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Use of *Cupriavidus basilensis*-aided bioabatement to enhance fermentation of acid-pretreated biomass hydrolysates by *Clostridium beijerinckii*

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Abstract Lignocellulose-derived microbial inhibitors (LDMICs) prevent efficient fermentation of *Miscanthus giganteus* (MG) hydrolysates to fuels and chemicals. To address this problem, we explored detoxification of pretreated MG biomass by *Cupriavidus basilensis* ATCC®BAA-699 prior to enzymatic saccharification. We document three key findings from our test of this strategy to alleviate LDMIC-mediated toxicity on *Clostridium beijerinckii* NCIMB 8052 during fermentation of MG hydrolysates. First, we demonstrate that growth of *C. basilensis* is possible on furfural, 5-hydroxymethyfurfural, cinnamaldehyde, 4-hydroxybenzaldehyde, syringaldehyde, vanillin, and ferulic, *p*-coumaric, syringic and vanillic acid, as sole carbon sources. Second, we report that *C. basilensis* detoxified and metabolized ~98 % LDMICs present in dilute acid-pretreated MG hydrolysates. Last, this bioabatement resulted in significant payoffs during acetone-butanol-ethanol (ABE) fermentation by *C. beijerinckii*: 70, 50 and 73 % improvement in ABE concentration, yield and productivity, respectively. Together, our results show that biological detoxification of acid-pretreated MG hydrolysates prior to fermentation is feasible and beneficial.

Keywords *Clostridium beijerinckii* · Butanol · *Cupriavidus basilensis* · Bioabatement · Lignocellulose

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Introduction

Due to the high cost of substrates resulting from the use of either refined sugars or food crop-related raw materials, there is an impetus to find cheap and sustainable non-food substrates for production of biofuels/biochemicals. These efforts have identified lignocellulosic biomass (LB; e.g., agricultural waste) and energy crops [e.g., *Miscanthus giganteus* (MG)] as low-cost fermentation substrates for the production of butanol and other biofuels, largely due to their abundance, affordability and fairly high sugar/carbon content [1–3]. Despite these benefits, the economic viability of LB to butanol conversion is limited by various factors including (i) carbon catabolite repression, a regulatory mechanism that imposes constraints on sugar utilization [4, 5], and (ii) lignocellulose-derived microbial inhibitory compounds (LDMICs) that are toxic to the fermenting microbes [6–9]. Overcoming the roadblock imposed by LDMICs is the motivation for this study.

Biomass deconstruction, the first step in biofuel production, leads to production of LDMICs. Lignin is a major component of lignocellulose (up to 40 %). Due to the free-radical mediated polymerization and non-enzymatic self-assembly reactions that are used during (bio) synthesis of lignin, there is inherent heterogeneity in this alkyl-aromatic polymer quite unlike any other in nature [10]. Thus, as might be expected, lignin depolymerization is difficult.

Although a microbial arsenal of oxidative enzymes is capable of overcoming this recalcitrance, there are no proven enzyme cocktails akin to those used for degradation of cellulose and hemicellulose. Therefore, LB deconstruction for biofuel production often begins with a thermochemical pretreatment to disrupt the lignin matrix and expose the cellulose/hemicellulose polysaccharides for subsequent depolymerization by glycosyl hydrolases to release fermentable sugars.

Regardless of the source of lignocellulose and the pretreatment approach employed, three main chemical groups of LDMICs—furans, phenolic compounds, and organic acids—are co-generated with fermentable sugars during pretreatment and hydrolysis [11]. These compounds impede the growth of solventogenic *Clostridium* species and butanol production by diverse mechanisms including disruption of the intracellular redox state, inhibition of glycolytic enzymes, decrease of intracellular pH, damage to the cell membrane and/or to nucleic acids [1, 9, 12, 13]. Because microbial growth and butanol production are significantly affected by these inhibitors, eliminating LDMICs from the fermentation medium is crucial to industrial-scale utilization of LB for biofuel production. Although LDMICs can be removed from lignocellulosic biomass hydrolysates (LBH) by physical and chemical methods, the price of and the fermentable sugars lost during these processes increase the overall butanol production cost [1, 14]. In this regard, microbial detoxification of LDMICs (bioabatement) holds significant promise as a cost-effective alternative.

A bioabatement strategy that employs living microorganisms to selectively metabolize LDMICs in the presence of fermentable sugars is considered ideal for removal of LDMICs. Several studies highlight the value of fungi for biological detoxification [15–18]; however, loss of fermentable sugars due to their uptake by the bioabatement microbial agent offsets some of the gains related to removal of inhibitors. Because bacteria are generally more tractable to genetic manipulations than fungi, it is appealing to consider approaches in which a bacterium is engineered to facilitate bioabatement to remove LDMICs without depleting fermentable sugars.

Here, we focus our attention on *Cupriavidus basilensis*, which is capable of metabolizing (i) furanic aldehydes in the presence of fermentable sugars [19], (ii) kraft lignin, a polymeric by-product of pulp and paper industry [20], and (iii) a wide range of phenolic compounds such as toluene, benzene, chlorobenzene, phenol, *ortho*-/*para*-substituted mono- and dichlorophenol isomers [21]. On account of these attributes, we first evaluated the ability of *C. basilensis* ATCC®BAA-699 (hereafter referred to as *C. basilensis*) to detoxify and metabolize LDMICs either alone or as mixtures present in dilute acid-pre-treated MG hydrolysates. We then validated the idea that *C. basilensis* could be used to detoxify dilute acid-pretreated MG hydrolysates (prior to enzymatic saccharification) and greatly aid fermentation of these hydrolysates to acetone, butanol and ethanol (ABE) by *Clostridium beijerinckii* NCIMB 8052 (hereafter referred to as *C. beijerinckii*). To our knowledge, this is the first report of butanol production from biologically-detoxified, dilute acid-pretreated LBH.

