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Conversion and assimilation of furfural and 5-(hydroxymethyl)furfural by *Pseudomonas putida* KT2440

Michael T. Guarnieri, Mary Ann Franden, Christopher W. Johnson, Gregg T. Beckham*

National Bioenergy Center, National Renewable Energy Laboratory, Golden CO 80401

A B S T R A C T

The sugar dehydration products, furfural and 5-(hydroxymethyl)furfural (HMF), are commonly formed during high-temperature processing of lignocellulose, most often in thermochemical pretreatment, liquefaction, or pyrolysis. Typically, these two aldehydes are considered major inhibitors in microbial conversion processes. Many microbes can convert these compounds to their less toxic, dead-end alcohol counterparts, furfuryl alcohol and 5-(hydroxymethyl)furfuryl alcohol. Recently, the genes responsible for aerobic catabolism of furfural and HMF were discovered in *Cupriavidus basilensis* HMF14 to enable complete conversion of these compounds to the TCA cycle intermediate, 2-oxo-glutarate. In this work, we engineer the robust soil microbe, *Pseudomonas putida* KT2440, to utilize furfural and HMF as sole carbon and energy sources via complete genomic integration of the 12 kB *hmf* gene cluster previously reported from *Burkholderia phytofirmans*. The common intermediate, 2-furoic acid, is shown to be a bottleneck for both furfural and HMF metabolism. When cultured on biomass hydrolysate containing representative amounts of furfural and HMF from dilute-acid pretreatment, the engineered strain outperforms the wild type microbe in terms of reduced lag time and enhanced growth rates due to catabolism of furfural and HMF. Overall, this study demonstrates that an approach for biological conversion of furfural and HMF, relative to the typical production of dead-end alcohols, enables both enhanced carbon conversion and substantially improves tolerance to hydrolysate inhibitors. This approach should find general utility both in emerging aerobic processes for the production of fuels and chemicals from biomass-derived sugars and in the biological conversion of high-temperature biomass streams from liquefaction or pyrolysis where furfural and HMF are much more abundant than in biomass hydrolysates from pretreatment.

1. Introduction

Glucose and xylose are the two most abundant building blocks of the polysaccharides cellulose and hemicellulose in plant cell walls. As most biomass conversion schemes for the production of fuels and chemicals employ a high-temperature processing step, the fate of glucose and xylose is of critical relevance at process temperatures. These unit operations range from thermochemical pretreatment to make the cell wall more amenable for enzymatic polysaccharide deconstruction (Chundawat et al., 2011) to liquefaction (Akhtar and Amin, 2011) or pyrolysis (Czernik and Bridgwater, 2004), which aim to rapidly break down biomass for downstream catalytic upgrading. In all cases, the high temperatures that biomass is exposed to, often in the simultaneous presence of catalysts, such as mineral acids, or via autohydrolysis by liberated acetic acid in biomass, can lead to the dehydration of glucose and xylose into the corresponding furans, 5-(hydroxymethyl)furfural (HMF) and furfural, respectively. Depending on the intended downstream application, these molecules are either a common target, e.g., for fuel or chemical production (Chheda et al., 2007; Tong et al., 2010), a major undesired inhibitor for downstream microbial conversion of biomass sugars (Mills et al., 2009; Palmqvist and Hahn-Hägerdal, 2000; Pienkos and Zhang, 2009), or simply a by-product of high-temperature processing in pyrolysis and liquefaction approaches.

From a microbial conversion perspective, furfural and HMF are most commonly studied as inhibitors introduced during thermochemical pretreatments that employ acid or autohydrolytic mechanisms (Mills et al., 2009; Palmqvist and Hahn-Hägerdal, 2000; Pienkos and Zhang, 2009). As such, there is an enormous body of research on optimization of pretreatment schemes to minimize the formation of furfural and HMF, on various hydrolysate conditioning schemes to remove these aldehydes and other inhibitors after pretreatment, and on the evolution and metabolic engineering of multiple model and non-model microbes for enhanced tolerance. In both aerobic and anaerobic microbes, the most commonly observed, native mechanism of detoxifying furfural and HMF employs reducing equivalents (e.g., NADH or

* Corresponding author.
E-mail address: gregg.beckham@nrel.gov (G.T. Beckham).

