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RESEARCH ARTICLE

Feedstock variability impacts the bioconversion of sugar and lignin streams derived from corn stover by *Clostridium tyrobutyricum* and engineered *Pseudomonas putida*

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Abstract

Feedstock variability represents a challenge in lignocellulosic biorefineries, as it can influence both lignocellulose deconstruction and microbial conversion processes for biofuels and biochemicals production. The impact of feedstock variability on microbial performance remains underexplored, and predictive tools for microbial behaviour are needed to mitigate risks in biorefinery scale-up. Here, twelve batches of corn stover were deconstructed via deacetylation, mechanical refining, and enzymatic hydrolysis to generate lignin-rich and sugar streams. These batches and their derived streams were characterised to identify their chemical components, and the streams were used as substrates for producing muconate and butyrate by engineered *Pseudomonas putida* and wildtype *Clostridium tyrobutyricum*, respectively. Bacterial performance (growth, product titers, yields, and productivities) differed among the batches, but no strong correlations were identified between feedstock composition and performance. To provide metabolic insights into the origin of these differences, we evaluated the effect of twenty-three isolated chemical components on these microbes, including three components in relevant bioprocess settings in bioreactors, and we found that growth-inhibitory concentrations were outside the ranges observed in the streams. Overall, this study generates a foundational dataset on *P. putida* and *C. tyrobutyricum* performance to enable future predictive models and underscores their resilience in effectively converting fluctuating lignocellulose-derived streams into bioproducts.

INTRODUCTION

Feedstock variability poses a significant challenge in lignocellulosic biorefineries, impacting operational reliability, conversion performance, and product quality (Carpenter et al., 2014; Kenney et al., 2013; Ray et al., 2020; Yan et al., 2020). This variability leads to the inability to predict performance, which increases the inherent risk associated with each new feedstock batch in large-scale operations. The sources of variability are very diverse. Variability can be inherent to the feedstock, such as the specific variety of corn stover (Kenney

et al., 2013; Williams et al., 2016), and enhanced by differences in geographical region, year-to-year precipitation (Carpenter et al., 2014; Ray et al., 2020; Templeton et al., 2009), or biomass collection methods. Different agronomic processes, use of harvesting equipment, and feedstock collection strategies have been shown to affect feedstock ash and moisture levels (Ray et al., 2020; Williams et al., 2016), which in turn impacts the concentrations of metals, salts, and inorganic ions in the streams (Li, Aston, et al., 2016). In addition, the usage of different harvesting equipment can result in different proportions of crop anatomical fractions recovered, which introduces further compositional variability (Chen et al., 2007; Williams et al., 2016). Storage methods also affect the moisture content of the bales and, thus, the level of bale degradation from microbial activity and self-heating (Li et al., 2020; Montross & Crofcheck, 2004). This variability has an effect on the lignocellulose deconstruction processes. For instance, acetyl and lignin content have been shown to impact cellulase accessibility during enzymatic hydrolysis processes, altering sugar yields (Li, Aston, et al., 2016). In brief, feedstock variability permeates throughout the entire process chain (Ray et al., 2020), and is likely to impact the overall microbial efficiency during the bioconversion of lignocellulose-derived streams to biofuel and biochemical precursors.

Lignocellulose deconstruction methods are diverse (Guo et al., 2022), leading to varied compositions in the streams utilised for bioconversion processes. An extensively studied deconstruction process is dilute-acid pre-treatment combined with enzymatic hydrolysis. This process normally generates lignin streams that are recalcitrant to depolymerisation and upgrading (Katahira et al., 2016) and sugar streams that contain furfural, 5-hydroxymethylfurfural, and acetate (Chen et al., 2012; Franden et al., 2013). Some of these compounds are recognised to inhibit biotechnological chassis, such as *Zymomonas mobilis* (Franden et al., 2013; Lawford & Rousseau, 2003), *Saccharomyces cerevisiae* (Banerjee et al., 1981), *Pseudomonas putida* (Guarnieri et al., 2017), *Escherichia coli* (Zaldivar et al., 1999), and *Clostridium tyrobutyricum* (Suo et al., 2023). In contrast, low concentrations of these compounds promoted growth in some bacterial species, such as *Cupriavidus basilensis* (Koopman et al., 2010) *and Clostridium acetobutylicum* (Zhang et al., 2012). The acid pre-treatment of spruce wood (Alriksson et al., 2011) also revealed the production of formic acid, which inhibited the growth of wild-type *P. putida* at concentrations as low as 1.0 g/L (Horlamus et al., 2019). Another deconstruction process, combining deacetylation, mechanical refining, and enzymatic hydrolysis (DMR-EH) (Figure 1), has been demonstrated to not generate furan-derived inhibitors (Chen et al., 2014). This method utilises equipment comparable to that established in the pulping industry, while avoiding high pressures and temperatures (Chen et al., 2014). To the best of our knowledge, the effect of feedstock variability on microbial performance using DMR-EH deconstruction processes has not yet been assessed.

The deconstruction of corn stover via DMR-EH involves a series of steps to generate lignin and sugar-containing streams (Figure 1). The first step, deacetylation with a mild NaOH treatment, generates a

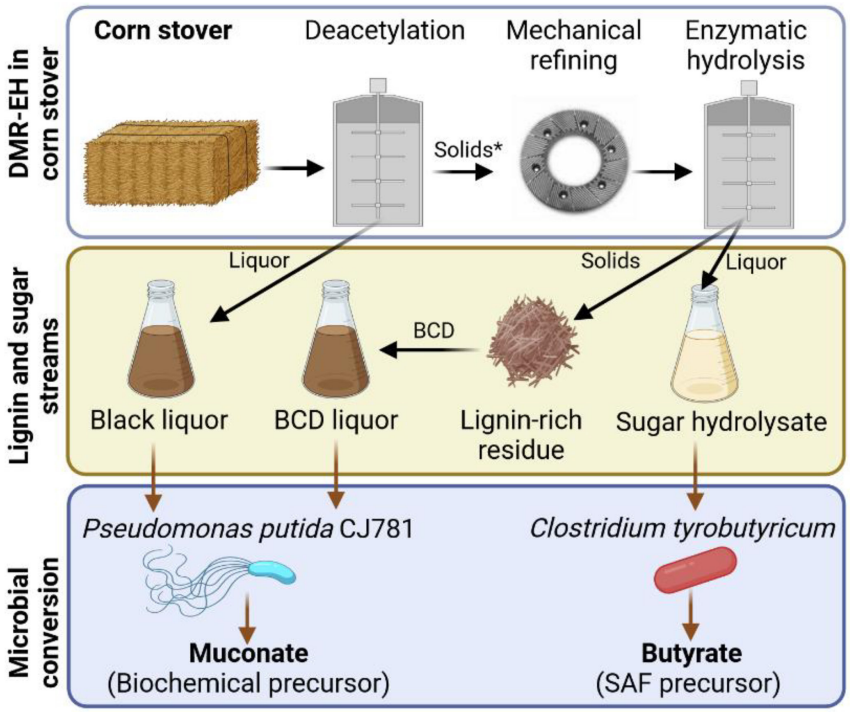

FIGURE 1 Bioconversion of corn stover to biochemicals and biofuel precursors. The deconstruction process (DMR-EH) of corn stover (top), the generation of lignin-rich and sugar streams (middle), and biocatalysts used in this study (bottom) are shown. BCD, base-catalysed depolymerisation. * Solids were washed with H_2O. Figure created with BioRender.com.

lignin-rich liquor (referred to as black liquor) and a solid material that is further milled and hydrolysed with cellulases and hemicellulases (Figure 1) (Chen et al., 2014). After enzymatic hydrolysis, two streams are produced: one soluble, containing sugars, and one insoluble, with a high lignin content. Because solid lignin cannot be easily upgraded biologically, this stream can be further solubilised through base catalysed depolymerisation (BCD) (Figure 1). Lignin streams have been used as substrates for muconate production by engineered *P. putida* (Almqvist et al., 2021; Rodriguez et al., 2017; Salvachúa et al., 2018; Vardon et al., 2015), an aerobic bacterium capable of tolerating and efficiently catabolising lignin-related aromatic compounds at a high rate compared to other microbes (Rodriguez et al., 2017; Salvachúa et al., 2015, 2020). Muconate is a C6-dicarboxylic acid that holds potential for conversion into commercial commodity chemicals, such as adipic acid and terephthalic acid (Curran et al., 2013; Draths & Frost, 1994), and can be directly used for the production of performance-advantaged composites (Rorrer et al., 2016). Sugar-derived streams from DMR-EH have also been utilised as a substrate for butyrate production by wild-type *C. tyrobutyricum* (Salvachúa et al., 2021). This bacterium is an anaerobe and generates butyrate as nearly the sole fermentative product (Salvachúa et al., 2021; Zhu et al., 2002). Butyrate has been identified as a sustainable aviation fuel (SAF) precursor (Kwon & Han, 2021), but it also finds utility in the food (Shu et al., 2011), medicine (Berni Canani et al., 2004), agriculture (Browning et al., 2006), and chemical sectors (Cao et al., 2011). Despite the promising bioconversion results of DMR-EH-derived streams to biofuel and biochemical precursors, our understanding of the resilience of these microbes in variable streams from different feedstock batches remains unexplored.

In this study, we aimed to examine the impact of feedstock variability on the performance of engineered *P. putida* KT2440-CJ78 (Kuatsjah et al., 2022) (thereafter *P. putida* CJ781) and wild-type *C. tyrobutyricum* in terms of growth, product titers, yields, and productivities. To achieve this goal, we deconstructed 12 corn stover batches via DMR-EH (Figure 1) and both the original corn stover and the resulting DMR-EH-derived streams were extensively characterised to identify their chemical components (hereafter, material attributes (MAs)). The lignin-rich streams were upgraded by *P. putida* CJ781 to muconate and the sugar streams by *C. tyrobutyricum* to butyrate. The MAs identified in the original feedstocks and DMR-EH-derived streams were correlated with microbial performance and the impact of isolated MAs was tested in both microbes. Overall, the performance of both microbes was influenced by stream variability, but both successfully tolerated higher MA concentrations than those typically found in DMR-EH streams, a positive trait for mitigating the adverse effects of feedstock variability during bioconversion processes.