Materials and methods

Microorganisms and culture conditions

Cupriavidus basilensis was obtained from the American Type Culture Collection, Manassas, VA. Lyophilized cells were revived by inoculation in 3 % (w/v) Trypticase Soy Broth (TSB; Becton–Dickinson and Company, Sparks, MD), followed by shake-flask incubation at 25 °C and 144 rpm for 24 h. Recovered cells were maintained as 30 % (v/v) glycerol stocks at −80 °C. Growth and detoxification experiments were performed in a mineral medium [21]. The modified mineral medium used in this study consisted of 0.02 g/L $(NH_4)_2HPO_4$, 50 mM phosphate buffer (pH 7.5; 1.3 g/L KH_2PO_4 and 10.9 g/L K_2HPO_4), 0.1 g/L yeast extract, 0.5 mL vitamin solution (mg/L: biotin, 2; nicotinic acid, 20; thiamine, 10; 4-aminobenzoate, 10; pantothenate, 5; cyanocobalamin, 20; pyridoxamine, 50), and 0.5 mL each of trace element solution I (6.5 mg/L $Na_2SeO_3{\cdot}5H_2O$ and 12.1 mg/L $Na_2WO_4{\cdot}2H_2O$ dissolved in 0.1 N NaOH) and trace element solution II (mg/L: $FeSO_4{\cdot}7H_2O$, 2250; H_3BO_3, 90; $MnSO_4{\cdot}H_2O$, 150; $CoSO_4{\cdot}7H_2O$, 210; $NiSO_4{\cdot}6H_2O$, 55; $ZnSO_4{\cdot}7H_2O$, 150; $CuSO_4{\cdot}5H_2O$, 10; and $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 80; in 0.001 N H_2SO_4). In addition to 50 mM phosphate, 2-(*N*-morpholino) ethanesulfonic acid (MES; 7 g/L) was added to MG hydrolysates to increase the buffering capacity during detoxification by *C. basilensis*. Filter-sterilized solution of LDMICs, vitamin and trace element stock solutions were added to the pre-autoclaved (121 °C for 15 min) phosphate buffer and yeast extract prior to inoculation with *C. basilensis*.

Clostridium beijerinckii ATCC 51743 (same strain as NCIMB 8052) was obtained from the American Type Culture Collection, Manassas, VA. *C. beijerinckii* stocks were routinely maintained as spore suspension in sterile double-distilled water at 4 °C. Spores for inoculation (200 μL) were heat-shocked at 75 °C for 10 min, cooled on ice for 2 min, and then inoculated into 10-mL anoxic tryptone–glucose–yeast extract (TGY) medium [9]. The culture was incubated anaerobically at 35 °C and grown until OD_{600} ~0.9–1.1 (usually ~12 h from time of inoculation; cuvette path length = 1 cm). Subsequently, actively growing *C. beijerinckii* culture (10 %, v/v) was transferred into fresh TGY medium (90 mL) and incubated for 3 h

(until OD_{600} ~1.1). Butanol fermentation was conducted in loosely-capped 50 ml Pyrex culture bottles using 6 % (v/v) of the pre-culture in 30 mL P2 medium, buffered with MES (7 g/L) [22]. Cultures were grown in the anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, MI) with a modified atmosphere of 82 % N_2, 15 % CO_2, and 3 % H_2. All experiments were conducted in triplicate.

Growth of *C. basilensis* on pure LDMICs as sole carbon sources

Cupriavidus basilensis pre-culture was prepared by inoculating a 500-μL glycerol stock in 50 mL 3 % (w/v) TSB medium followed by incubation at 25 °C and 144 rpm for 24 h. *C. basilensis* was harvested by centrifugation at 1500×*g* and 4 °C for 5 min, and then inoculated to an initial OD_{600} ~0.2 (cuvette path length = 1 cm) in the mineral medium. Carbon sources consisted of 0.1–3 g/L furans, furfural and 5-hydroxymethylfurfural (HMF), lignocellulose-derived phenolic compounds (syringic acid, syringaldehyde, *p*-coumaric acid, vanillin, vanillic acid, ferulic acid, 4-hydroxybenzaldehyde [4-HBD], and cinnamic acid), or mixed solution of the compounds (0.3 g/L each of furfural and HMF, and 0.1 g/L each of *p*-coumaric acid, vanillin, and vanillic acid), all of which make up the LDMICs. Cultures were incubated at 25 °C and 144 rpm for either 84 h or until LDMICs were completely metabolized. Samples were taken every 12 h to measure cell growth, and decrease in the concentration of LDMICs. In the case of furfural and HMF, furfuryl/HMF alcohols and furoic acid, intermediate products of furan metabolism, were also quantified.

Adaptation of *C. basilensis* to furfural

Because furans are the most abundant LDMICs in dilute acid-pretreated lignocellulosic biomass hydrolysates, they exert significant inhibitory effects on fermenting microbes. To enhance the ability of *C. basilensis* to detoxify furan-rich LBH, *C. basilensis* was first adapted to furfural. A 500 μL glycerol stock of *C. basilensis* was initially grown in a 500-mL conical flask containing 100 mL 3 % (w/v) TSB medium at 25 °C and 144 rpm for 24 h. Subsequently, *C. basilensis* was harvested by centrifugation at 1500×*g* for 5 min. *C. basilensis* was used to inoculate 250 mL conical flasks containing 50 ml of mineral medium supplemented with 0.1 g/L yeast extract and 0.5 g/L furfural to OD of ~0.2. After 12 h of incubation when the furfural concentration in the culture had decreased to half the initial concentration, the cells were harvested and inoculated into fresh mineral medium containing 1 g/L furfural. Growth was allowed to proceed for another 24 h after which cells were transferred to fresh mineral medium containing 1.5 g/L furfural. One additional round of incubation (post-cell harvest and transfer to a fresh medium containing furfural), was conducted with 2 g/L furfural. Finally, furfural-adapted *C. basilensis* cells were harvested at mid-exponential phase, plated on TSB agar plates supplemented with 1 g/L furfural. Single colonies were picked and inoculated into mineral medium (10 mL) containing 0.5 g/L furfural. *C. basilensis* cells growing at mid-exponential phase were harvested and stored as 30 % (v/v) glycerol stocks at −80 °C. Samples were taken during the adaptation experiment to monitor cell growth and furfural reduction. The rate of furfural utilization by furfural-adapted *C. basilensis* was compared to the non-adapted cells.

Pretreatment of *Miscanthus giganteus*

Miscanthus giganteus (MG) biomass used for this experiment was obtained from Dr. Stephen Long, Department of Plant Biology and Institute for Genomic Biology, University of Illinois Urbana-Champaign, Illinois. The MG biomass, which consists mainly of cellulose (40–60 % dry wt), hemicelluloses (20–40 % dry wt), and lignin (10–30 % dry wt; [23]) was ground to fine particles (1 mm) using a Thomas–Wiley Mill (Thomas Scientific, Swedesboro, NJ, USA). The moisture content of ground MG biomass was measured gravimetrically using a TempCon Oven (American Scientific Products, McGaw Park, IL, USA). Approximately 120 g of MG biomass with a moisture content of 10 % was mixed with 600 ml 2 % (v/v) sulfuric acid to obtain 15 % (w/v) solids loading. Acid pretreatment was carried out using a customized biomass pretreatment reactor at 180 °C and 150 psi for 1 h. After cooling to 25 °C room temperature, the MG slurry was aseptically adjusted to pH 5 (using ammonium hydroxide) and then stored at −20 °C.