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NADPH) to convert the aldehydes into the corresponding alcohols, furfuryl alcohol and 5-(hydroxymethyl) furfuryl alcohol, which are less toxic. However, this approach is non-optimal as it consumes reducing equivalents and leads to dead-end compounds that are then not useful for conversion to target products and, instead, persist as impurities. Alternatively, microbial conversion of these compounds to carbon and energy sources would be much more desirable (Wierckx et al., 2011).

To that end, in 2009, Koopman, Wierckx, and co-workers reported the exciting discovery of a metabolic pathway in the soil isolate *Cupriavidus basilensis* HMF14 (Wierckx et al., 2010) for the complete catabolism of furfural and HMF into the tricarboxylic acid (TCA) cycle via a common intermediate, 2-furoic acid. *In silico* and physiological analyses identified a series of microbes with putative homologous gene clusters, including *Burkholderia phytofirmans* (Koopman et al., 2010b). As shown in Fig. 1, in both cases, the aldehydes are converted to the corresponding acids (2-furoic acid for furfural and 2,5-furan dicarboxylic acid for HMF) by oxidoreductases. A 2,5-furan dicarboxylic acid decarboxylase generates 2-furoic acid as a common intermediate, which is then converted to 2-furoyl-CoA that is ultimately incorporated into the TCA cycle via 2-oxo-glutarate. In the same report, the authors engineered this pathway into a plasmid-bearing *Pseuodomonas putida* S12 to demonstrate conversion of these compounds to microbial biomass (Koopman et al., 2010b). In a separate study, the same authors also developed a strain capable of producing high yields and titers of 2,5-furandicarboxylic acid, a highly sought replacement for terephthalic acid, via the use of this pathway (Koopman et al., 2010a). Overall, this seminal discovery led the way for aerobic conversion of furfural and HMF into usable carbon for both biomass growth and for target product formation (Koopman et al., 2010b).

Besides being prevalent in biomass hydrolysates from thermochemical pretreatment, furfural and HMF are also common intermediates in biomass pyrolysis and liquefaction processes. The aqueous streams from fast pyrolysis, in particular, are usually rich in levoglucosan, cellobiosan, HMF, furfural, and small acids (e.g., acetate) (Black et al., 2016; Johnston and Brown, 2014; Patwardhan et al., 2009; Pollard et al., 2012; Remón et al., 2014; Rover et al., 2013; Valle et al., 2013; Vispute and Huber, 2009; Vispute et al., 2010). The microbial conversion of these types of substrates, dubbed “hybrid processing” by Brown, Jarboe, and colleagues, is far less studied than the conversion of biomass-derived sugars (Bacik and Jarboe, 2016; Brown, 2005, 2007; Jarboe et al., 2011b; Shen et al., 2015). The use of biological approaches to convert pyrolysis-derived compounds has almost solely focused to date on the conversion of levoglucosan and acetate, both highly prevalent intermediates from pyrolysis, into ethanol or natural carbon storage products such as fatty acids or polyhydroxyalkanoates in green algae, oleaginous yeast, model microbes such as *Escherichia coli*, or robust soil microbes such *P. putida* KT2440 (Chi et al., 2013; Dalluge et al., 2014; Lian et al., 2010, 2016, 2013, 2012; Liang et al., 2013; Linger et al., 2016; Rover et al., 2014; Zhao et al., 2016). The conversion of furfural and HMF, in addition to levoglucosan, acetate, and cellobiosan, would enable a more comprehensive microbial conversion process for pyrolysis and liquefaction-based substrates via a hybrid processing approach. Indeed, prior metabolic engineering efforts in *P. putida* KT2440 have established this strain as a robust biocatalyst for conversion of an array of such substrates.

