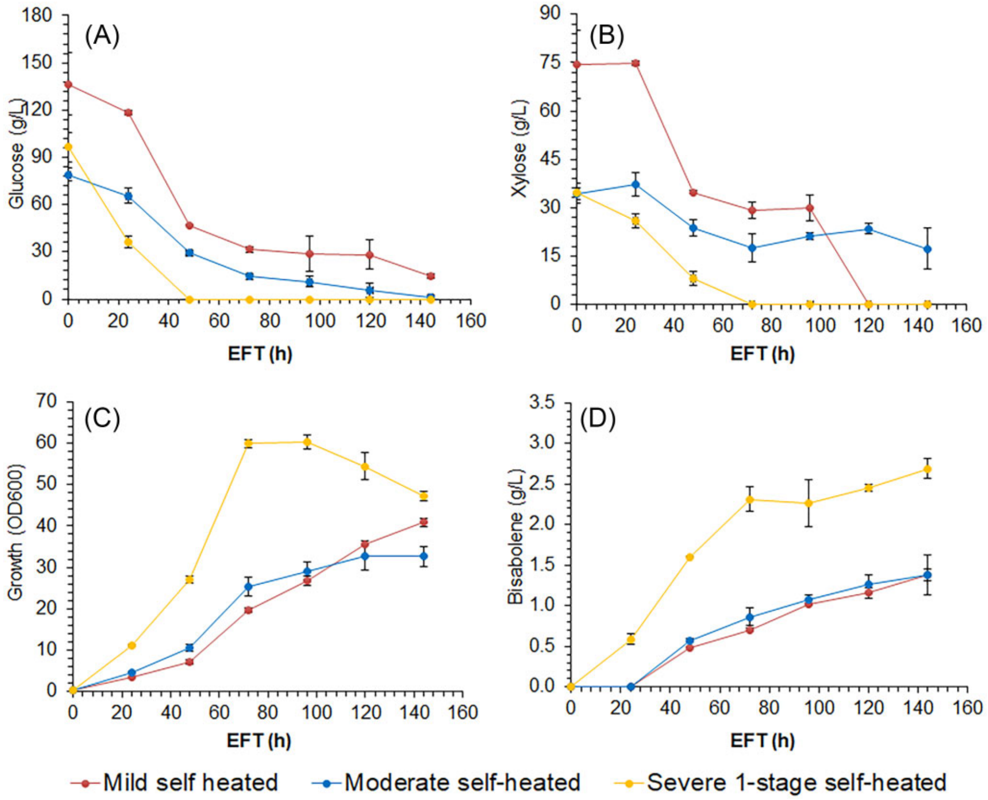

Fig. 7. Sugar consumption, growth, and bisabolene production by *R. toruloides* in corn stover hydrolysates under varying levels of self-heating. (A) Glucose consumption. (B) Xylose consumption. (C) Growth of *R. toruloides* on different levels of self-heated feedstock. Sugar consumption was more rapid in severe self-heated hydrolysate compared to either mild or moderate self-heated hydrolysate. Severe > moderate > mild. (D) Bisabolene production by *R. toruloides* on hydrolysates with varying degrees of self-heating. Severe > moderate > mild.

mild or moderate self-heated hydrolysates (Fig. 7C). Similarly, the bisabolene titers with severe self-heated hydrolysate were significantly higher ($p < .05$) than mild or moderate self-heated hydrolysate (2.69 ± 0.12 g/L, 1.38 ± 0.24 g/L, and 1.38 ± 0.08 g/L for severe, mild, and moderate self-heated hydrolysate, respectively). Moderate and severe self-heated had similar sugar profiles compared to mild self-heated. However, the bisabolene trend in moderate self-heated was similar to mild self-heated.

While severe and moderate self-heating conditions can lead to lower sugar yields due to degradation and formation of inhibitors and volatilization, under these same conditions, the self-heating process may have significantly disrupted the bulk structure of plant tissue due to microbial enzyme secretion, thereby improving enzyme accessibility and resulting in overall enhanced conversion performance. The severe self-heating of corn stover biomass thus points to potentially important considerations in trading the total potential sugar yield of biomass with the ease of accessibility of those sugars, per the strengths and weaknesses of a given hydrolysis method and the level sensitivity of a given biocatalyst to the inhibitory compounds, which may arise as a function of storage-derived self-heating. Also, the somewhat surprising outcome of improved hydrolysate performance from severely self-heated corn stover brings into consideration other logistics advantages that longer-term storage or on-field storage may offer.