Bioabatement to remove LDMICs from acid-pretreated *Miscanthus giganteus*

The MG biomass slurry was thawed at room temperature for 2 h and then 100 mL was mixed with 100 μL each of filter-sterilized trace element solutions I and II, and vitamin solution [21]. Previously sterilized yeast extract was added to a final concentration of 0.1 g/L. The medium was further buffered by addition of MES (7 g/L), and the pH was adjusted to 7.5 using ammonium hydroxide. Preculture for the detoxification experiment was prepared by inoculating furfural-adapted *C. basilensis* (30 % glycerol stock) into 1 L 3 % TSB. After 24 h of incubation at 25 °C and 144 rpm, *C. basilensis* cells were harvested by centrifugation at 2000 rpm for 5 min (4 °C), and then inoculated at ~1.4×10^{13} CFU/ml into 100 mL of the MG biomass slurry supplemented with 0.1 g/L yeast extract, mineral and vitamin solutions. The culture was incubated

at 144 rpm and 25 °C for 12 h to minimize loss of fermentable sugars. Samples were taken every 4 h for analyses of cell growth (colony forming units/ml), sugars (glucose, xylose, and arabinose), and LDMICs concentrations.

Enzymatic hydrolysis

After detoxification by *C. basilensis*, the slurry was autoclaved at 121 °C for 5 min to kill live cells and then cooled to 25 °C. The MG slurry was adjusted to pH 5 using undiluted NH_4OH. Subsequently, Cellulase (15 FPU/g cellulose), Viscozyme (2.4 g/100 mL), and Xylanase (0.4 g/100 mL) (Sigma-Aldrich, St. Louis, MO, USA) were added to the detoxified and un-detoxified MG biomass. Enzyme-biomass mixtures were incubated in a water-bath shaker at 50 °C and 80 rpm for 120 h. Samples were taken every 12 h to monitor saccharification. After hydrolysis, the MG hydrolysate was centrifuged to remove solid debris, and filtered with Whatman filter (11 μm pore size, 110 mm diameter; Whatman International Ltd, Maid Stone, England) to further remove solid residues. Subsequently, clear hydrolysates were filter-sterilized by passing through a 0.2 μm sterile filter (250 mL volume; Corning Inc., NY). The filter-sterilized MG hydrolysate was stored in a sterile screw-capped Pyrex bottle at −20 °C for subsequent butanol fermentation studies.

Fermentation of *Miscanthus giganteus* hydrolysates

The pH of sterile *C. basilensis*-detoxified MG hydrolysate was between 4.8 and 5.1. Fermentation media were prepared using different volume ratios of detoxified MG hydrolysates to P2 medium, by mixing appropriate volumes of MG hydrolysate with P2 Medium. The ratios of detoxified MG hydrolysate to P2 medium used included 10:0, 80:20, and 60:40, each of which was supplemented with 1 g/L yeast extract, glucose (to a final total sugar concentration of 60 g/L), and P2 stock solutions as previously described [2]. The un-detoxified MG hydrolysate was also prepared accordingly in P2 medium. The fermentation media were adjusted to pH 6.2 using 5 M NH_4OH and inoculated with 6 % *C. beijerinckii* pre-culture. The un-detoxified hydrolysates and P2 medium were used as controls. All cultures were buffered with MES (7 g/L). Fermentation was conducted in loosely capped 50 mL culture bottles containing 30 mL fermentation medium at 35 ± 1 °C for 84 h in the anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, MI). Two mL samples were taken every 12 h for analyses of cell growth, and to determine the concentrations of butanol, acetone, ethanol, acetic and butyric acids, sugars, and LDMICs.

Analytical methods

Cell growth was determined by counting colony forming units on agar plates inoculated with samples from fermentation cultures or by measuring optical density at 600 nm (OD_{600}) using a DU® 800 spectrophotometer (Beckman Coulter Inc., Brea, CA, USA). Changes in the concentrations of LDMICs were determined by monitoring changes in wavelength using a DU® 800 spectrophotometer. The maximum absorption spectra of quantified LDMICs were at 275, 220, 282, 222, 252, 260, 278, 285, 304, 315, 333, 273, and 291 nm for furfural, furfuryl alcohol, HMF, HMF alcohol, vanillic acid, syringic acid, vanillin, *p*-coumaric acid, syringaldehyde, ferulic acid, hydroxybenzaldehyde, cinnamic acid, and cinnamaldehyde, respectively. Furoic acid was measured at 245 nm. The concentrations of LDMICs were further validated by high performance liquid chromatography (HPLC) using a Waters 2796 Bioseparation Module equipped with Photodiode Array Detector (PDA; Waters, Milford, MA) and a 3.5-μm Xbridge C18, 150 mm × 4.6 mm column (Waters, Milford, MA, USA) [2]. Samples were eluted using a gradient mobile phase of acetic acid [0.3 % (v/v) in HPLC-grade water] and HPLC-grade methanol, operated at a flow rate of 0.6 mL/min as previously described [7]. Sugar yield following hydrolysis of MG biomass was estimated as the amount of glucose, arabinose, and xylose obtained in hydrolysates per gram of biomass. Total reducing sugar concentrations in the MG hydrolysates and fermentation media were measured using the 3,5-dinitrosalicylic acid-based assay [24]. The concentration of individual sugars was quantified by HPLC using Waters 2796 Bioseparation module equipped with Evaporative Light Scattering Detector (ELSD; Waters, Milford, MA) and a 9-μm Aminex HPX-87P, 300 mm × 7.8 mm column maintained at 65 °C in series with an Aminex deashing guard column (4.6 mm internal diameter × 3 cm long; Bio-Rad, Hercules, CA, USA). The mobile phase was HPLC-grade water at a flow rate of 0.6 mL/min [7]. The concentration of the fermentation products, acetone, butanol, ethanol, acetic acid, and butyric acid, was quantified using a 7890A Agilent gas chromatograph (Agilent Technologies Inc., Wilmington, DE, USA) equipped with a flame ionization detector (FID) and 30 m (length) × 320 μm (internal diameter) × 0.50 μm (HP-INNOWax film) J × W 19091 N-213 capillary column as described previously [9]. ABE yield was defined as total ABE produced (in grams) per grams of sugar utilized. Productivity was calculated as the concentration (g/L) of ABE produced per hour.