Here, we utilize the previously discovered pathways (Koopman et al., 2010b) to engineer a genome-integrated strain of *P. putida* KT2440 to utilize furfural and HMF as sole carbon and energy sources. The resulting engineered strain exhibits enhanced growth rates and a reduced lag phase on biomass hydrolysate with representative concentrations of furfural and HMF; this demonstrates a general strategy of increased carbon conversion to biomass and target metabolites in emerging processes for aerobic conversion of biomass-derived substrates. Moreover, when combined with previously engineered strains of *P. putida* KT2440 that incorporate stable, genome-integrated expression of new catabolic genes (Linger et al., 2016), this work will ultimately enable more complete conversion of pyrolysis-derived substrates.

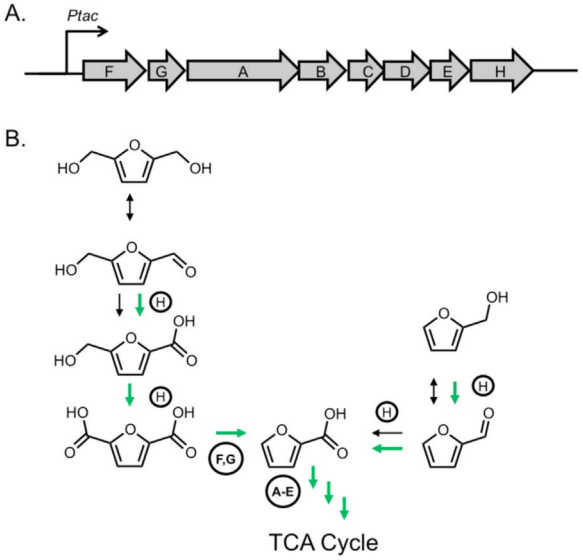

Fig. 1. Schematic representation of the (A) Ptac-driven *hmf* gene cluster from *B. phytofirmans* heterologously expressed in *P. putida* KT2440 and the (B) proposed furfural and HMF catabolic routes in wild-type (black arrows) and engineered (green arrows) strains of *P. putida* KT2440. Encircled letters indicate enzymatic components of the *B. phytofirmans hmf* gene cluster displayed in (A), as follows: H, furfural/HMF oxidoreductase; F, G, 2,5-furan-dicarboxylic acid decarboxylase; A, B, C, furoyl-CoA dehydrogenase; D, 2 furoyl-CoA synthetase; E, 2-oxoglutaryl-CoA hydrolase. Black arrows (wild-type) indicate putative non-specific dehydrogenase activity. Adapted from Koopman et al. (2010b). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2. Materials and methods

2.1. Plasmid and strain construction

Fragments of the *hmf*-encoding gene cluster (2180–2187) were amplified from *Burkholderia phytofirmans* PsJN (DSM #17436) genomic DNA using NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs) and assembled into a complete tac-driven operon using recursive Gibson assembly following manufacturer's instructions (New England Biolabs). The resultant operon was subcloned into a pk18mobsacB derivative (Marx, 2008), as described previously (Johnson and Beckham, 2015; Linger et al., 2016), with the addition of two 1-kb regions from the *P. putida* KT2440 genome targeting integration in the intergenic region between *fpvA* and PP_4218, which encodes a putative lipase/esterase family protein. The specific genome integration coordinates were 4766833–4767832 and 4767833–4768832, corresponding to 5’ and 3’ targeting regions, respectively; the resultant Ptac-hmf operon was ligated via restriction enzyme-mediated subcloning in between these 1 kb homology regions using SbfI and *Not*I restriction sites and transformed into NEB® 10-beta Competent *E. coli* (New England Biolabs) for plasmid assembly and amplification. Following sequence verification of the integration cassette, operon integration into the genome of *P. putida* KT2440 (ATCC 47054) was achieved by transforming the suicide integration vector into the target strain via electroporation and directly selecting for recombination of the plasmid into the genome on M9 minimal media plates containing 6.78 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 2 mM $MgSO_4$, 100 μM $CaCl_2$, 18 μM $FeSO_4$ and 15 g/L agar supplemented with 1 g/L furfural as the sole carbon source. Transformants were further screened for kanamycin resistance