EXPERIMENTAL PROCEDURES

Deconstruction of corn stover for the generation of lignin-rich and sugar streams via DMR-EH

Deacetylation and mechanical refining (DMR)

The DMR process was carried out using the FY18 National Renewable Energy Laboratory (NREL) State of Technology conditions (70 kg NaOH/ton of biomass at 92°C for 2 h) in a 90 L reactor followed by disk refining using an in-house 12″ disk refiner and Szego mill. More specifically, deacetylation was carried out in a 90 L paddle-type deacetylation reactor using 5.3 kg of each corn stover batch (5.0 kg dry) in a final concentration of 10 wt% slurries consisting of ~45 kg of NaOH solution at 70 kg NaOH/metric ton biomass (oven dried) and at 90°C for 2 h. The deacetylated corn stover was then pressed to ~30% solids with a Vincent press (Model 10), washed with 60 kg of water, pressed again to ~40% solids with the Vincent press, washed with 60 kg of water and titrated with sulphuric acid to pH ~5.3, and then pressed with the Vincent press again to ~40 wt% solids. The disk refining was carried out in the 12″ disk refiner with a gap of 10/1000 inch using the deacetylated corn stover at 30% solids. The disk-refined corn stover was then milled using the Szego mill at 5% solids. The DMR-treated biomass was then dewatered to 30% solids using the Vincent press.

Enzymatic hydrolysis (EH)

The EH was carried out using a 9-L roller bottle made of 316 stainless steel. The biomass (4 kg) was loaded in sterilised vessels at the required solids loadings. Prior to enzyme hydrolysis, the deacetylated and mechanically refined corn stover batches were titrated with sodium hydroxide to a pH of 5.3 in an industrial dough mixer (Presto Planetary Mixer, Model PM-30). Then 4 kg of the pH-adjusted substrate was autoclaved at 121°C for 30 min. The cellulase/hemicellulase enzyme cocktail and additional water were added to the final required total solids concentration of 20%. As previously detailed (Salvachúa et al., 2021), a concentration of 16 mg/g of Novozymes Cellic® Ctec3 and 4 mg/g of Novozymes Cellic® HTec3 (Franklinton, NC, USA) were added and the hydrolysis occurred at 50°C for 5 days. The hydrolysate slurries were then harvested and centrifuged to remove lignin-rich solids from the liquor phase.

Solubilisation of lignin-rich solids remaining after the enzymatic hydrolysis step

Lignin-rich solids (Figure 1) were subjected to base-catalysed deconstruction (BCD) as previously reported (Rodriguez et al., 2017). Briefly, 3 g of dry residue and 30 mL of 1 wt% NaOH in deionised water were added to a 75 mL batch reactor. The reactor was sealed, purged of air, leak checked, heated to 120°C, and held for 40 min. The reactor was quenched in cool water and the solids were filtered using a 60 mL Chemrus funnel.

Analysis of feedstocks and DMR-EH-derived streams

Compositional analyses of feedstocks and black liquors to quantify polysaccharides and lignin content

Compositional analyses of feedstocks and black liquors were conducted using NREL's Laboratory Analytical Procedure (LAP) 'Structural Carbohydrates and Lignin in Biomass' (Sluiter et al., 2008). We note that aqueous black liquors (from controls and microbial cultivations) were first centrifuged for 15 min at 6400 *g* (to remove cells) and 20 mL of the supernatants were lyophilised for compositional analyses. Carbohydrates were quantified via High Performance Liquid Chromatography (HPLC) using an Agilent 1100/1200 and Shodex SP0810 column. Acetic acid was quantified using an Agilent 1260 and Aminex HPX-87H column. Parameters for both HPLC methods are outlined in the NREL LAP. Calibration standards were purchased from Absolute Standards. Results are shown in Table 1, Tables S1 and S2.

TABLE 1 Total sugar concentrations in hydrolysates from twelve corn stover sources.

Sample description	Total sugars (g/L)
Feedstock 1	187.3
Feedstock 2	188.5
Feedstock 3	125.6
Feedstock 4	127.6
Feedstock 5	136.8
Feedstock 6	166.5
Feedstock 6_cob	214.9
Feedstock 6_stalk	193.5
Feedstock 6_husk	157.9
Feedstock 7_mild	149.2
Feedstock 7_moderate	103.1
Feedstock 7_severe	90.4

Note: These values include monomers of glucose, xylose, and arabinose. Results are derived from single corn stover batches.

Analyses of sugars, butyric acid, and other carboxylic acids in mock black liquor, black liquors, and sugar hydrolysates by HPLC-RID

We note that before analysing carboxylic acids in black liquors via HPLC, samples were acidified with 72% sulphuric acid (to a final concentration of 3.36% sulphuric acid to precipitate oligomeric lignin and avoid interferences with the analytical method), vortexed for 30 s, and re-filtered using 0.2-micron PTFE filters. All other samples (mock media and sugar hydrolysates) were analysed for carboxylic acids without any acidification. Monomeric sugars, butyric acid, and other small carboxylic acids were analysed by HPLC coupled to a refractive index detector (RID) as previously described (Alt et al., 2024). Briefly, samples and standards were analysed using a Bio-Rad Aminex HPX-87H column and a mobile phase of 0.01 N aqueous sulphuric acid.

Analysis of muconic acid isomers and aromatic compounds in mock black liquor and black liquors by HPLC-DAD, UHPLC-DAD and UHPLC–MS/MS

Muconic acid and aromatic compounds were analysed by HPLC coupled with a diode array detector (DAD) as detailed in the published protocol (Michener et al., 2024). In particular instances, other methodologies were employed to enable more sensitive detection or alternative chromatographic separations. To achieve lower detection limits samples were analysed by ultra-HPLC tandem mass spectrometry (UHPLC–MS/MS) detection as previously detailed (Haugen et al., 2023). To enable the expansion of aromatic compounds and intermediates, the method was revised to allow for better chromatographic separation of analytes of interest. These changes are illustrated in a published protocol (Woodworth et al., 2024).

Analyses of traces in black liquors and sugar hydrolysates via ICP

Aluminium, iron, potassium, magnesium, sodium, phosphorous, silicon, sulphur, and titanium quantifications were conducted via inductively coupled plasma optical emission spectroscopy (ICP) analysis, performed as previously described (Stetson et al., 2022). The samples were also analysed for Cl-, Br-, NO_3-, SO_4^{2-}, and PO_4^{3-} by ion chromatography. The ammonia analysis was done by steam distillation of the sample into boric acid and quantitative titration with hydrochloric acid to quantify ammonia.

Evaluation of *Pseudomonas putida* CJ781

Evaluation of *P. putida* CJ781 in lignin-rich streams in shaken flasks

Pseudomonas putida CJ781 (*P. putida* KT2440 Δ*catRBCA*::*Ptac*:*catA* Δ*pcaHG*::*Ptac*:*aroY*:*ecdBD* Δ*crc* Δ*pobAR fpvA*::*Ptac*:*pral*:*vanAB*) was constructed as previously described (Kuatsjah et al., 2022). To prepare seeds, *P. putida* CJ781 stocks were revived from glycerol stocks by scraping the surface of the glycerol stock and inoculating in a 250 mL baffled flask with 50 mL LB media. Seed cultures were incubated at 30°C and 225 rpm for around16 h. Cells were centrifuged at 6400 *g*, 5 min, and resuspended in modified M9 minimal media (13.55 g/L Na_2HPO_4, 6 g/L KH_2PO_4, 1 g/L NaCl, 2.25 g/L $(NH4)_2SO_4$, all from Sigma-Aldrich, St. Louis, MO) for inoculation in shaken flasks, at an initial OD_{600} of 0.2. Lignin streams obtained from the deacetylation step were defrosted at 4°C, homogenised by vigorous mixing, centrifuged at 12,000 × *g* for 15 min to spin out the solids, pH adjusted to neutral using 4 N H_2SO_4 with vigorous shaking throughout, centrifuged again as above, and filter-sterilised using 0.2-micron nylon filters. Cultivation media was prepared with 90% of either black liquor or BCD liquor, 10% of 10X modified M9, and traces (2 mL/L of 1 M $MgSO_4{\cdot}7H_2O$, 0.1 mL/L of 1 M $CaCl_2{\cdot}7H_2O$, and 1 mL/L of 18 mM $FeSO_4{\cdot}7H_2O$). *P. putida* CJ781 performance in black liquor was evaluated in 250 mL baffled flasks with 50 mL of cultivation media and in BCD liquors in 50 mL baffled flasks with 10 mL of cultivation media. Performance was tracked over 72 h of cultivation at 30°C and 225 rpm, with periodic sampling to measure metabolites and bacterial growth (optical density at 600 nm, OD_{600}), assessed on a NanoDrop (ThermoFisher Scientific).

Evaluation of the effect of individual MAs on *P. putida* CJ781 growth in microtiter plates

The list of selected MAs that may be critical to the biological conversion of feedstocks was compiled using previously published data (Chen et al., 2007). We initially identified twenty-seven material attributes, but four of these compounds were not analysed due to issues with stability in the media (magnesium sulphate, ferrous sulphate), or quantification (citric acid, isocitric acid). *P. putida* growth evaluations were carried out in a BioScreen C microtiter plate platform (Oy Growth Curves AB, Finland) in a cultivation medium amended with increasing concentrations of MAs. The cultivation medium included a mock black liquor solution, which was formulated to represent a typical black liquor and consisted of 1.44 g/L *p*-coumaric acid (AK Scientific, Union City, CA), 0.56 g/L ferulic acid (AK Scientific, Union City, CA), and 2.67 g/L acetic acid (Sigma-Aldrich, St. Louis, MO), and the pH was adjusted to neutral with 4 N KOH. This mock medium was supplemented with 10X M9 (and traces) as described above. The use of mock liquor was necessary for microtiter plate experiments because lignin liquors are too dark to be assessed optically. The following compounds were tested for inhibitory effects: sodium chloride, sodium sulphate, sodium nitrate, ammonium chloride, ammonium sulphate, potassium chloride, potassium phosphate, sodium acetate, potassium acetate, ammonium acetate, glucose, xylose, galactose, glycerol, fructose, sucrose, and arabinose (Table 2); all purchased from Sigma-Aldrich (St. Louis, MO). The following compounds were prepared from the acid form and neutralised with the indicated base: potassium formate, potassium lactate, potassium malate (Sigma-Aldrich, St. Louis, MO), potassium ferulate, and potassium *p*-coumarate (AK Scientific, Union City, CA). Each tested MA concentration (see concentrations in Figure S1) was evaluated in quadruplicate. Four to eight control wells, in which the cultivation medium was not amended with any MA, were included in each BioScreen C plate. Wells were filled with 200 μL of media and inoculated with 15 μL of *P. putida* seed culture (prepared as detailed above) to an initial OD_{600} of 0.2. BioScreen C plates were incubated at 30°C and maximum agitation for 96 h. Biocatalyst performance was assessed by calculating growth rates and lags from the resulting data using an in-house Python script. Specifically, the growth rates were calculated from every 8 consecutive data points (spanning 2 hours of growth) in a continuous manner. The highest calculated growth rate was reported as the maximum growth rate and the completion of the lag phase was considered as the time at which OD_{600} rose above 0.3.