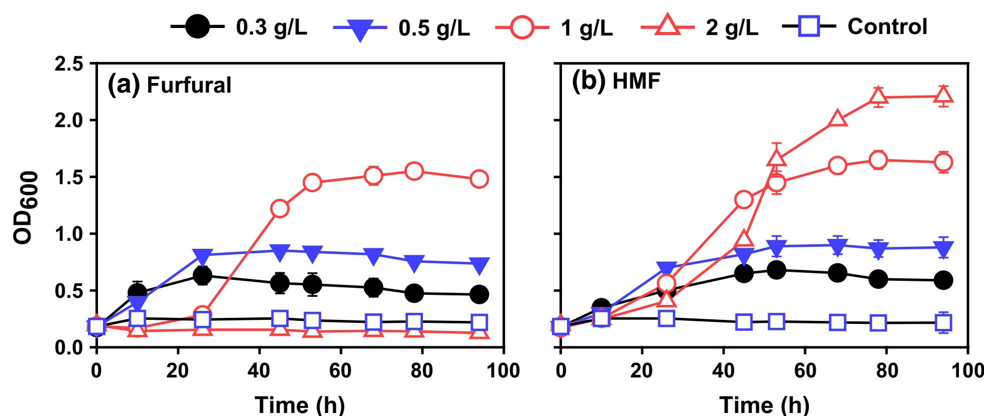

Fig. 1 The growth profile of *C. basilensis* challenged with different concentrations of furfural and HMF. **a** 0.3, 0.5, 1 or 2 g/L furfural; **b** 0.3, 0.5, 1 or 2 g/L HMF. In addition to LDMICs, cultures were supplemented with 0.1 g/L yeast extract. The control contained 0.1 g/L yeast extract only. *Error bars* represent standard deviations of means ($n = 3$)

Statistical analysis

The general linear model of Minitab version 17 (Minitab Inc., State College, PA, USA) was used for all statistical analyses to compare the differences between treatments as they pertain to growth, product yields and productivities, rate of reduction of LDMICs, and concentrations of sugars, LDMICs, and ABE. ANOVA was conducted at different fermentation time points. Tukey's test at 95 % confidence interval was applied to pair-wise comparison to determine level of significance.

Results and discussion

Mineralization of model solution of lignocellulose-derived microbial inhibitors by *C. basilensis*

LBH detoxification by *C. basilensis* prior to fermentation is desirable due to the deleterious effects of LDMICs on fermenting microbes that result in decreased generation of the product of interest. Bioabatement as a strategy for detoxifying LDMICs in LBH is potentially cheaper than physical and chemical methods of detoxification [15]. Here, our overarching objective was to test the utility of *C. basilensis* for detoxification of LDMICs in MG hydrolysates prior to saccharification, and the associated payoffs to ABE fermentation by *C. beijerinckii*. Our approach entailed: (i) testing LDMICs (furans and phenolics) as carbon sources for *C. basilensis*, (ii) using this microbe for detoxifying biomass hydrolysates currently used in industry, and (iii) finally assessing if the bioabatement did improve solventogenesis by *C. beijerinckii* during ABE fermentation. For all growth and substrate utilization studies, *C. basilensis* was pre-grown in 3 % (w/v) tripticase soybroth at 25 °C and 144 rpm for 24 h followed by centrifugation at 1500×*g* and 4 °C for 5 min. The supernatant was discarded and the pellet was used to inoculate the mineral medium to OD_{600} ~0.2 (cuvette path length = 1 cm).

Utilization of furanic aldehydes

We first tested the ability of un-adapted *C. basilensis* to metabolize LDMICs. Figure 1 depicts the time-dependent growth profile of *C. basilensis* during growth on 0.3, 0.5, 1, and 2 g/L furfural or HMF. The growth of *C. basilensis* on furfural and HMF was concentration dependent. At 0.3–1.0 g/L each of furfural and HMF, *C. basilensis* growth was significantly ($p < 0.05$) greater than the control culture in which these furans were not included. However, while 2 g/L furfural resulted in complete growth inhibition, 2 g/L HMF supported the growth of *C. basilensis*.

Aerobic mineralization of furans proceeds via an initial detoxification step where furans are rapidly converted to their corresponding alcohols [19, 25]. Therefore, we examined the levels of furfuryl/hydroxymethyl furfuryl alcohols and 2-furoic acid (Fig. 2). As expected, a decrease in furfural and HMF concentrations was accompanied by the generation of their respective alcohols and furoic acid in the growth medium (Fig. 2). These alcohols are reconverted to furfural and HMF prior to conversion to 2-furoic acid via the furan catabolic pathway (Fig. 3; Trudgill pathway) [25]. Initial reduction of both furanic aldehydes to their less toxic alcohols is consistent with previous studies using *C. beijerinckii* [2], *C. acetobutylicum* ATCC 824 [7], and *C. basilensis* HMF14 [19]. The rapid generation of the less toxic alcohol forms ensures an immediate cessation of cell damage even while preserving the carbon sources as substrate reservoirs for subsequent gradual dissimilation via

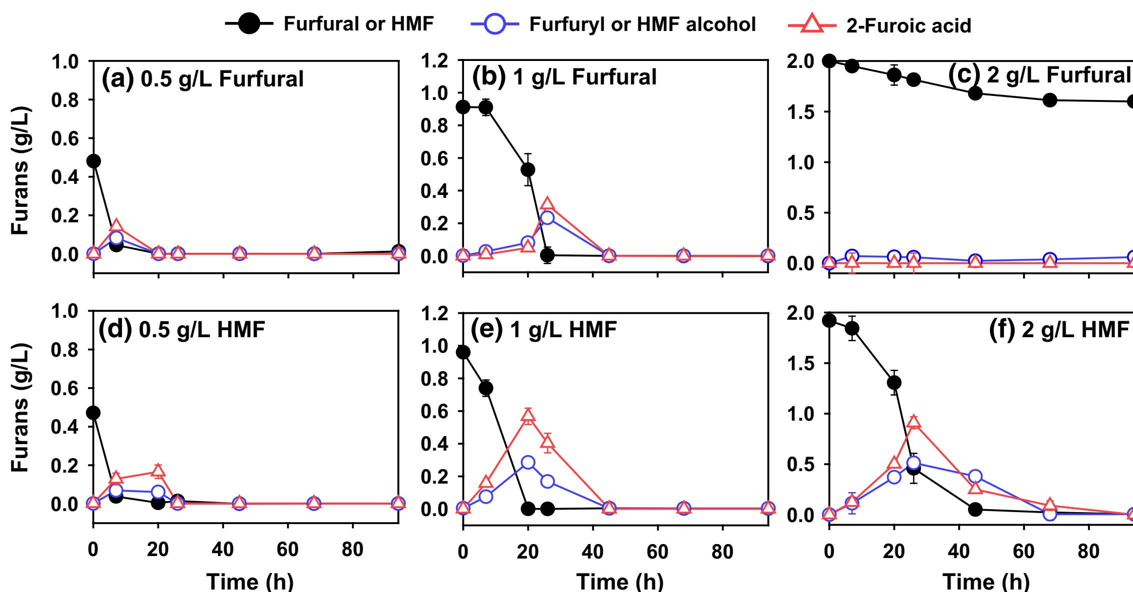

Fig. 2 Utilization of furfural and HMF by *C. basilensis*. Furfural and HMF degradation involved the production of furfuryl/HMF alcohols and furoic acid. Furoic acid formation signals degradation via the Trudgill pathway. **a**, **b** 0.5, 1, and 2 g/L furfural; **d**–**f** 0.5, 1, and 2 g/L HMF, respectively. In addition to LDMICs, cultures were supplemented with 0.1 g/L yeast extract. The control contained only 0.1 g/L yeast extract. *Error bars* represent standard deviations of means ($n = 3$)