on LB (Lennox) plates containing 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, and 15 g/L agar, supplemented with 50 μg/mL kanamycin. Marker removal was achieved via counter-selection for recombination of the plasmid out of the genome on YT+25% sucrose plates containing 10 g/L yeast extract, 20 g/L tryptone, 250 g/L sucrose, and 18 g/L agar, as described previously (Johnson and Beckham, 2015). Plasmid maps and primer sequences can be found in Fig. S1 and Table S1, respectively.

2.2. Media and growth conditions

2.2.1. Cultivation on aldehydes

Overnight cultures of *P. putida* KT2440 were generated on LB medium at 30°C with continuous shaking at 225 rpm. Overnight cultures were pelleted via centrifugation for 5 min at 4500 rpm and washed and resuspended in modified M9 medium, as described previously (Franden et al., 2009; Linger et al., 2014). Modified M9 medium was supplemented with 1g/L HMF (Sigma Aldrich, W501808), 1g/L furfural (Sigma Aldrich, 185914) or 1g/L each of HMF and furfural. The engineered strain was adapted to M9 medium supplemented with 1g/L HMF and 1g/L furfural via 10 rounds of serial dilution (1:10) and outgrowth (approximately 30 generations), prior to sample harvest and analysis (described below). All bacterial cultivation was conducted at 30°C, 25 mL volume in 125 mL shake-flasks with continuous shaking at 225 rpm, with initial inoculation at an optical density at 600 nm (OD_{600}) of 0.2, unless otherwise stated. All cultivation trials were performed in triplicate.

2.2.2. Cultivation on mock hydrolysate

Growth curves were generated using a Bioscreen C automated microbiology growth curve analysis system from Growth Curves USA, as described previously (Linger et al., 2016). Briefly, overnight cultures of *P. putida* cultivated in modified M9 medium supplemented with 1% glucose were pelleted, washed in modified M9 medium with no supplemental carbon, and diluted to OD_{600} =0.2 in a total volume of 300 μL of mock hydrolysate supplemented with increasing quantities of HMF and furfural. Mock hydrolysate contained 75 g/L glucose, 44 g/L xylose, 2.45 g/L galactose, 6.5 g/L arabinose, 0.14 g/L glycerol, and 0.82 g/L acetic acid diluted 1:1 in modified M9 minimal medium, reflective of previously generated hydrolysate compositions (Chen et al., 2014; Chen et al., 2012). Cultivation was conducted at 30°C with continuous shaking at maximum intensity and turbidity measurements ($OD_{420–580}$) were collected every 15 min for the duration of the experiments. All cultivations were performed in triplicate.

2.3. Generation of enzymatically hydrolyzed, pretreated corn stover and cultivation thereon

Hydrolyzed, saccharified corn stover was generated via dilute acid pretreatment, as described previously (Chen et al., 2014; Chen et al., 2012). The hydrolysate was concentrated before distribution by bench-scale rotary evaporation, and thus contained lower than normal concentration of aldehydes. A neutralized (pH =7.0), 50% hydrolysate stream was generated via 1:1 dilution of above hydrolysate with M9 minimal medium supplemented with 5 mM NH_4Cl, and a final concentration of 2g/L furfural and 1g/L HMF, approximating previously reported concentrations for lignocellulosic hydrolysates (Chen et al., 2014; Chen et al., 2012). Overnight cultures of *P. putida* KT2440 were generated as described above, and inoculated at OD_{600}=0.2 in the 50% hydrolysate solution. Cultivation proceeded as described above at 25 mL scale. 1 mL culture aliquots were harvested via centrifugation, resuspended and washed in minimal media, as described above, prior to measurement of OD_{600}.