Evaluation of the effect of critical material attributes (CMAs) on *P. putida* CJ781 performance in bioreactors

The evaluation of CMAs was conducted at concentrations of CMA that reduced the growth rate by 25% and 75% in microtiter plates. The effect of the top three CMAs on *P. putida* performance was assessed in constant fed-batch cultivations in BioStat-Q Plus 0.5 L bioreactors (Sartorius Stedim Biotech). The batch medium consisted of mock black liquor (prepared as described above) amended with either an EC25 or an EC75 concentration of one of the three CMAs (sodium chloride, sodium sulphate, and sodium nitrate), along with a no-CMA-added control. The EC25 and EC75 concentrations, respectively, of each CMA were: 122 and 349 mM and for sodium nitrate, 133 and 268 mM sodium sulphate, 156 and 450 mM sodium chloride. *P. putida* seed culture (prepared as detailed above) was inoculated to an initial OD_{600} of 0.2. The initial batch volume was 250 mL. Feeding began when the cells fully utilised the acetate in the batch phase, as indicated by a sharp

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TABLE 2 Identification of critical material attributes (CMAs) in black liquors (BL) for *P. putida* KT2440 and sugar hydrolysates (SH) for *C. tyrobutyricum*.

Potential material attribute in BL	EC25 (mM)	Quantified in BL (mM)		EC25 in BL (%)[a]	CR[b]	Potential material attribute in SH	EC25 (mM)	Quantified in SH (mM)		EC25 in SH (%)[a]	CR[b]
Sodium chloride	156	Sodium	176.6	113.4	4	Sodium chloride	233	Sodium	24.4	10.5	7
		Chloride	13.2	8.5				Chloride	3.0	1.3	
Sodium sulphate	133	Sodium	176.6	132.9	3	Sodium sulphate	130	Sodium	24.4	18.8	6
		Sulphate	3.0	2.2				Sulphate	22.4	17.2	
Sodium nitrate	122	Sodium	176.6	144.9	2	Sodium nitrate	284	Sodium	24.4	8.6	NA
		Nitrate	2.6	2.1				Nitrate	0.0	0.0	
Ammonium chloride	51	Ammonium	0.8	1.5	9	Ammonium chloride	189	Ammonium	1.3	0.7	12
		Chloride	13.2	25.9				Chloride	3.0	1.6	
Ammonium sulphate	166	Ammonium	0.8	0.5	14	Ammonium sulphate	105	Ammonium	1.3	1.2	4
		Sulphate	3.0	1.8				Sulphate	22.4	21.3	
Potassium chloride	148	Potassium	22.1	14.9	10	Potassium chloride	257	Potassium	1.7	0.6	13
		Chloride	13.2	8.9				Chloride	3.0	1.2	
Potassium phosphate	148	Potassium	22.1	14.9	11	Potassium phosphate	554	Potassium	1.7	0.3	14
		Phosphate	1.6	1.1				Phosphate	2.6	0.5	
Potassium formate	83	Potassium	22.1	26.8	8	Sodium formate	26	Sodium	24.4	93.8	NA
		Formate	20.2	24.5				Formate	0.0	0.0	
Potassium acetate	183	Potassium	22.1	12.0	7	Potassium acetate	210	Potassium	1.7	0.8	9
		Acetate	70.8	38.6				Acetate	12.1	5.7	
Sodium acetate	162	Sodium	176.6	109.2	5	Sodium acetate	248	Sodium	24.4	9.8	8
		Acetate	70.8	43.8				Acetate	12.1	4.9	
Ammonium acetate	84	Ammonium	0.8	0.9	6	Ammonium acetate	220	Ammonium	1.3	0.6	10
		Acetate	70.8	83.9				Acetate	12.1	5.5	
Potassium lactate	230	Potassium	22.1	9.6	13	Sodium lactate	257	Sodium	24.4	9.5	5
		Lactate	17.9	7.8				Lactate	52.1	20.3	
Potassium malate	149	Potassium	22.1	14.8	12	Sodium malate	165	Sodium	24.4	14.8	NA
		Malate	3	2.0				Malate	0.0	0	
Potassium coumarate	14	Potassium	22.1	153.4	1	Sodium coumarate	2	Sodium	24.4	1109.1	1
		Coumarate	11.7	81.3				Coumarate	0.1	4.5	
Potassium ferulate	48	Potassium	22.1	45.9	6	Sodium ferulate	2	Sodium	24.4	1161.9	NA
		Ferulate	4.4	9.1				Ferulate	0.0	0.0	
Glucose	NT	0.0		-		Glucose	760	607.3		79.9	3
Galactose	NT	0.0		-		Galactose	659	0.0		0.0	NA

TABLE 2 (Continued)

Potential material attribute in BL	EC25 (mM)	Quantified in BL (mM)	EC25 in BL (%)[a]	CR[b]	Potential material attribute in SH	EC25 (mM)	Quantified in SH (mM)	EC25 in SH (%)[a]	CR[b]
Fructose	NT	0.0	-		Fructose	677	0.0	0.0	NA
Xylose	NT	0.0	-		Xylose	671	605.0	90.2	2
Arabinose	NT	0.0	-		Arabinose	800	44.5	5.6	10
Sucrose	NT	0.0	-		Sucrose	492	21.9	4.5	11
Glycerol	973	7.6	0.8	15	Glycerol	2600	0.0	0.0	NA
Xylitol	NT	0.0	-		Xylitol	739	0.0	0.0	NA

Note: EC25 values (calculated from Figures S1 and S2) and concentrations of 23 potential material attributes in BL and SH streams. NA, not applicable, compound was not present in the stream; NT, not tested in the inhibition studies.

[a]MA concentration in the stream was divided by the calculated EC25 value. Values of ≥100% indicate that the stream contains a concentration of that compound (at least one anion or cation) sufficient to reduce the growth rate by ≥25%.

[b]CR = criticality ranking based on the EC25 values in BL and SH.

increase of the dissolved oxygen (DO) in the bioreactor (up to 70%). The feed medium consisted of a concentrated (20X) mock black liquor solution, 250 μL antifoam 204 (Sigma-Aldrich, St. Louis, MO), and either an EC25 or an EC75 concentration of the CMA to maintain the same CMA concentration during the fed-batch phase. Feeding was constant and adjusted to feed acetate at a rate of 0.35 g/L/h. Feeding ended when feed medium was depleted (250 mL) or the cells died (indicated as DO over 30%). Bioreactors were controlled at 30°C, pH 7 with 4 N KOH and 4 N H_2SO_4, and sparged with air at 1.2 vvm. The initial DO was 100% and agitation was 350 rpm. DO was then controlled at 30% via a cascade that varies the agitation automatically. Each experimental condition was assessed at least in duplicate, and the control was included with each independent bioreactor campaign. Bioreactors were sampled during the batch and fed-batch phases to analyse OD_{600} and metabolite concentrations.

Evaluation of *C. tyrobutyricum*

Evaluation of *C. tyrobutyricum* in sugar hydrolysates in bioreactors

Clostridium tyrobutyricum ATCC® 25755™ was obtained from the American Type Culture Collection (ATCC). *C. tyrobutyricum* seeds were prepared by adding 1 thawed frozen glycerol stock vial to a 100 mL-serum bottle containing 50 mL of anoxic modified Reinforced Clostridial Medium (RCM) (Becton Dickenson, Franklin Lakes, NJ), supplemented with 10 g/L glucose and 20 g/L xylose after the RCM was autoclaved. Seed cultures were vented via a needle and a 0.2 μm filter inserted through the septum and incubated at 37°C and 100 rpm for 18 h to an OD_{600} of 5.0. Clostridial Media (CM) was the base media utilised for the fermentations in this study. CM consisted of 5 g/L yeast extract, 10 g/L peptone, 0.5 g/L L-Cysteine·HCl, 3 g/L $(NH_4)_2SO_4$, 3.26 g/L KH_2PO_4, 0.3 g/L $MgSO_4{\cdot}7H_2O$, 0.02 g/L $CaCl_2{\cdot}7H_2O$, 0.03 g/L $FeSO_4{\cdot}7H_2O$, and 0.02 g/L $MnSO_4{\cdot}H_2O$. Enzyme hydrolysate was diluted to 75 g/L (sum of glucose, xylose, and arabinose) in CM. Evaluations were conducted in 0.5-L bioreactors, in batch mode with 300 mL of CM, inoculated via ~10% direct transfer of the seed to target an initial OD_{600} of 2.0, controlled at pH 6.0 with 4 N NaOH, at 37°C, and 150 rpm. Nitrogen was sparged at 0.2 vvm to ensure anaerobic conditions. Samples were periodically taken to measure OD_{600} and metabolites.