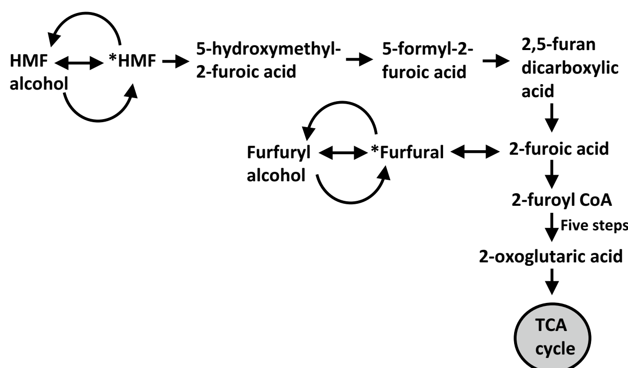

Fig. 3 Trudgill pathway illustrating catabolism of furfural and HMF by *C. basilensis*. *Curved arrows* indicate the initial detoxification of furfural and HMF by reduction to their respective alcohols. *Starting substrate—furfural and HMF

the Trudgill pathway to produce 2-furoyl-CoA (via furoic acid and three other intermediates in the case of HMF alcohol), and 2-oxoglutaric acid, which is fed into the TCA cycle (Fig. 3).

At 0.3 and 0.5 g/L, the rates of furfural and HMF utilization by un-adapted *C. basilensis* were not significantly different ($p > 0.05$). However, at 1 g/L, the rate of HMF utilization (0.03 g/Lh^{-1}) was significantly higher than that of furfural (1.9×10^{-4} g/Lh^{-1}, $p < 0.05$), suggesting a preference for HMF as a carbon source. At 2 g/L furan, while HMF was completely metabolized after 48 h, less than 25 % furfural was utilized during the same period (Fig. 2f vs. c). Notably, ≥1 g/L furfural or HMF and 2 g/L furfural (Fig. 1a) resulted in extended lag phase and severe growth inhibition, respectively, of un-adapted *C. basilensis*. Nevertheless, higher inoculum size of the un-adapted *C. basilensis* (OD_{600} ~2.0) decreased the lag phase and consequently, greater than 2 g/L furfural was completely metabolized (data not shown). Interestingly, when furfural-adapted *C. basilensis* (OD_{600} ~0.2) was challenged with 2 g/L furfural, it showed robust growth (Fig. S1), which is likely a reflection of adaptive tolerance, a phenomenon well documented with other microbes including endophytic fungi [17, 25].

Utilization of lignin-derived phenolic compounds

To ascertain the ability of un-adapted *C. basilensis* to mineralize lignin-derived phenolic compounds (LDPCs), it was grown in mineral medium supplemented with 0.1 g/L yeast extract and 0.1–0.5 g/L *p*-coumaric acid, cinnamic acid, syringaldehyde, vanillin, vanillic acid or hydroxybenzaldehyde as the sole carbon source (Fig. 4). The control was mineral medium supplemented with 0.1 g/L yeast extract only. Whereas *C. basilensis* metabolized 2 g/L HMF and 1 g/L furfural, its growth was slower in media with >0.5 g/L of LPDCs (data not shown). In media containing different concentrations (0.1–0.5 g/L) of *p*-coumaric acid, vanillin, vanillic acid or 4-hydroxybenzaldehyde (4-HBD) as the sole carbon source, the optical density of un-adapted *C. basilensis* decreased by at least 2- and 2.7-fold, respectively, relative to the cells grown on

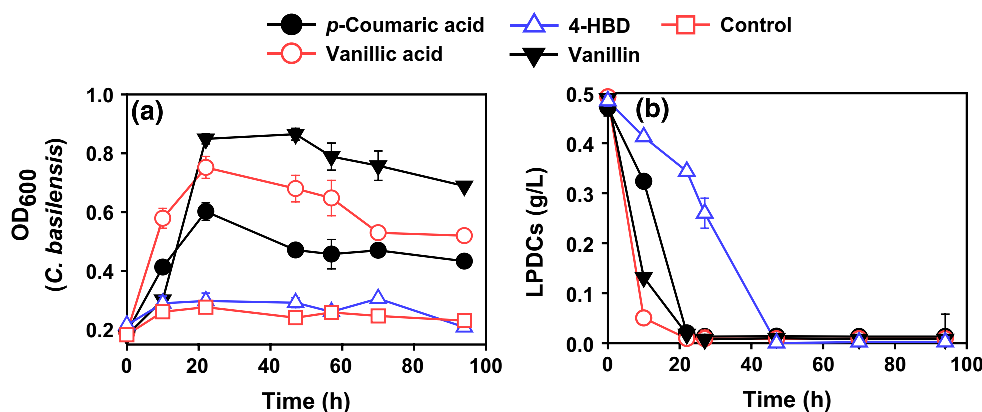

Fig. 4 Utilization of phenolic compounds by *C. basilensis*. In addition to 0.1 g/L yeast extract, separate cultures contained 0.5 g/L *p*-coumaric acid, 4-HBD, vanillic acid or vanillin as a carbon source. The control was supplemented with 0.1 g/L yeast extract only. **a** Cell growth; **b** concentrations of phenolic compounds. *Error bars* represent standard deviations of means ($n = 3$)

furfural or HMF as the sole carbon source. Figure 4 shows the patterns of cell growth and substrate consumption when *C. basilensis* was grown on 0.5 g/L of different LPDCs. Interestingly, LBHs contain higher concentration of furans than LPDCs after pretreatment and hydrolysis [7]; thus, making *C. basilensis* an ideal bioabatement agent to decrease LDMICs (both furans and phenolic compounds) in LBHs. The growth rate of *C. basilensis* in medium containing vanillic acid (0.083 h^{-1}) was significantly higher ($p < 0.05$) than its growth rate in medium containing vanillin (0.044 h^{-1}) or *p*-coumaric acid (0.038 h^{-1}). The extent to which phenolic substrates are favorably metabolized by *C. basilensis* appears to depend on the degree of hydroxylation of the phenyl ring, as evidenced by higher consumption of *p*-coumaric acid than cinnamic acid. Similarly, there is a transformation bias in favor of acids than aldehydes (for instance, cinnamic acid was easier to degrade than cinnamaldehyde (data not shown). These findings are expected since phenol hydroxylases activate aromatic intermediates using electron-rich substituents through the *meta-* or *ortho-* cleavage reactions for subsequent ring opening/cleavage (Fig. S2) [26, 27].