2.4. Aldehyde conversion intermediate analysis

0.5 mL of bacterial culture supernatant was harvested from cultivation vessels along a time course and filter-sterilized using a 0.2-μm filtration unit. Concentrations of substrates and products were determined from filtered sample supernatants by high performance liquid chromatography (HPLC) on an Agilent1100 series system (Agilent USA, Santa Clara, CA) utilizing a Phenomenex Rezex RFQ-Fast Fruit H + column (Phenomenex, Torrance, CA) and cation H+ guard cartridge (Bio-Rad Laboratories, Hercules, CA) operating at 85°C. Dilute sulfuric acid (0.01 N) was used as the isocratic mobile phase at a flow rate of 1.0 mL/min. Refractive index and diode array detectors were used for compound detection. By-products were identified by co-elution at the same retention time with pure compounds as well as having matching spectral profiles as that of pure compounds. Initial aldehyde conversion rates were determined via linear regression analysis of aldehyde concentration following 8 h growth.

3. Results

3.1. Incorporation of heterologous furfural and HMF utilization pathways in P. putida KT2440

In light of the growing interest in developing *P. putida* KT2440 as a workhorse for biological conversion processes (reviewed in Nikel et al., 2016; Nikel et al., 2014), particularly those involving non-traditional, toxic feedstocks, we sought to engineer this organism to assimilate HMF and furfural. Prior reports have identified the presence of HMF and furfural assimilatory machinery in *P. putida* strains Fu1 and F2 (Koenig and Andreesen, 1990; Trudgill, 1969). However, to date, there have been no reports of *P. putida* KT2440 harboring such metabolic capacity. Further, efforts to cultivate *P. putida* KT2440 on HMF and furfural as sole carbon and energy sources were unsuccessful and the strain displayed substantial growth inhibition in the presence of these aldehydes (discussed further below). In an effort to confer HMF and furfural utilization capacity in *P. putida* KT2440, putative genes encoding HMF and furfural assimilatory enzymes were isolated from *B. phytofirmans* PsJN, a readily cultivatable, biosafety level 1 bacterium, via amplification from genomic DNA, and subcloned for expression behind the strong and constitutively active hybrid Ptac promoter (Fig. 1). *P. putida* KT2440 was transformed with the resultant Ptac-hmf construct and the selection marker was removed to yield a markerless and scarless genome-integrated strain heterologously expressing HMF and furfural conversion machinery.

Following ten rounds of serial adaptation in M9 medium supplemented with 1 g/L each of HMF and furfural, engineered *P. putida* strains were comparatively assessed against wild-type for cultivation capacity on these aldehydes as a sole carbon and energy source. Engineered *P. putida* demonstrated minimal lag and relatively robust cultivation capacity on furfural, HMF, and the mixture as sole carbon and energy sources (Fig. 2). Conversely, wild-type *P. putida* displayed little-to-no growth after 24 h, with negligible increase in culture optical density thereafter, possibly due to evaporative losses. Additionally, wild-type cells cultivated in liquid culture media yielded no colonies when outgrown on solid media containing hmf and/or furfural as sole carbon sources (data not shown).