Evaluation of the effect of individual MAs on *C. tyrobutyricum* growth in microtiter plates

Clostridium tyrobutyricum growth evaluations were conducted in a LogPhase 600 instrument (Agilent, Santa

Clara, CA). This instrument simultaneously reads 4 standard 96-well plates. The 96-well plates (Cat# FB012931, Fisher Scientific, St. Louis, MO) were prepared by adding 135 μL of media. The CM base media was combined with 25 g/L mock hydrolysate (16 g/L glucose, 8 g/L xylose, 0.25 g/L galactose, and 1 g/L arabinose, in proportions found in typical enzyme hydrolysates) and the MA, then sterile filtered in an anaerobic chamber. To this, antifoam 204 (Sigma-Aldrich, St. Louis, MO) was added at 1/1000 (v/v). The following compounds were tested for inhibitory effects: sodium chloride, sodium sulphate, sodium nitrate, ammonium chloride, ammonium sulphate, potassium chloride, potassium phosphate, sodium formate, potassium acetate, sodium acetate, ammonium acetate, sodium lactate, sodium malate, sodium *p*-coumarate, sodium ferulate, glucose, galactose, fructose, xylose, arabinose, sucrose, glycerol, and xylitol; all were purchased from Sigma-Aldrich (St. Louis, MO). The following MAs were prepared from the acid and neutralised with the indicated base: sodium formate, sodium lactate, potassium acetate, sodium malate, sodium ferulate, and sodium *p*-coumarate (AK Scientific, Union City, CA). The tested concentrations can be found in Figure S2. For replication, all 8 wells of each column contained the same media condition. Prior to inoculation, the seed culture pH was adjusted to 6.0 with NaOH. The wells were then inoculated with 15 μL of seed culture (prepared as detailed above) in an anaerobic chamber and the plate was covered with sealing tape (Cat# 6050185, Perkin Elmer, Waltham, MA). The instrument settings were 37°C, gradient on, no agitation, and 20-min sample interval.

Evaluation of the effect of CMAs on *C. tyrobutyricum* performance in bioreactors

Cultivations were conducted as described in the section 'Evaluation of *C. tyrobutyricum* in sugar hydrolysate in bioreactors', with some differences. These cultivations were conducted in fed-batch mode. The batch medium was CM base media with a sugar concentration of 50 g/L (mock sugar hydrolysate, containing 49 g/L glucose, 23 g/L xylose, 0.75 g/L galactose, and 3 g/L arabinose) and the CMA to be investigated. The feeding solutions contained mock hydrolysate (330 g/L glucose, 150 g/L xylose, 5 g/L galactose, and 19 g/L arabinose) combined with the CMA to be investigated at the following EC25 and EC75 concentrations, respectively: 113 and 312 mM for sodium lactate, 105 and 304 mM for ammonium sulphate, and 2.2 and 7.8 mM for sodium *p*-coumarate. Media were sterilised via filtration (0.2 μM filters) in an anaerobic chamber. Seeds from serum bottles were used to inoculate 0.5 L bioreactors containing 300 mL of Clostridial Media (CM) with 10 g/L glucose and 40 g/L xylose to an initial OD_{600} of 0.1. The feeding rate was manually adjusted to maintain glucose concentrations above zero.

RESULTS

Origin and characterisation of twelve corn stover batches

The corn stover (*Zea mays*) bales utilised and analysed in the current study had been collected from fields in Iowa at different locations and milled using various strategies (Figure 2A,B). One of the bales (Feedstock 6) was also hand-sorted and milled to analyse the effect of enriching the samples with different anatomical fractions (cob, husk, and stalk, which together account for the entirety of the whole stover) (Figure 2C). Lastly, another bale (Feedstock 7) was deconstructed into segments and sampled at different sections representing different stages of degradation (mild, moderate, or severe) (Figure 2D) to analyse the effect of degradation level on the overall bioconversion process. The composition of the corn stover was analysed as previously described (Sluiter et al., 2008) and the variability among samples was assessed (Figure 2B). Glucan and xylan, the major polysaccharides within these twelve feedstocks, show a variability of 6.7% and 14.9% in content, respectively. However, we note that the sample enriched with cobs (F6_cob) contributes to 36% of the variation in xylan content. Variability in lignin content, another major component in corn stover, is low, spanning 3.2%. Minor polysaccharides (galactan, arabinan), ethanol extractives, and acetyl concentrations also exhibit low variation among the samples (<1.0%, 2.3%, 2.2%, and 2.0%, respectively). Variability is higher for structural and non-structural inorganics, spanning 8.6% and 6.8%, respectively. Interestingly, the highest variability is observed in the per cent of total ash and water-soluble extractives (14.6% and 17.2%, respectively), though 61% of the variation in the latter is due to the two most degraded bales, F7_moderate and F7_severe. This result suggests that the degradation process in corn stover bales (biological and/or chemical) favours the release of small soluble molecules from the original feedstock. The three samples with the lowest ash content corresponded to the batches enriched in anatomical fractions (F6_cob, F6_husk, F6_stalk). The low ash content in these three samples may be explained by some ash being lost during the sorting of the anatomical fractions because the parental feedstock (F6) does not exhibit low ash values.

Generation and characterisation of lignin-rich and sugar streams derived from DMR-EH of corn stover

The corn stover samples were fractionated using a DMR-EH process to generate 12 aqueous black liquor streams, 12 aqueous sugar hydrolysates, and 12 solid lignin-rich streams (Figure 1). The composition of the

(A)

Sample description	County (Iowa)	Size reduction
Feedstock 1	Poweshiek	Method 1
Feedstock 2	Poweshiek	Method 1
Feedstock 3	Poweshiek	Method 1
Feedstock 4	Poweshiek	Method 1
Feedstock 5	Story	Method 3
Feedstock 6	Hardin	Method 2
Feedstock 6_cob	Hardin	Method 2
Feedstock 6_husk	Hardin	Method 2
Feedstock 6_stalk	Hardin	Method 2
Feedstock 7_mild	Story	Method 3
Feedstock 7_moderate	Story	Method 3
Feedstock 7_severe	Story	Method 3

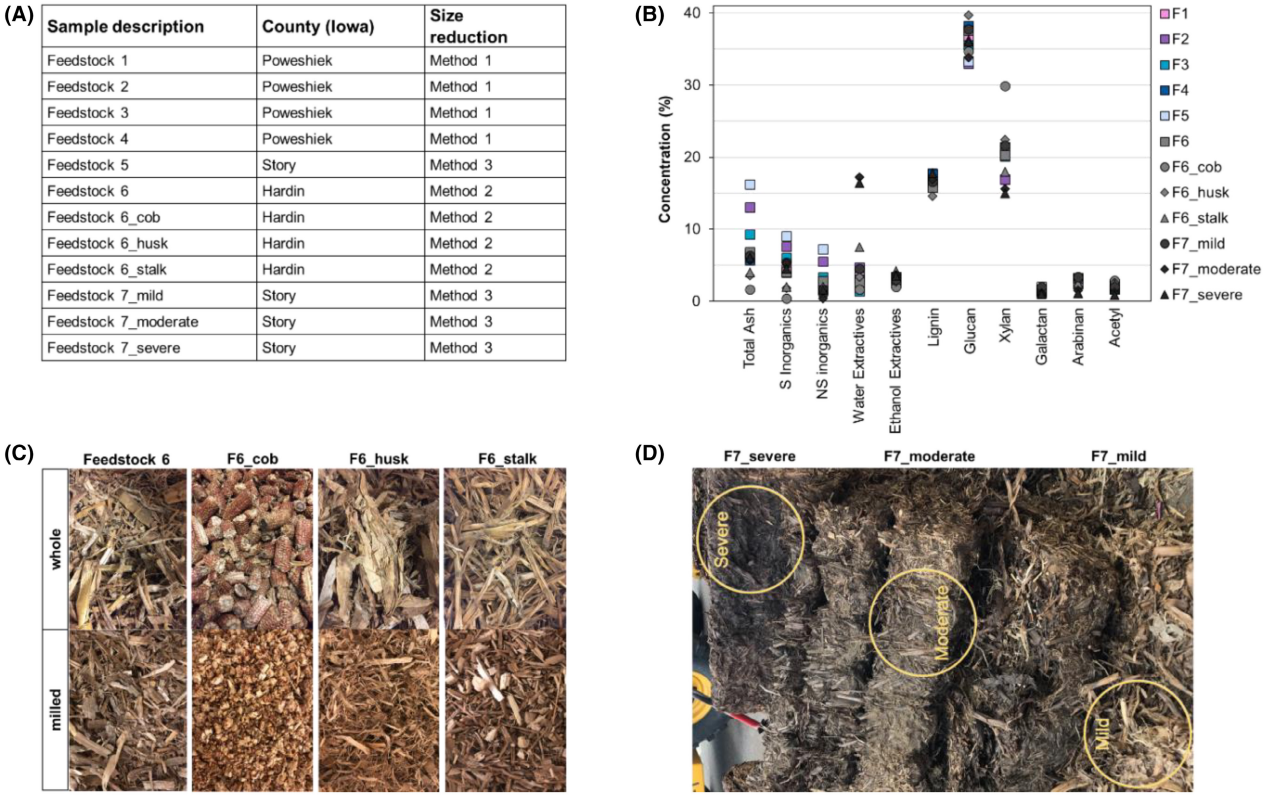

FIGURE 2 Origin and characterisation of corn stover bales utilised in the current study. (A) Description of field location and size reduction strategy. The size reduction methods are as follows: Method 1: Hammer mill, 3″ screen, followed by Knife mill, 1″ screen; Method 2 = Knife mill, 3/4″ screen; Method 3 = Hammer mill, 3/4″ screen. (B) Characterisation of the twelve feedstocks. Numeric data are also provided in Table S1. (C) Illustration of samples enriched with different anatomical fractions (from whole material, F6) utilised in the current study (F6_cob, F6_husk, F6_stalk, F6) before and after milling. (D) Illustration of corn stover bales representing different stages of degradation from which samples F7_mild, F7_moderate, and F7_severe were obtained.

streams was analysed, and we grouped the identified compounds as traces, aromatic compounds, carboxylic acids, and sugars (Figure 3). These analyses were not conducted in the 12 solid lignin streams because these streams were further subjected to BCD (Figure 1) to be made bioavailable to the microbes, which introduces high concentrations of sodium to the stream (Rodriguez et al., 2017).

All the black liquors contain high sodium concentrations (2.67–4.06 g/L) compared to other trace elements (Figure 3), which was expected due to the addition of sodium hydroxide in the deacetylation step. Sodium varies by 40% between the highest and lowest concentrations, which is smaller than the variation observed for the other traces identified (details in Text S1). Black liquors are also rich in aromatic monomeric aromatic compounds, specifically *p*-coumaric acid (0.25–2.03 g/L) and ferulic acid (0.10–1.02 g/L), with the lowest concentrations found in the two most degraded bales, F7_moderate and F7_severe. Organic acids are abundant in the streams and very diverse (10 identified). Acetate is the most abundant (1.87–3.84 g/L) and is the main carbon source in these streams. Interestingly, some organic acids, such as oxalic, malonic, and malic acid, are more abundant in the most degraded feedstocks. Lastly, sugars are not detected in the lignin streams, excluding the sugar alcohol glycerol (Figure 3). These results suggest that the degradation stage (in Feedstock 7) significantly affects the composition of black liquors, which is likely related to biological activity in those bales. For example, during the degradation process, fungi could be utilising various compounds within the feedstocks for growth and generating byproducts, such as organic acids (Liaud et al., 2014).