Utilization of mixture of LDMICs

Another interesting attribute that we studied relates to the ability of *C. basilensis* to simultaneously metabolize furans and phenolic compounds (Fig. 5). We tested the ability of un-adapted *C. basilensis* to grow on media containing a mixture of LDMICs. The growth medium consisted of 0.3 g/L each of furfural and HMF, and 0.1 g/L each of *p*-coumaric acid, vanillic acid, and vanillin as carbon sources. Figure 5 shows the growth and substrate utilization profiles of *C. basilensis* in these LDMIC-rich media in which the maximum growth of *C. basilensis* was 1.8-fold ($p < 0.05$) greater than the control with no LDMICs as carbon source (Fig. 5a). Utilization of furfural and HMF was accompanied by the production of furfuryl and HMF alcohols, which were rapidly converted to furoic acid (Fig. 5b). Similar to the mineralization profile of single substrates above, vanillic acid was utilized faster than vanillin and *p*-coumaric acid (Fig. 5c). Remarkably, all the inhibitors tested in the mixed-inhibitor medium were simultaneously utilized by *C. basilensis*, although the furanic aldehydes were preferred over the LPDCs. Results obtained from the utilization of LDMICs mixture experiment motivated us to explore a bioabatement strategy for LBH detoxification, specifically dilute acid-pretreated *Miscanthus giganteus*.

Pretreatment and detoxification of *Miscanthus giganteus* biomass hydrolysate

Acid pretreatment of MG biomass produced glucose, xylose, and arabinose with yields of 0.06, 0.07, and 0.04 g, respectively, per gram of MG biomass. To ensure maximum generation of LDMICs from MG biomass, the duration of pretreatment was extended to 1 h. The goal was to demonstrate the ability of *C. basilensis* to detoxify dilute acid-pretreated lignocellulosic biomass to levels that are tolerated by *C. beijerinckii* during butanol fermentation. The concentration of LDMICs after acid pretreatment of MG biomass was 4.62 g/L furfural, 1.1 g/L HMF, and 1.5 g/L total phenolic compounds. At these concentrations, the growth of *C. beijerinckii* was severely inhibited. However, we reasoned that the furfural-adapted *C. basilensis* culture could be used to detoxify pretreated MG hydrolysate.

Although the growth of *C. basilensis* was initially delayed likely due to high concentration of LDMICs in the culture medium, the growth increased considerably after 24–36 h depending on the inoculum size used and the time at which the concentration of the LDMICs in the medium had decreased (Fig. 5d). The initial lag in growth coincided with the detoxification phase during which LDMICs were rapidly transformed to their less toxic intermediates (i.e., conversion of furans to their respective alcohols) [7] (Fig. 2). As shown in Table 1, the initial concentration of furfural, HMF, vanillin, *p*-coumaric acid, and vanillic acid was depleted after 8 h of bioabatement. Furthermore, the

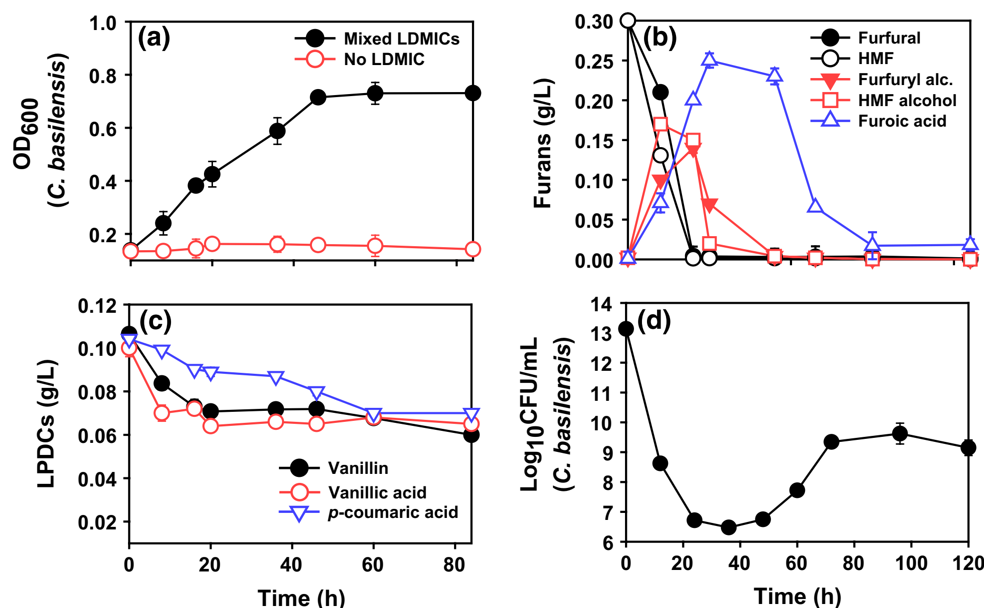

Fig. 5 Co-utilization of mixtures of microbial inhibitory compounds by *C. basilensis* and extended growth profile during the bioabatement of MG**. a** Cell growth on mixtures of LDMICs containing furfural (0.3 g/L), HMF (0.3 g/L), and 0.5 g/L each of vanillin, vanillic acid, and *p*-coumaric acid; **b** furfural and HMF reduction involved the production of furfuryl/HMF alcohol and furoic acid; **c** utilization of phenolic compounds; and **d** *C. basilensis* growth profile during 120 h of MG bioabatement. Note that **a**–**c** represent measurements carried out in mineral medium, while **d** was evaluated in MG hydrolysate. The mineral medium control contained only 0.1 g/L yeast extract. *Error bars* represent standard deviations of means ($n = 3$)

Table 1 Decrease in the concentrations of LDMICs during bioabatement of acid-pretreated *Miscanthus giganteus* biomass by *C. basilensis*

LDMICs (mg/L)	Time (h)				% Utilization
	0	4	8	12	
Furfural	3,810 ± 2.4	1124 ± 1.1	ND	ND	100
HMF	899 ± 0.01	456.18 ± 0.12	ND	ND	100
Cinnamaldehyde	30.1 ± 0.01	28.32 ± 0.05	27.04 ± 0.1	25.02 ± 0.3	16.9
Ferulic acid	78.6 ± 0.09	76.19 ± 0.1	74.28 ± 0.5	58.96 ± 0.5	25
p-Coumaric acid	50 ± 0	38.67 ± 0.02	ND	ND	100
Syringaldehyde	179 ± 0.06	161 ± 0	71.27 ± 0.2	ND	100
Vanillin	118.6 ± 0.04	91.31 ± 0.05	ND	ND	100
Syringic acid	204.78 ± 0.1	185.78 ± 0.13	122.91 ± 0.2	ND	100
Vanillic acid	258.5 ± 0.02	189.56 ± 0.2	ND	ND	100
4-HBD	14 ± 0.02	13.74 ± 0.1	10.69 ± 0.05	ND	100

ND not detected

initial concentration of syringaldehyde, syringic acid and 4-HBD was depleted after 12 h of bioabatement while ferulic acid and cinnamaldehyde decreased by 25 and 17 %, respectively, possibly feeding intermediates to the TCA cycle. Collectively, significant amounts of LDMICs were depleted after 12 h of bioabatement (Fig. S3). Nonetheless, it is likely that the amount of LDMICs in the MG hydrolysates is insufficient to sustain robust growth of *C. basilensis* since we observed a decrease in total reducing sugar concentration during the bioabatement.