3.2. Catabolic intermediate analyses

HMF and furfural catabolic and assimilatory routes have been proposed in a subset of microbes, proceeding through a common 2-furoic acid intermediate, as described above. To examine conversion intermediates generated via furfural and HMF catabolism in wild type and engineered *P. putida* KT2440 strains, time course metabolite analyses were conducted. Wild-type and engineered *P. putida* were cultivated in M9 medium supplemented with 1 g/L each of HMF and

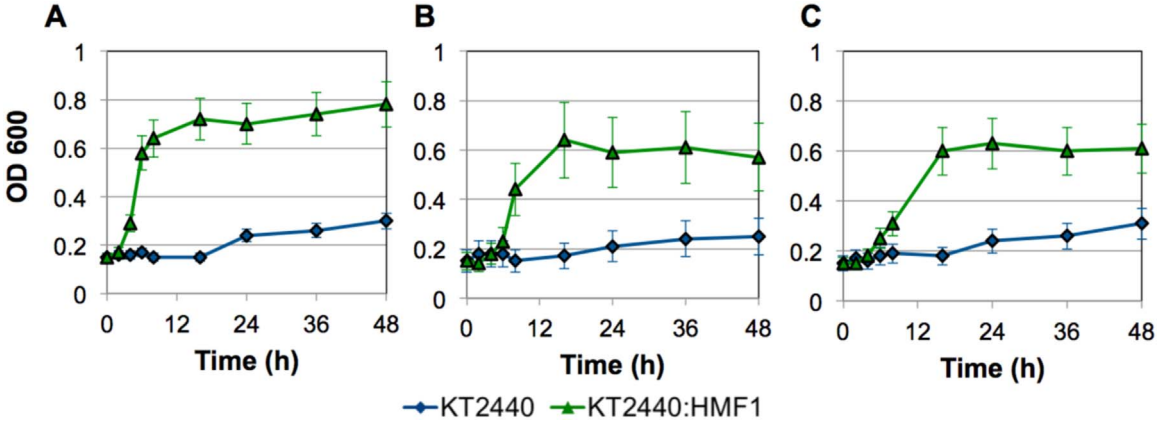

Fig. 2. Engineered *P. putida* KT2440 is capable of growth on aldehydes as a sole carbon and energy source. Growth analyses of wild-type (blue) and engineered (green) *P. putida* strains cultivated in M9 minimal medium supplemented with (A) 1 g/L HMF, (B) 1 g/L furfural, and (C) 1 g/L HMF +1 g/L furfural. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

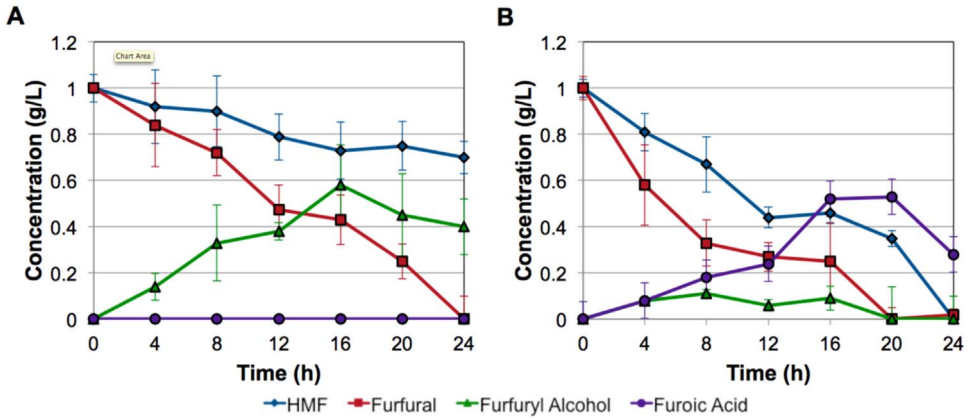

Fig. 3. Metabolite analyses implicate differential aldehyde catabolic routes in wild-type and engineered *P. putida* strains. Intermediate metabolites detected in culture supernatants for (**A**) wild-type and (**B**) engineered *P. putida* KT2440 cultivated in M9 medium supplemented with 1 g/L HMF and furfural as sole carbon and energy sources were examined via HPLC analyses.

furfural (corresponding to cultivation conditions seen in Fig. 2C).