The content of monomeric sugars in sugar-derived hydrolysates is significantly different among the samples (Table 1), even though all the samples were subjected to the same conditions of pre-treatment and enzymatic hydrolysis. Hydrolysates from the two most degraded bales, F7_moderate and F7_severe contained the lowest content of sugar monomers (33.1% and 39.9% lower than the mildly degraded sample from the same bale, respectively). This result correlates with the lowest xylan content in the feedstocks but not the lowest glucan content (Figure 2B). The highest sugar content was identified in the hydrolysate from the cobs-enriched stream, F6_cob (24.6% higher than the unsorted feedstock, F6), which also contained the highest xylan content (Figure 2B). The concentration of glucose

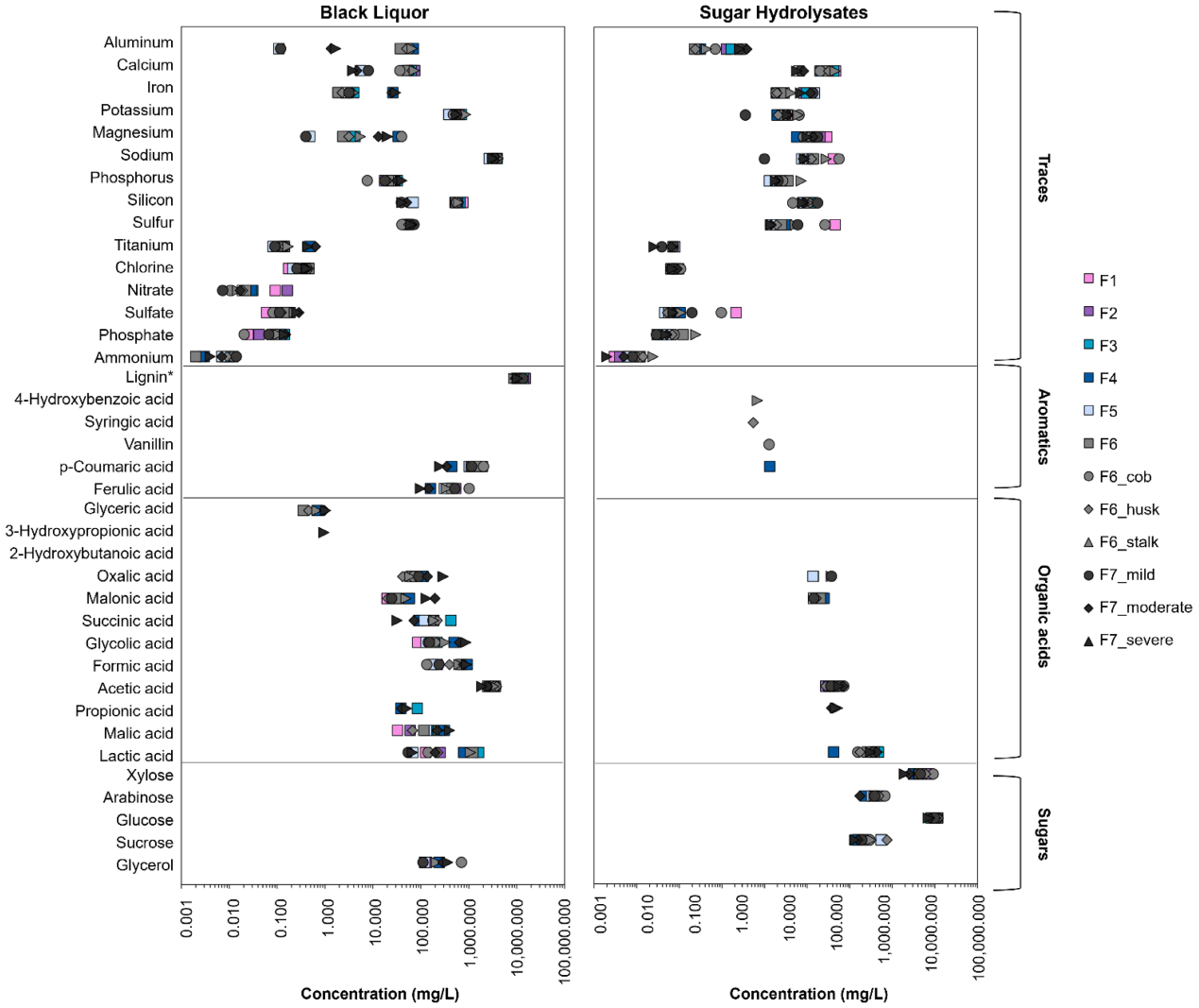

FIGURE 3 Chemical characterization of black liquors and sugar hydrolysates derived from DMR-EH of twelve corn stover samples. Data were obtained by high-performance liquid chromatography (HPLC), inductively coupled plasma optical emission spectroscopy (ICP-OES), anion scan, or ammonia analyses, depending on the analyte (see Materials and Methods). Numerical data are also provided in Tables S2–S4.

ranged from 70 to 121 g/L and xylose from 19 to 91 g/L (Table S3). The ratio of the major sugars (glucose and xylose) ranged from 1.3 to 3.6 g/L, which corresponds to ~100% difference (Table S3). These and subsequent per cent difference values refer to the absolute difference between the highest and lowest values detected divided by the average of those values. Regarding traces in sugar hydrolysates, the content of sodium is an order of magnitude lower than that in the black liquors. However, the sodium content exhibits a higher sample-to-sample variation, with a 310% difference between the highest and lowest sodium concentrations (Figure 3), which can originate from differences in the washing/pressing process after the deacetylation step (Figure 1). Details about minor traces in these hydrolysates are shown in Text S2. Overall, in terms of percentage of variation, these finer-scale analyses reveal that the concentration of most of the components analysed in both black liquors and sugar hydrolysates span more than one order of magnitude. These concentration variations may significantly influence microbial downstream conversion and – most importantly – differentially (Figure 3).

Biocatalyst performance in DMR-EH-derived streams

The DMR-EH-derived streams (12 black liquors, 12 sugar hydrolysates, and 12 BCD liquors) were utilised to test the effect of stream variability on performance of the biocatalysts *P. putida* CJ781 and *C. tyrobutyricum* (Figure 1). The metrics we used to evaluate performance are substrate utilisation, bacterial growth rate, maximum cellular turbidities (measured as optical density at 600 nm (OD_{600})), and product generation (titer,

productivity, and yield). *P. putida* CJ781 was utilised for the bioconversion of black liquors and BCD liquors (as the sole carbon source, at 90% lignin stream and 10% minimal media) to muconate in shaken flasks. Among the 12 black liquors, we identified the largest differences in maximum OD_{600} (163% difference, $p < 0.001$) (Figure 4A), growth rate (130% difference, $p < 0.001$) (Table S5), final muconate titers (136% difference, $p < 0.001$) (Figure 4B), and muconate yield (77% difference, $p < 0.0001$) (Table S6). Acetate (the major organic acid in black liquors) was fully utilised in each cultivation (Figure 5) and its initial concentration in the black liquors was positively correlated with the maximum OD_{600} ($R^2 = 0.74$, $p < 0.001$), which suggests that acetate is the major carbon source in the streams. The lowest maximum growth is observed in Feedstock 5 and Feedstock 7_mild. However, this cannot be attributed only to lower acetate concentrations, especially in Feedstock 5 (Figure 5A). Thus, we hypothesise that other components in this stream are inhibiting growth. Based on the chemical composition (Figure 3), black liquor derived from Feedstock 5 does not contain any outstanding characteristics compared to the other streams. However, we note that the initial feedstock contains the highest concentrations of ash and inorganics (Figure 2). Muconate titers are correlated with the initial concentration of aromatic compounds (total *p*-coumarate and ferulate) in the black liquors ($R^2 = 0.90$, $p < 0.00001$). Black liquor derived from Feedstock 6_cobs generates the highest titers while Feedstock 4 and the two most degraded bales, F7_moderate and F7_severe, exhibit the lowest titers. As productivity was calculated from titers (over the 72-h cultivation), the trends in productivity mirrored those of titers. The highest muconate yields (calculated from *p*-coumarate and ferulate, Figure 4C,D) were achieved in the two degraded bales, F7_moderate and F7_severe (145% and 164%, respectively) (Table S6). Considering that the maximum theoretical yield is 100%, we hypothesised that the degraded streams could contain other lignin-derived aromatic monomers that contribute to muconate production that we are not detecting and/or identifying. Indeed, follow-up analyses revealed that 4-hydroxybenzaldehyde is present in these two streams, which can be catabolised by *P. putida*. When additionally considering the concentration of this compound, muconate yields are slightly lower (139% and 155%, respectively). Lignin content was also quantified at the end of the experiment to evaluate if the increase in yields (over the theoretical maximum) could be due to depolymerisation of residual lignin solids and utilisation of these new (previously unquantified monomers) during bioconversion. However, we could not accurately measure lignin to confirm this hypothesis due to the small fraction of lignin utilised and the presence of a larger analytical variance (Figure 5B).

Growth rate, maximum OD_{600}, and final muconate titers were also assessed for *P. putida* CJ781 in BCD liquors. Due to volume limitations of BCD liquors from Feedstock 5 and the three streams derived from Feedstock 7 (mild, moderate, and severe), *P. putida* performance was not tested in these, and the sampling volume was also decreased in the remaining 8 samples, limiting the resolution of the analyses to only initial and final time points. Maximum differences of 28% in maximum OD_{600}, 73% in growth rate, and 75% in final muconate titers ($p < 0.001$) are observed at the final time point of cultivations in these 8 BCD liquors (Figure 4E–H). There is no correlation identified between maximum OD_{600} reached in black liquors and in BCD liquors ($R^2 = 0.0122$). Indeed, growth in BCD liquors is in general lower than that in black liquors due to the potentially lower concentrations of organic acids that can be used as carbon sources (Rodriguez et al., 2017). As observed in black liquors, maximum muconate titers are also correlated with aromatic monomer content in the BCD liquors ($R^2 = 0.9566$, $p < 0.0001$). However, the concentration of aromatic monomers in BCD liquors and black liquors is not correlated ($R^2 = 0.0237$), and thus, neither are maximum muconate titers ($R^2 = 0.0021$) among these lignin streams. These results suggest that microbial performance should be independently monitored in both lignin streams if both are going to be biologically upgraded using this deconstruction strategy. We note that the overall performance variability in BCD liquors is lower than that in black liquors. This can be attributed to the additional process steps to generate BCD liquors (Figure 1), which could mask some of the intrinsic feedstock variability.