Bioabatement of the acid-pretreated MG biomass was followed by the addition of saccharolytic enzymes to release fermentable sugars from the pretreated MG biomass. Table 2 shows the concentration of sugars before and after *C. basilensis*-mediated detoxification and enzymatic hydrolysis. As expected, the un-detoxified MG hydrolysate (control) contained significantly higher concentrations of LDMICs (p <0.05) than the detoxified hydrolysate. While *C. basilensis* was effective at removing LDMICs from acid-pretreated MG biomass, it metabolized some sugars during

Table 2 Concentrations of sugars and microbial inhibitory compounds in the MG hydrolysate pre- and post-detoxification, and after enzymatic saccharification

Sugars/inhibitors	Acid-pretreated MG		After enzymatic hydrolysis	
	Before bioabatement (0 h)	After bioabatement (12 h)	Un-detoxified MG	Detoxified MG
Glucose (g/L)	6.67 ± 0.01[a]	ND[b]	10.21 ± 1.34[b]	5.97 ± 0.15[a]
Xylose (g/L)	8.13 ± 0.01[a]	6.82 ± 0.11[b]	9.12 ± 0.11[b]	7.84 ± 0.32[a, b]
Arabinose (g/L)	4.36 ± 0.21[a]	4.33 ± 0.31[a]	5.01 ± 0.29[a]	5.34 ± 0.25[b]
Furfural (g/L)	3.81 ± 2.4[a]	ND	4.12 ± 0.51[a]	ND
HMF (g/L)	0.9 ± 0.01[a]	ND	1.01 ± 0.02[a]	ND
Cinnamaldehyde (mg/L)	30.1 ± 0.01[a]	25.02 ± 0.01[b]	35.22 ± 0.01[a]	23.2 ± 0.01[b]
4-HBD (mg/L)	14 ± 0.02[a]	ND	16.54 ± 0.01[a]	ND
Vanillic acid (mg/L)	258.5 ± 0.02[a]	ND	279.7 ± 0.6[a]	ND
Vanillin (mg/L)	118.6 ± 0.04[a]	ND	126.12 ± 0.02[a]	ND
p-Coumaric acid (mg/L)	50 ± 0[a]	ND	55 ± 0.13[a]	ND
Syringic acid (mg/L)	204.78 ± 0.10[a]	ND	218.75 ± 0.56[a]	ND
Syringaldehyde (mg/L)	179 ± 0.06[a]	ND	182.22 ± 0.01[a]	ND
Ferulic acid (mg/L)	78.6 ± 0.09[a]	58.96 ± 0[b]	88.7 ± 0.21[a]	50.2 ± 0.28[b]

Parameters with different letters in each row are significantly different ($p < 0.05$)

ND not detected

[a,b,c,d] Tukey's pairwise comparisons between means in each row for acid-pretreated MG (before and after bioabatement), and after enzymatic hydrolysis (detoxified and un-detoxified MG)

bioabatement process, which resulted in up to 21 % sugar loss when compared to un-detoxified saccharified acid-pretreated MG biomass hydrolysates (Table 2). A major challenge with bioabatement is to facilitate elimination of toxic inhibitors without siphoning away valuable sugars—in our case, a 12-h bioabatement of MG biomass led to removal of 98 % of the total LDMICs (Table 1) and the sugar loss was cut down from 45 to 21 %.

Comparative growth and ABE profiles of *C. beijerinckii* grown using either *C. basilensis*-treated or untreated MG hydrolysates

Solventogenic *Clostridium* species are capable of detoxifying up to 2 g/L furans without any adverse effect on butanol production [7]. However, acid-pretreated LBHs contain a higher concentration, and to make matters worse, these furans are present with other LDMICs. Indeed, the toxic cocktail in MG hydrolysates inhibits microbial growth and severely undermines butanol production by *C. beijerinckii* [2]. To investigate the fermentability of *C. basilensis*-detoxified LBH, both detoxified and untreated MG hydrolysates were used as carbon source for ABE fermentation by *C. beijerinckii*.

Figure 6 shows the growth and ABE production profiles of *C. beijerinckii* grown in different concentrations (60–100 %) of MG hydrolysates. In all treatments, the growth of *C. beijerinckii* and ABE production using detoxified MG hydrolysates feedstock were significantly higher ($p < 0.05$) than that observed with un-detoxified MG hydrolysates. The growth of *C. beijerinckii* in MG hydrolysates and ABE production increase with decreasing concentration of MG hydrolysates (60 > 80 > 100 %). This finding indicates some inhibition from the residual LDMICs present post-bioabatement. Importantly, detoxification of the MG hydrolysates resulted in ~70 % increase in total ABE concentration when compared to the untreated un-detoxified control. Hence, ABE yields for the detoxified hydrolysates (0.31–0.34 g/g) were significantly ($p < 0.05$) higher than that of the un-detoxified controls (0.15–0.19 g/g), and remarkably quite similar to that for the P2 control containing pure glucose and no LDMIC (0.33 g/g; Table 3). Further, ABE productivities for *C. beijerinckii* grown in detoxified MG hydrolysates (0.1–0.15 g/Lh^{-1}) were at least 73 % higher than those of the untreated cultures (0.01–0.04 g/Lh^{-1}; Table 3).