Furfural conversion in wild-type *P. putida* cultures was linear, with 50% reduction in furfural observed in culture media following 16 h of growth. The decrease in furfural was accompanied by accumulation of furfuryl alcohol in culture supernatants (Fig. 3A). Steady furfuryl alcohol accumulation continued for approximately 18 h, after which detection steadily decreased, possibly due to evaporative losses. Furfural was undetected in culture supernatants following 24 h of cultivation. HMF conversion proceeded more slowly, with nearly 75% of initial HMF concentration still detected in culture media following 24 h of cultivation. No HMF alcohol was detected above concentrations of 5 mg/L in wild-type or engineered strains (data not shown). No 2-furoic acid was detected in wild type culture supernatant. These data suggest that furfural and HMF conversion primarily proceeds via non-specific dehydrogenase activity to corresponding alcohol intermediates in wild type (Fig. 1, (Koenig and Andreesen, 1990; Koopman et al., 2010b). The possibility that conversion proceeds through a non-secreted 2-furoic acid intermediate cannot be ruled out, though inability to grow on HMF and furfural suggest this is unlikely.

Engineered *P. putida* exhibited a much different conversion profile (Fig. 3B) compared to the wild type strain. Initial aldehyde conversion rates were faster in the engineered strain (0.041 g/h vs. 0.013 g/h and 0.084 g/h vs. 0.035 g/h for HMF and furfural, respectively), with complete conversion of furfural observed following 20 h of cultivation, and complete HMF conversion observed following 24 h cultivation. Unlike wild-type *P. putida*, little-to-no furfuryl alcohol accumulation was detected in the engineered strain culture supernatants. Following approximately 12 h of cultivation, HMF and furfural conversion was halted in the engineered strain, while 2-furoic acid steadily accumulated. After an additional 4 h, HMF, furfural, and 2-furoic acid concentrations decreased, suggesting a possible flux bottleneck leading to 2-furoic acid accumulation and secretion, preventing further conversion of HMF and furfural. Thereafter, 2-furoic acid concentrations continued to decrease, and a corresponding decrease in HMF and furfural was also observed. Combined, these data implicate different aldehyde conversion pathways in wild-type and engineered strains, with the latter proceeding through a 2-furoic acid conversion intermediate which is ultimately assimilated into the TCA cycle, conferring growth on HMF and furfural as sole carbon and energy sources, as seen in the native organism(s) harboring homologous *hmf* gene clusters. Additionally, 2-furoic acid is implicated as a flux bottleneck in engineered *P. putida* expressing the *hmf* gene cluster heterologously under the control of the constitutively strong Ptac promoter.

3.3. Examination of aldehyde toxicity in mock lignocellulosic hydrolysate

HMF and furfural toxicity limits the lignocellulosic cultivation and/or detoxification capacity of *P. putida* KT2440. To examine potential cultivation enhancements conferred via heterologous expression of the HMF gene cluster in *P. putida* KT2440, growth trials were conducted in the Bioscreen C using mock hydrolysate supplemented with increasing