Lastly, *C. tyrobutyricum* was utilised for the bioconversion of sugar hydrolysates to butyric acid in 0.5 L bioreactors in batch mode. Initial total sugar (glucose, xylose, and arabinose) concentrations were normalised to 75 g/L. Growth and butyric acid production is less variable in the twelve sugar hydrolysate streams than that observed for *P. putida* in the lignin streams, with a maximum difference of 35% ($p < 0.0001$) in maximum OD_{600}, 79% ($p < 0.0001$) in growth rate (Table S5), 24% ($p < 0.01$) in butyric acid titer (Figure 4I–L), 11% ($p < 0.1$) in butyric acid yield, and 33% ($p < 0.0001$) in productivity at the end of the cultivation. One potential reason for this lower variability is that the total sugar content of each stream was normalised prior to cultivation and sugar concentration is a major variable in these streams (Table 1). While sugar ratios (see ratios in Table S3) have been shown to affect sugar utilisation profiles in *C. tyrobutyricum* (Luo et al., 2017; Salvachúa et al., 2021), we did not detect a correlation between glucose and xylose ratio and final butyrate titer either. Nevertheless, a maximum difference of 33% in productivity is significant from an industrial point of view, where productivity is a major cost driver.

Correlation between the composition of feedstocks, black liquors, sugar hydrolysates, and microbial performance

We investigated whether a correlation between the original composition of feedstocks (Figure 2B), black liquors, or sugar hydrolysate streams (Figure 3; Table S4) and biocatalyst performance (Figure 4) could be identified (Table S7). Linear least-squares regressions were performed between feedstock composition attributes and microbial performance metrics; a conservative significance cutoff of $p < 0.001$ was used to identify correlations (Table S7). Historically, ash has been identified as a crucial factor that affects process economics and/or biocatalyst performance because it can reduce pretreatment effectiveness, increase equipment wear and disposal costs, and contain other fermentation inhibitors (Bonner et al., 2014; Kenney et al., 2013; Li, Aston, et al., 2016; Williams et al., 2016). Additionally, ash can inhibit enzymatic hydrolysis, reducing efficacy and increasing enzyme costs (Hörhammer et al., 2018). The total ash portion in a feedstock includes compounds like alkaline and alkali earth metals, chlorine, sulphur, and silica (Yan et al., 2020). In this study, a correlation between the percentage of ash in the original feedstocks (Table S1) or in black liquors (Table S2) with *P. putida* CJ781 and *C. tyrobutyricum* performance metrics was not identified (Table S7), which suggests that ash is not a critical factor in a lignocellulosic biorefinery when using DMR-EH and these biocatalysts.

In the case of *P. putida* CJ781, we hypothesised that feedstock composition may also correlate with the properties of the streams (e.g., higher lignin or acetyl content in the feedstock could lead to higher concentrations of aromatics or acetate in the black liquors), which, in turn, would correlate with biocatalyst performance. Components in the black liquors are indeed correlated with biocatalyst performance. More specifically, final cell densities are positively correlated with acetate concentration in black liquor ($R^2 = 0.70$, $p < 0.001$; Table S7), and final muconate titers and productivities are positively correlated with aromatic monomer content ($R^2 = 0.90$, $p < 0.00001$). However, strong correlations between the percentage of acetyl in the original feedstock and the concentration of acetate in the black liquors ($R^2 = 0.50$, $p < 0.01$) or final cell density ($R^2 = 0.15$) are not identified. Similarly, the concentration of aromatic monomers in black liquors neither depends on the lignin content of the original feedstocks

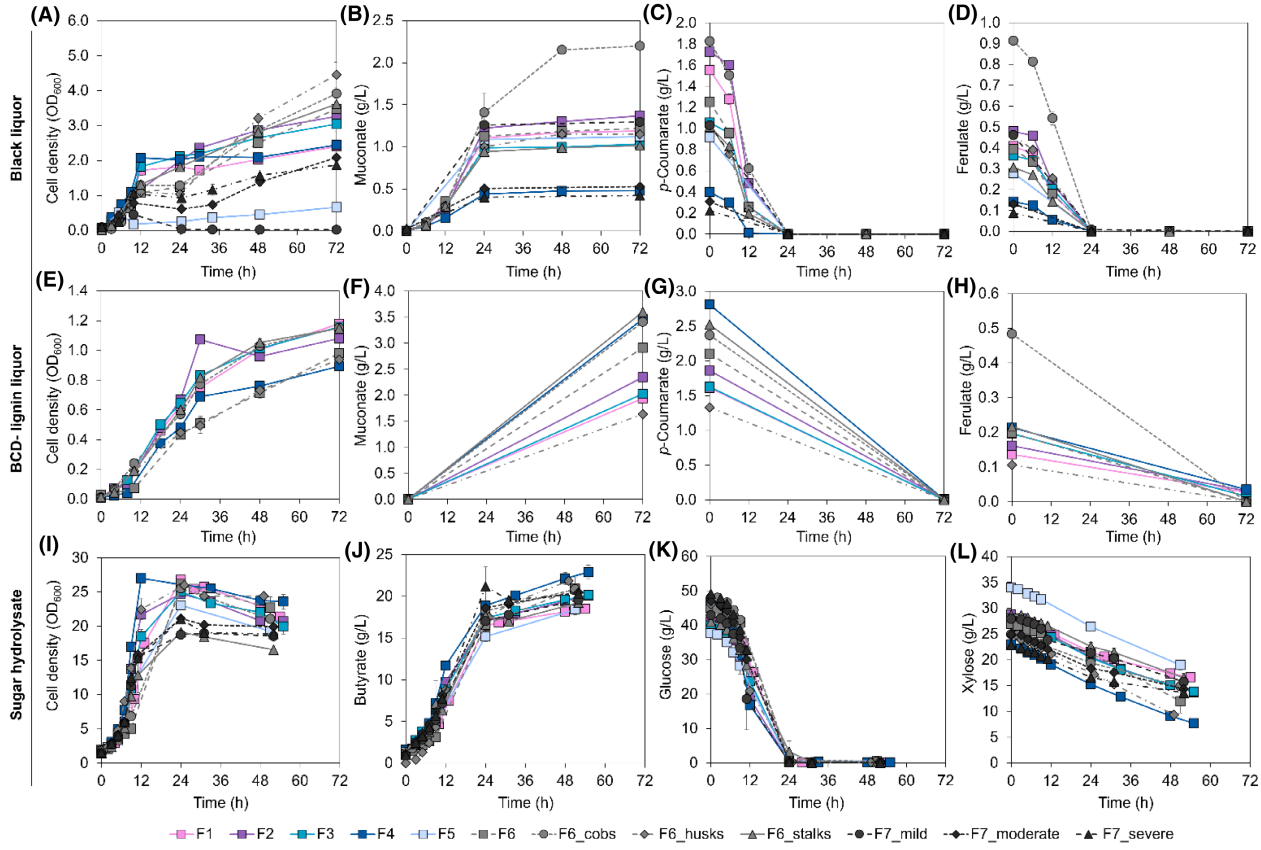

FIGURE 4 Bioconversion of black liquor, BCD liquor, and sugar hydrolysates. Cultivation profiles of (A–D) *P. putida* CJ781 in black liquor, (E–H) *P. putida* CJ781 in BCD liquor, and (I–L) *C. tyrobutyricum* in sugar hydrolysates. (A, E, I) Microbial growth, (B, F, J) product accumulation, and (C–D, G–H, K–L) product precursor utilisation are shown. Data represent the average of three biological replicates and error bars show ±SEM.

($R^2 = 0.09$) nor the lignin content of the black liquors ($R^2 = 0.29$). Therefore, lignin content in black liquors and final muconate titer ($R^2 = 0.21$) do not correlate, which indicates that lignin content in corn stover is not a predictor of the concentration of aromatic compounds released in the deacetylation process. The only other MAs that exhibit a positive correlation with a *P. putida* performance metric are oxalic acid and glycolic acid, both of which have a positive impact on muconic acid yield specifically. However, this correlation disappears if the two most degraded bales, F7_mod and F7_severe, are excluded from the analysis. As implied earlier, muconate yield in those samples may be enhanced due to unidentified aromatic monomers rather than an increase in organic acid concentrations. We note that using a cutoff of $p < 0.01$, we could identify four additional correlations with the feedstock and fifteen with the black liquors (Table S7). However, we limited our analysis to more conservative significant differences.

Correlations ($p < 0.001$) between sugar content and *C. tyrobutyricum* performance are not identified (Table S7). This result was expected because sugar concentrations were normalised for the experiments (Figure 4), but it also indicates that different sugar ratios do not impact performance. Similarly, *C. tyrobutyricum* performance did not depend on the concentration of any investigated component in the feedstock or sugar stream (Table S7). The only significative finding was a negative correlation at $p < 0.01$ between iron concentration and final growth (Table S7).

Effect of individual material attributes (MAs) on microbial growth

Cultivations in black liquor and sugar hydrolysate streams demonstrated that variability in corn stover feedstocks significantly impacts biocatalyst performance. Because correlations between biocatalyst performance and specific characteristics of the streams were either limited (to acetate and aromatic monomer content in *P. putida*) or nonexistent (in *C. tyrobutyricum*), we next sought to investigate the effect of individual MAs on performance. For this purpose, we formulated mock media that were representative of a typical black liquor or sugar hydrolysate stream for *P. putida* CJ781 and *C. tyrobutyricum*, respectively. Then, these media were amended with increasing concentrations of MAs (Figures S1 and S2) that we previously identified in black liquors and sugar hydrolysates (Figure 3). Growth rates and lags were monitored in microtiter plates and utilised as a proxy for bacterial performance (e.g. the higher the growth rate, the higher the expected productivity).