Though the sugar concentration in severe self-heated corn stover subjected to a two-stage (severe two-stage self-heated) process (where sodium carbonate was used before DMR-EH) was about 10% higher than severe self-heated corn stover pretreated with only DMR-EH (severe one-stage self-heated), no significant difference in bisabolene titers was observed. However, the sugar consumption was largely different between both conditions, as shown in Fig. 8. We observed higher consumption rate with severe one-stage self-heated compared to severe two-stage self-heated as well as slightly higher bisabolene production rate. Higher production rate and substrate consumption can improve the economics of the bioprocess by reducing the cost per unit per run and thus increasing the overall production capacity. The similarities in the fermentation performance between the severe one-stage self-heated and severe two-stage self-heated corn stover-pretreated samples (Fig. 8) can be attributed to similar levels of deacetylation. Furthermore, the efficiency of enzymatic hydrolysis might be similar for both methods. Additionally, *R. toruloides* may have a broad tolerance range to variations in hydrolysate composition resulting from the difference in pretreatment methods. The lack of significant difference in fermentation performance can be attributed to the similar effectiveness of both pretreatment methods in producing fermentable sugars and the tolerance of the fermentation organism to variations in substrate composition. A two-stage pretreatment process might be detrimental to improving sugar yield; however, in the case of severe self-heated corn stover, a two-stage pretreatment might not be necessary because self-heating of such degree results in the accessible and porous material for enzymatic activity.

Conclusions

Corn stover is a leading candidate and an important model for lignocellulosic biomass-to-ethanol processing and SAF conversion.