Since *C. beijerinckii* can transform sub-lethal concentrations of LDMICs (e.g. <4 g/L furfural [7]), we analyzed the concentration of LDMICs during ABE fermentation of the detoxified and un-detoxified MG hydrolysates. We observed that *C. beijerinckii* completely transformed residual LDMICs present in the detoxified MG hydrolysates medium into less microbial inhibitory compounds during ABE fermentation unlike the untreated MG hydrolysate control (Table S1). Using detoxified MG hydrolysates as substrate, *C. beijerinckii* exhibited its typical biphasic fermentation characteristic as indicated by an initial decrease in the culture pH during the exponential growth phase (due

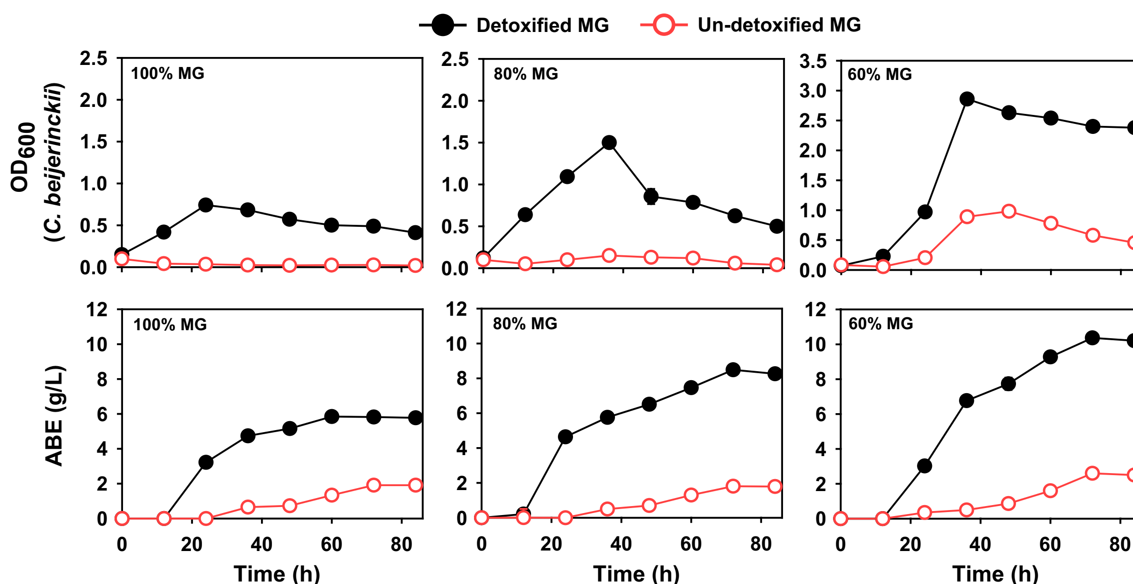

Fig. 6 Cell growth and ABE production profiles of *C. beijerinckii* grown in detoxified and un-detoxified MG hydrolysate-based medium. Fermentations were conducted in 100, 80, and 60 % MG hydrolysates. *Error bars* represent standard deviations of means ($n = 3$)

Table 3 The fermentation profile of *C. beijerinckii* in *C. basilensis*-detoxified and un-detoxified MG hydrolysates depicting maximum product concentrations, total sugar utilized, and ABE productivities and yield

Sample	MG (%)	Maximum product concentration (g/L)				Total sugar consumed (g/L)*	ABE productivity (g/Lh^{-1})	ABE yield (g/g)
		Acetone	Ethanol	Butanol	Total ABE			
Detoxified MG	100	2.01 ± 0.01^a	1.76 ± 0^a	2.33 ± 0.1^a	6.1 ± 0.11^a	18.61 ± 0.02^a	0.1 ± 0.02^a	0.31 ± 0.04^a
	80	2.11 ± 0.05^c	2.69 ± 0.05^c	3.78 ± 0.3^c	8.58 ± 0.4^c	24.69 ± 0.01^c	0.12 ± 0.13^c	0.34 ± 0.01^c
	60	2.33 ± 0.12^e	2.26 ± 0.22^e	5.87 ± 0.2^e	10.5 ± 0.5^e	36.46 ± 1.10^e	0.15 ± 0.04^e	0.30 ± 0.08^e
Un-detoxified MG	100	0^b	1.91 ± 0.1^b	0^b	1.91 ± 0.1^b	11.33 ± 0.50^b	0.02 ± 0.01^b	0.17 ± 0.33^b
	80	0^d	1.81 ± 0.3^d	0^d	1.81 ± 0.3^d	11.89 ± 0.21^d	0.03 ± 0.01^d	0.15 ± 0.01^d
	60	0^f	2.6 ± 0.53^f	0^f	2.6 ± 0.53^f	13.8 ± 0^f	0.04 ± 0^f	0.19 ± 0^f
P2	0	3.53 ± 0.02	2.38 ± 0.35	9.74 ± 0.5	15.7 ± 0.9	47.53 ± 2.0	0.26 ± 0.10	0.33 ± 0.22

Hydrolysates were supplemented with glucose to yield a total 60 g/L sugar pre-fermentation

Tukey's pairwise comparisons within parameters and between each volume ratio (^{a,b} 100 %, ^{c,d} 80 %, or ^{e,f} 60 %) for detoxified and un-detoxified MG hydrolysate

Values with different letters, for each volume ratio, are significantly different ($p < 0.05$)

* Total sugar comprises glucose, arabinose and xylose [32]. The theoretical yield of ABE/g of glucose is 0.415 g/g [33]

to acid production) followed by an increase in pH (as a result of acid re-assimilation and ABE production; Fig. S4). In contrast, *C. beijerinckii* grown in a medium containing untreated MG hydrolysates did not show this biphasic pH shift; in fact, it accumulated higher concentrations of acetic and butyric acids and did not transition to solventogenesis (Fig. S5). *C. beijerinckii* grown in LDMIC-replete LBHs are typically characterized by accumulation of high concentrations of acetic and butyric acids [8]. This finding is attributed to LDMICs, especially the furans, which drain the cell of ATP [28, 29], and the counter-response of solventogenic *Clostridium* species to increase the biosynthesis of acetic and butyric acids to boost ATP generation [8].

Conclusions

While our study highlights the feasibility and profitability of exploiting *C. basilensis* for cheap and sustainable removal of LDMICs from LBHs, translating this important advance from laboratory- to industrial-scale fermentation will require a few additional steps. First, disruption

of genes involved in glucose uptake in *C. basilensis* ATCC®BAA-699 is needed to reduce or eliminate loss of glucose during bioabatement. In fact, this goal is attainable given precedents, such as the enhanced uptake of pentoses concomitant with down-regulation of a glucose transporter [30]. Although our data indicate that *C. basilensis* preferentially uses hexoses than pentoses, disrupting hexose uptake and relieving carbon catabolite repression might lead to some re-wiring with respect to sugar utilization but unlikely to undermine bioabatement. Second, it is important to determine the upper threshold in terms of LDMIC concentration that can be tolerated by the fermentation microbe of choice. This information is vital to minimize sugar losses since bioabatement can be terminated upon meeting this threshold. However, if utilization of LDPCs by *C. basilensis* generates β-ketoadipate pathway intermediates (Fig. S2), which are known to scavenge reactive oxygen species [31] and therefore valuable in alleviating stress caused by LDMICs, we will need to establish the ideal bioabatement landscape where such benefits are maximized and sugar losses are minimized. Overall, bioabatement offers exciting prospects for exploiting agricultural waste as feedstock to generate valuable chemicals and fuels.

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