Both microbes display significant growth rate reduction in response to all salts tested (sodium chloride, sodium sulphate, sodium nitrate, ammonium chloride, ammonium sulphate, and potassium chloride) (Figures S1 and S2). In the case of *P. putida* CJ781, the concentrations of salts that negatively impact growth rate fall within the range of concentrations that may be present in black liquors (Table 2; Table S4). This is particularly true for black liquors produced by DMR-EH, which tend to have elevated salt levels, especially sodium (Figure 3). Based on this observation, it is possible that the negative effect of sodium on *P. putida* performance in black liquors was not identified at the stringency of $p < 0.001$ (Table S7) due to the relatively high sodium levels in all 12 streams, thereby reducing performance variability. The aromatic compounds *p*-coumarate and ferulate exhibit inhibitory effects on both organisms (Figures S1 and S2), although not at concentrations typically found in the streams (Figure 3). As anticipated, *P. putida* displays higher tolerance to aromatic compounds than *C. tyrobutyricum*, and the latter is inhibited by concentrations of *p*-coumaric or ferulic acid as low as 2 mM (Table 2). The most inhibitory organic acid to *P. putida* is acetate, and formate to *C. tyrobutyricum*. However, these organic acids are inhibitory only at concentrations higher (Figures S1 and S2) than those identified in the streams (Figure 3). Sugars also reduce growth rates at very high concentrations (above ~75 g/L for *P. putida* and 150 g/L for *C. tyrobutyricum*, Figures S1 and S2), most likely due to osmotic stress. In summary, although growth inhibition becomes evident at sufficiently high concentrations of all tested compounds, many of these growth-inhibitory concentrations fall outside the typical ranges found in black liquors and sugar hydrolysates.

Identification of critical material attributes (CMAs) for *P. putida* CJ781 and *C. tyrobutyricum*

Because growth inhibition is generally observed at MA concentrations higher than those identified in DMR-EH-derived streams, we considered two factors to further rank their criticality: (1) the inhibitory effect of the compound on microbial growth rate (specifically, the MA concentration that decreases growth rate by 25%, from here on referred to as "EC25", as compared to the control, without MA), and (2) the maximum concentration of that compound in any of the studied black liquor or sugar hydrolysate streams (Table 2). A growth reduction of 25% was selected arbitrarily, considering the variation in the controls (Figures S1 and S2), which is within 10%; therefore, a 25% reduction was deemed significant. Then, to calculate the criticality of the MAs present in the streams, the MA concentration in the stream was divided by the calculated EC25 value. Values of ≥100% indicate that the stream contains that compound (or at least one anion or cation in compounds that are salts) at a concentration sufficient to reduce the growth rate by ≥25%. In general, the most critical MAs for *P.*

putida were aromatic compounds and some sodium-containing compounds, and aromatic compounds and sugars for *C. tyrobutyricum* (Table 2; Text S3).

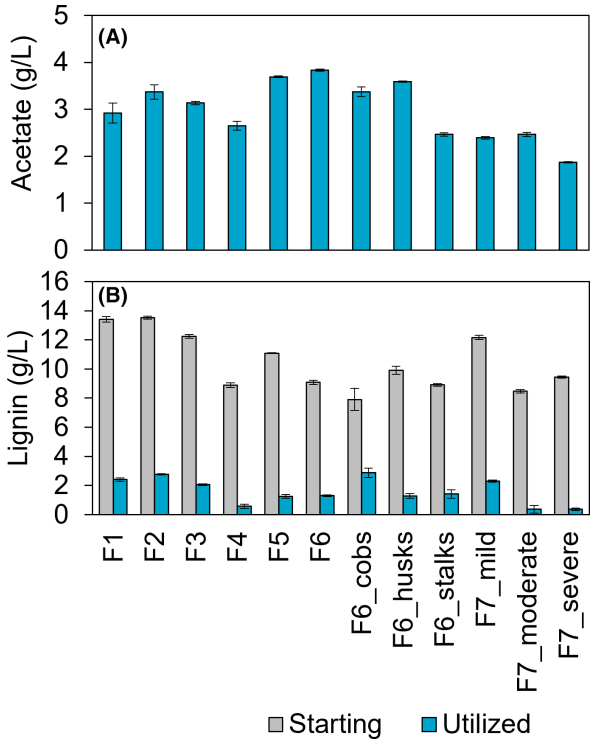

FIGURE 5 Quantification of (A) acetate and (B) lignin content in black liquors. Analyses were conducted in black liquors from 12 corn stover batches before and after 72-h cultivation with *P. putida* CJ781. Results show the average of three biological replicates, and the error bars indicate ±SEM.

Effect of CMAs on microbial performance in bioreactors

The impact of three CMAs on product generation (titers, yields, productivities) by each microbe, as opposed to only growth (Figures S1 and S2), was then examined in bioreactors. Cultivations were performed in fed-batch mode, which is a process-relevant cultivation approach, and CMAs were added at two concentrations corresponding to 'low' and 'high' concentrations (i.e. EC25 and EC75) in the batch and feeding media (Figure 6). Mock black liquor and sugar hydrolysate were used to simplify the test, aiming to get a better understanding of the specific effects of the selected MAs on the microbes.

Interestingly, some of the main CMAs for both microbes are carbon substrates, or in other words, product precursors (e.g., *p*-coumarate for *P. putida* CJ781 and glucose and xylose for *C. tyrobutyricum*). Because substrate concentrations are typically limited in bioreactor cultivations to not exceed the maximum tolerable amount for the biocatalysts, these compounds were excluded from this experiment. With this exclusion, the top 3 CMAs for *P. putida* CJ781 are sodium chloride, sodium sulphate, and sodium nitrate and for *C. tyrobutyricum, p*-coumarate, ammonium sulphate, and ammonium lactate (Table 2). While lactate is not normally found in high concentrations in hydrolysates, we would like to note here that lactate concentration can be higher in sugar hydrolysates that are not properly stored and biologically contaminated, suggesting a mechanism by which contamination could inhibit overall productivity (Serate et al., 2015).

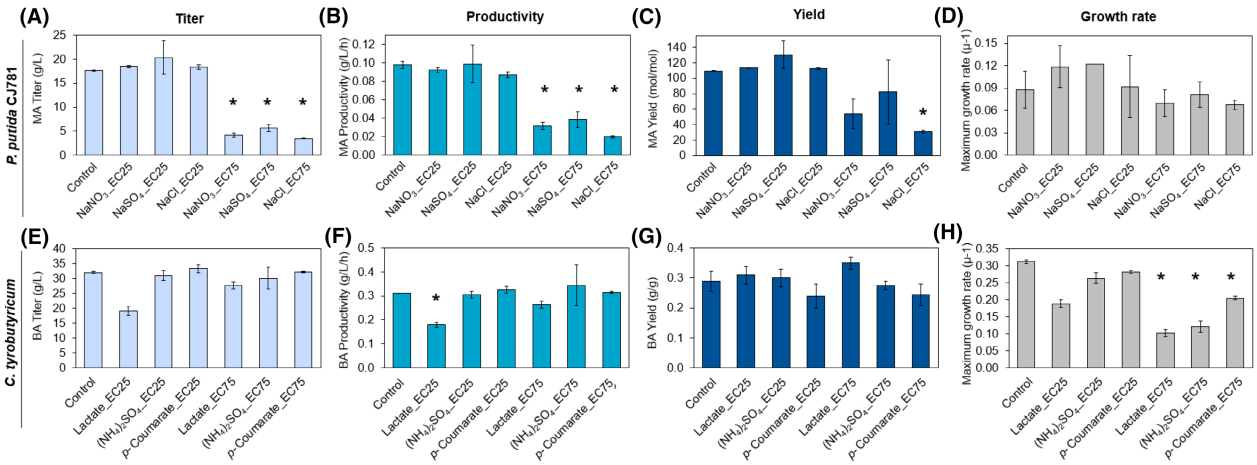

FIGURE 6 Performance metrics of *P. putida* CJ781 and *C. tyrobutyricum* in 0.5-L bioreactors in the presence of CMAs. Performance of (A–D) *P. putida* in mock black liquor and (E–H) *C. tyrobutyricum* in mock sugar hydrolysates, both amended with corresponding EC25 or EC75 concentrations of CMAs. EC25 and EC75 concentrations were (respectively): 122 and 349 mM for sodium nitrate ($NaNO_3$), 133 and 268 mM for sodium sulphate ($NaSO_4$), 156 and 450 mM for sodium chloride (NaCl), 113 and 312 mM for sodium lactate (LAC), 105 and 304 mM for ammonium sulphate (AS), and 2.2 and 7.8 mM for sodium *p*-coumarate (pCA). (A, E) Maximum titer, (B, F) productivity, (C, G) yield, and (D, H) maximum growth rate are shown. Full bioreactor profiles are shown in Figures S3 and S4. Bars represent the average of two biological replicates, except in the cases of *P. putida* CJ781 Control and $NaNO_3$_EC25, which were performed in triplicate. Error bars show absolute differences between duplicates or ±SEM for triplicates. Asterisks denote conditions that are significantly different from the control at $p < 0.01$.

Full bioconversion profiles in bioreactors in the presence of MAs are shown in Figures S3 and S4. *P. putida* KT2440-CJ781 cultivated in the unamended control produced an average of 17.7 g/L muconate (Figure 6; Figure S3) from *p*-coumarate and ferulate, using acetate as the sole carbon source, with no residual aromatic monomers or acetate remaining in the medium. Average maximum growth rate was 0.088 μ^{-1} and average productivity and yield (mol muconate/mol aromatic compounds added) were 0.098 g/L/h and 109.5%, respectively. Interestingly, these metrics were similar to those identified in media amended with an EC25 concentration of any of the tested compounds (Figure 6, Figure S3), which indicates that *P. putida* CJ781 performance in bioreactors may be more robust than in microplates in the presence of these CMAs (e.g. due to higher aeration and higher cell density). However, *P. putida* CJ781 performance is reduced in media amended with EC75 concentrations in all cases (Figure 6; Figure S3). Of the three salts tested, sodium chloride (at the EC75 concentration) resulted in the largest reduction in performance across all metrics, with a 26% difference in maximum growth rate, 134% in titer, 133% in productivity, and 112% in yield between this condition and the unamended control. These results confirm that *P. putida*, in the cultivation conditions tested, is not affected by CMAs at concentrations that may be present in black liquors (concentrations lower or equal to those corresponding to EC25).