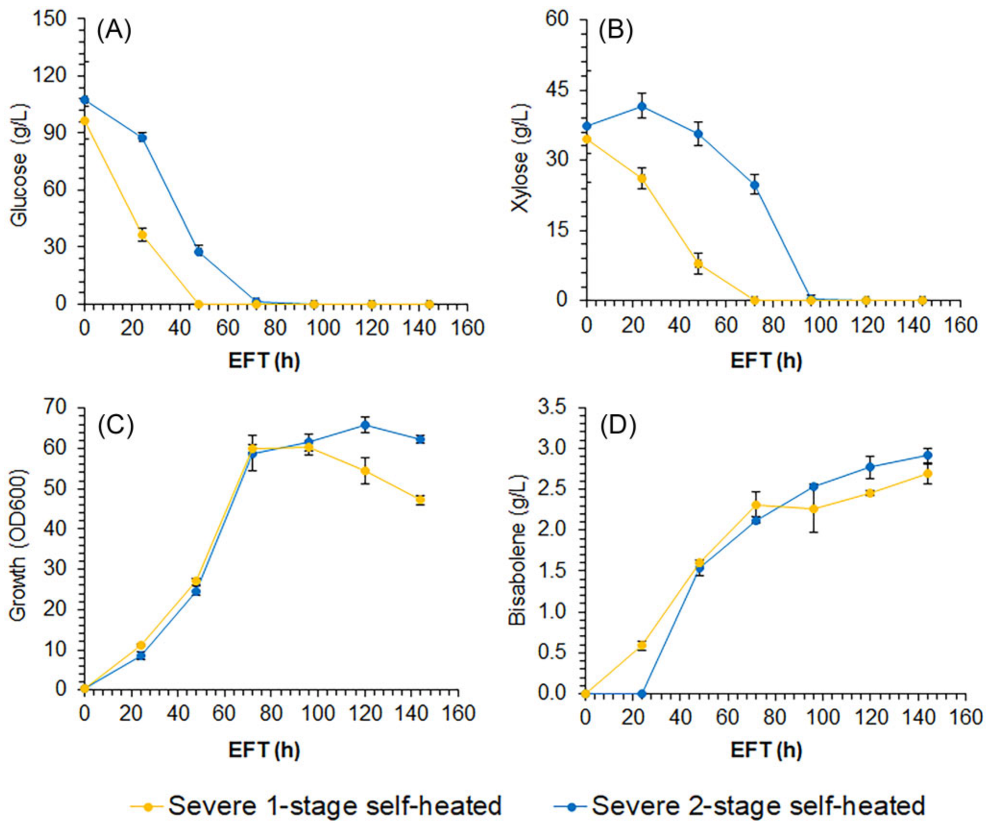

Fig. 8. Comparison of sugar consumption in severe self-heated corn stover that was subjected to a one-stage pretreatment prior to enzymatic hydrolysis and a two-stage pretreatment using sodium carbonate before deacetylation with sodium hydroxide. (A) Glucose consumption by *R. toruloides*. (B) Xylose consumption. (C) Growth of *R. toruloides* on corn stover anatomical fractions. Sugar consumption was faster in severe one-stage self-heated hydrolysate than in severe two-stage self-heated hydrolysate. Severe one-stage self-heated > severe two-stage self-heated. (D) Bisabolene production by *R. toruloides* on severe one-stage self-heated hydrolysate and severe two-stage self-heated hydrolysate. Result was not statistically significant ($p = .99$).

However, its economic and technical valuation depends on detailed knowledge of its chemical composition, which varies due to factors like source, growing conditions, storage, and processing methods. A growing body of publicly available data on the impact of feedstock variability on fermentation performance supports biorefineries faced with a choice to either accept the inherent performance risks associated with varying feedstocks or invest in generating proprietary data and knowledge. Corn stover exhibited significant heterogeneity within the plant, with different anatomical fractions (e.g., cob, husk, and stalk) showing varied chemical compositions, and as a function of degradation during storage. One primary measure of lignocellulosic efficacy is the ability of microbial strains to utilize available carbon sources, primarily glucose, which was the case in this study. The different corn stover feedstocks studied showed varying conversion performance, which suggested that critical material attributes present in the biomass influenced the conversion performance. Proteomic analysis revealed significant protein expression differences, especially in the expression of metal transport proteins and energy conversion, as well as lipid metabolism and transport, further indicating variations in hydrolysate composition. Understanding these interactions requires a multifaceted approach and further investigation. Overall, DMR-EH pretreatment was shown to be an effective pretreatment approach as *R. toruloides*-produced bisabolene on all feedstocks evaluated. However, there is a need to address feedstock heterogeneity to improve commercial-scale performance.

The United States has been exploring different strategies to sustainably produce aviation fuels, reduce GHG emissions, and meet growing market demand. Even though commercial viability is a major concern for large-scale deployment and commercialization, the ability of the aviation sector to meet SAF production and GHG reduction targets depends largely on the amount of sustainable feedstock that will be available for the sector (Chao et al., 2019; Nelson, 2018; Pasa et al., 2022). As is generally the case for the economics of commodity chemicals, the availability and cost of feedstocks are critical for the viability of virtually every biomass processing activity, regardless of the final product. Utilizing these feedstocks would be critical for achieving aviation fuel goals as well as providing sustainable feedstock for decarbonization. However, as this and other studies have shown, feedstock variability can lead to variability in product outcomes, which in turn introduces significant business risk and economic uncertainty. Understanding and, where possible, neutralizing variability in the biomass can protect product yield and allow biorefineries to focus their sensitivity analyses on factors over which they have less influence, allowing them to build more informed strategies for their business and day-to-day operations. Even though SAF production from lignocellulosic materials is still at the demonstration scale, researchers in the Feedstock Conversion Interface Consortium have made extensive efforts towards de-risking biomass variability to make the production of SAF economically feasible (Bose et al., 2020; Leal et al., 2020, 2022; Ray et al., 2020). This study elucidates the pronounced effects on conversion efficiency as a function of batch-to-batch variability of whole corn stover and corn stover anatomical fractions, as well as biological degradation during storage, emphasizing the importance of understanding feedstock variability to improve bioprocess operations and economics. Further studies should also include engineering the strain for increased robustness, enabling it to better withstand variations in feedstock composition. An effective solution will likely require a combined approach encompassing improved feedstock characteristics, enhanced strain robustness, and an optimized fermentation process. This highlights the critical importance of collaboration in addressing some of the most significant challenges.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

Data Availability

The proteomics data generated in this study have been deposited in the MassIVE database under accession MSV000095450. *Reviewers can access the data via FTP using: Server: massive.ucsd.edu, User: MSV000095450, Password: Corn5830*

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