Clostridium tyrobutyricum in the unamended control condition produced 32.0 g/L butyrate (Figure 6; Figure S4) from glucose and xylose. In this condition, maximum growth rate, yield (g butyrate/ g sugars utilised), and productivity were 0.308 μ^{-1}, 0.29 g/g, and 0.31 g/L/h, respectively (Figure 6). Most of the supplied glucose was utilised, while residual xylose remained in all tested conditions (Figures S4 and S5). Maximum growth rate is reduced in all experimental conditions as compared to the control, with the largest reductions seen in media amended with lactate (101% difference and 48% difference in the EC75 and the EC25 condition, respectively) (Figure 6). Butyrate titer and productivity are lower in media supplemented with an EC75 concentration of all compounds tested and the EC25 concentration of lactate. Interestingly, aside from the maximum cell growth rate, the performance in media containing an EC75 concentration of lactate is closer to that of the control than in media containing an EC25 concentration (Figure 6). Hypotheses about this result are discussed in the subsequent section. In summary, sodium lactate is the most detrimental CMA to *C. tyrobutyricum* performance and *p*-coumarate is the least detrimental (Figure 6E–H). In addition, despite the reduction in growth rates caused by CMAs (as was also observed in microplates), titers, produtivities, and yields do not decrease proportionally for ammonium sulphate and *p*-coumarate, underscoring the robustness of *C. tyrobutyricum* in bioreactors.

DISCUSSION

Feedstocks often exhibit variability, which can adversely affect the performance of biorefineries (Carpenter et al., 2014; Kenney et al., 2013; Ray et al., 2020; Yan et al., 2020). Therefore, predicting the overall bioconversion performance to fuels and chemicals based on the initial characteristics of the feedstock is desired. In this study, although no specific MAs have been identified in the original feedstocks to predict *P. putida* CJ781 performance, various characteristics of black liquors can, to some extent, predict bacterial growth and muconate production (such as % acetyl, and the concentration of acetate, *p*-coumarate, and ferulate). In addition, from our results, we can infer that feedstocks with a higher proportion of cobs and minimal in-field degradation are anticipated to exhibit better properties than other feedstocks for muconate production under similar conversion conditions. Lastly, it was notable that growth-inhibitory concentrations of MAs typically fall outside the MA ranges found in black liquors. However, if MA concentrations increase due to different process parameters (e.g. concentration of black liquors to increase titers in fed-batch processes), the performance may significantly decline. In the case of inhibition due to high salt concentrations, lignin streams may require desalting prior to bioconversion processes.

The performance of *C. tyrobutyricum* in sugar hydrolysates was not linked to any specific feedstock attribute either. It is important to note that process parameters, particularly in the later stages of a fractionation process like DMR-EH, can mask the inherent variability of the feedstock. Indeed, variability in lignin-rich streams had a more significant impact on biocatalyst performance than variability in sugar streams. While the black liquors already contain critical components from the process, such as sodium (from the deacetylation process), the solids undergo additional water washing before enzymatic hydrolysis. Therefore, this washing step becomes a crucial process parameter. Avoiding the washing step would result in higher concentrations of aromatic compounds in the sugar hydrolysates. However, based on our bioreactor experiments (Figure 6), and assuming that up to 10% black liquor is mixed with the sugar hydrolysate (v/v) without the washing step, the maximum concentration of *p*-coumaric acid reaching the sugar stream would not be inhibitory to *C. tyrobutyricum* (<0.19 g/L). This is a very positive result for reducing process costs by eliminating the washing step.

Bioreactor studies conducted in the presence of CMAs (Figure 6; Figures S3–S5) have also provided valuable insights into microbial metabolism. For *P. putida* CJ781, the three tested CMAs contained sodium. Although some sodium is used in microbial metabolism (Henriquez et al., 2021), the sodium added in the deacetylation step in black liquors is certain to be

in excess. Excess sodium can induce osmotic stress, prompting cells to adapt by accumulating osmotic solutes, and imposing an additional energetic burden (Ruhl et al., 2022), which leads to performance reductions. Salts can also destabilise DNA (Gruenwedel & Hsu, 1969) and interfere with enzyme structure and function (Warren & Cheatum, 1966). *P. putida* CJ781 was previously engineered to overcome a series of bottlenecks for the production of muconate from aromatic compounds, particularly in the hydroxylation step from 4-hydroxybenzoate to protocatechuate (Kuatsjah et al., 2022). In our current study, we observe that 4-hydroxybenzoate accumulates in cultivations with EC75 concentrations of sodium salts (Figure S3), a phenotype not previously observed in this strain. This finding suggests that salt toxicity may negatively impact the enzyme involved in the hydroxylation step, PraI. However, an enzymology and systems biology approach would be recommended to understand this result and phenotype in a holistic manner before targeting specific enzymes for strain improvement.

Acetate is inhibitory to *P. putida* CJ781 growth (Table 2). However, its toxicity can be significantly influenced by the conjugate cation. Notably, ammonium acetate exhibited a 2-fold lower EC25 value than either sodium or potassium acetate (Table 2), indicating that ammonium enhances the inhibitory effect of acetate. In bioreactor experiments, potassium was chosen as the base source for neutralising acetate during media preparation, but not for muconate neutralisation (which was neutralised with ammonium during the bioreactor run). This presents an opportunity for further bioprocess improvement. Because acetate serves as the primary carbon source in black liquors, it was selected as the carbon source in the bioreactor assays. Most previous studies reporting the conversion of aromatic compounds to products have utilised glucose as a carbon source (Kuatsjah et al., 2022; Niu et al., 2020; Salvachúa et al., 2018; Suzuki et al., 2021). Therefore, the feeding rates employed in this study are not optimised and were conservative to prevent acetate accumulation in the bioreactor. Bioprocess development and strain engineering to improve tolerance to acetate are warranted to enhance muconate productivity using acetate as a carbon source.

The metabolism of *C. tyrobutyricum* has been much less studied compared to that of *P. putida*. Regarding potential inhibitors in sugar hydrolysates, *C. tyrobutyricum* has been shown to have low tolerance to aldehydes (e.g. furfural) present in certain lignocellulose-derived streams (Suo et al., 2023). In contrast, we demonstrate that this microbe can tolerate aromatic compounds to a significant extent, higher than the levels identified in DMR-EH sugar hydrolysates. Notably, the bioreactor experiments conducted in the presence of *p*-coumarate also showed *p*-coumarate depletion at both the EC25 and EC75 levels (Figure S4), and an increase in formate production at the EC75 level (Figure S4). These findings suggest that *C. tyrobutyricum* is metabolising *p*-coumarate, which, to our knowledge, has not been reported to date. Additional experiments will be necessary to understand if *p*-coumarate is used as a carbon source or only modified. Lactate metabolism by this microbe is also worth highlighting. In the presence of lactate at EC25 and EC75 levels, propionate accumulated over time (Figure S4), an unreported result for this species. Although *C. tyrobutyricum* has been described to utilise lactate as a carbon source (through pyruvate towards acetyl-CoA and the tricarboxylic acid cycle), our finding suggests lactate conversion via the acrylate pathway, leading to propionate production (Li, Zhang, et al., 2016; Stams et al., 1998). When lactate is used as a carbon source, it can also result in the production of butyrate (Munier et al., 2023), potentially explaining the increased yield observed in the cultivations at the EC75 concentration of lactate compared to the control (lactate was not used for yield calculation). Overall, these findings pave the way for pathway discovery in *C. tyrobutyricum*.

The current study has several limitations. The relatively small number of tested samples may have restricted our ability to detect a higher number of statistically significant correlations. To increase the sample set enough for building predictive models related to feedstock variability and microbial performance, it would be necessary to increase the throughput of deconstruction by performing it at scales smaller than 5 kg (the scale used here), the number of microbial hosts, the species of feedstocks beyond corn stover, and the variety of deconstruction methods beyond DMR-EH. Exploring MAs in combinations would also reveal potential synergies. Additionally, correlating physical properties such as feedstock moisture, particle morphology, and microstructure, which are known to impact feedstock processing performance (Williams et al., 2016), with performance, would further enhance the predictive power. Lastly, growth served as a proxy for performance when evaluating MAs in high-throughput screenings. While productivity correlates with growth rate (Feng et al., 2022; Lipson, 2015; Venayak et al., 2015), yield, which is another crucial metric, was not monitored in these screenings. Including high-throughput sample collection and substrate/product analyses in this pipeline would be desirable.

In summary, our study reveals that while feedstock variability impacts microbial performance, *P. putida* CJ781 and *C. tyrobutyricum* exhibit high tolerance to the MAs commonly found in DMR-EH streams. This result is promising for lignocellulosic biorefineries aiming to implement this type of deconstruction strategy and utilise these biocatalysts. Going forward, it will be valuable to explore the performance of these microbes in streams obtained from different feedstocks and deconstruction

processes. Additionally, identifying beneficial phenotypes and genetic traits to cope with high diversity and levels of MAs will be warranted. This will enable us to further engineer and improve microbes to effectively manage stream variability and successfully produce fuel and chemical precursors from lignocellulosic wastes.

AUTHOR CONTRIBUTIONS

Ilona A. Ruhl: Investigation; writing – original draft; writing – review and editing; visualization; formal analysis; methodology. **Robert S. Nelson:** Investigation; methodology; writing – review and editing. **Rui Katahira:** Investigation; methodology; writing – review and editing. **Jacob S. Kruger:** Investigation; methodology; writing – review and editing. **Xiaowen Chen:** Investigation; methodology; funding acquisition; writing – review and editing. **Stefan J. Haugen:** Methodology; writing – review and editing. **Morgan A. Ingraham:** Methodology; writing – review and editing. **Sean P. Woodworth:** Methodology; writing – review and editing. **Hannah Alt:** Methodology; writing – review and editing. **Kelsey J. Ramirez:** Methodology; writing – review and editing. **Darren J. Peterson:** Methodology; writing – review and editing. **Ling Ding:** Methodology; writing – review and editing. **Philip D. Laible:** Conceptualization; writing – review and editing. **Jeffrey G. Linger:** Conceptualization; funding acquisition; writing – review and editing; supervision. **Davinia Salvachúa:** Writing – review and editing; writing – original draft; visualization; conceptualization; funding acquisition; supervision.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data are available in the manuscript.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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