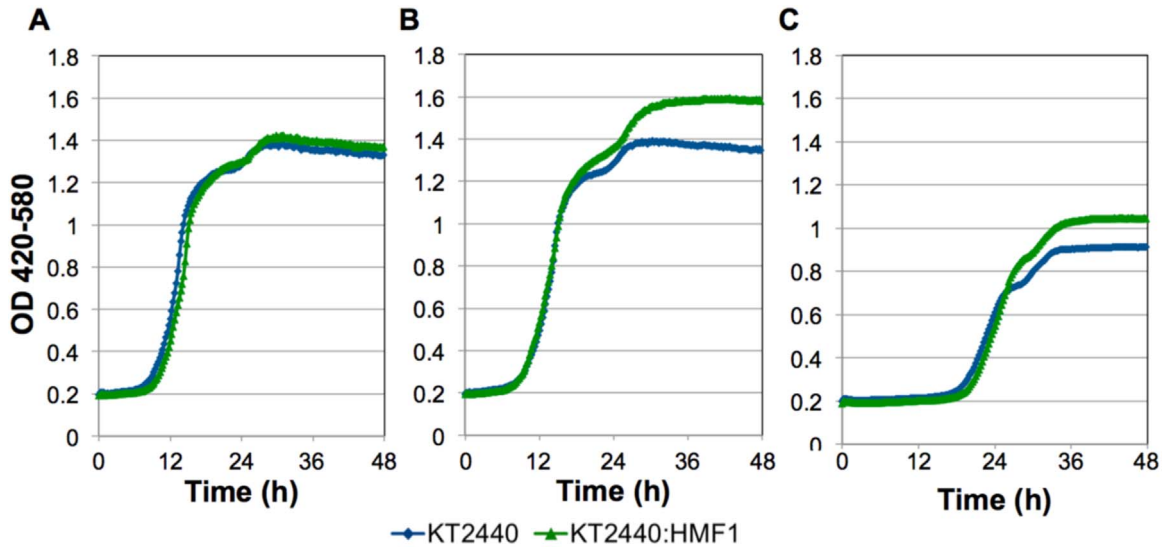

Fig. 4. Heterologous expression of the *hmf* cluster confers enhanced growth capacity and aldehyde tolerance in mock lignocellulosic hydrolysate. Growth of wild type (green) and engineered (blue) strains of *P. putida* KT2440 was monitored in the Bioscreen C using a wide-band filter (420–580 nm) in the presence of mock hydrolysates with increasing concentration of supplemented furans: (A) 0.1 g/L furfural, 0.05 g/L HMF, (B) 1 g/L furfural, 0.5 g/L HMF, (C) 2.0 g/L furfural, 1 g/L HMF. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

concentration of furfural and HMF. Minimal growth rate and final cell density differences were observed in cultures supplemented with these aldehydes in concentrations approximating 0.1X those observed in lignocellulosic hydrolysates, indicating negligible impact of aldehydes at dilute concentrations (Fig. 4A). Growth rate remained unaffected in strains supplemented with process-relevant concentrations of furfural and HMF (Fig. 4B); however, a difference in final cell density was observed. Cells heterologously expressing aldehyde utilization genes achieved 15–20% higher final non-linear cell densities compared to wild-type and cells cultivated on 0.1X aldehyde concentration (Fig. 4A, B), indicating additional carbon was assimilated into microbial biomass. This effect was also observed in cultures supplemented with aldehydes at higher concentration, approximating 2X process relevant concentrations. When the aldehyde concentration was 2-fold higher, an extended lag phase was observed in both wild-type and engineered strains, and the final cell density in these cultures was nearly 30% lower than that achieved in 1X aldehyde concentration (Fig. 4C), indicating persistent aldehyde toxicity and an inability to assimilate additional sugar- or aldehyde-derived carbon in the presence of higher furfural and HMF concentrations. All growth curves displayed a minor growth delay at approximately 24 h, possibly due to cell settling in Bioscreen C wells, though diauxic carbon utilization cannot be ruled out.

3.4. Cultivation on lignocellulosic hydrolysate

We next sought to examine the potential for furfural and HMF detoxification of and/or cultivation on corn stover-derived lignocellulosic hydrolysate. Wild type and engineered strains of *P. putida* KT2440 were cultivated in shake flasks in a 50% hydrolysate stream containing 2 g/L furfural and 1 g/L HMF. As shown in Fig. 5, the wild type strain displayed a lag (> 24 h), whereas the engineered strain entered logarithmic growth within 10–12 h and ultimately achieved nearly a 1.5-fold higher cell density compared to wild-type, following 60 h of cultivation. Notably, the former observation differed from cultivation in mock hydrolysate supplemented with similar concentration of furfural and HMF, in which an equivalent lag was observed between wild type and engineered strains. No HMF or furfural catabolic intermediates were detected in endpoint culture supernatant analyses (data not shown).

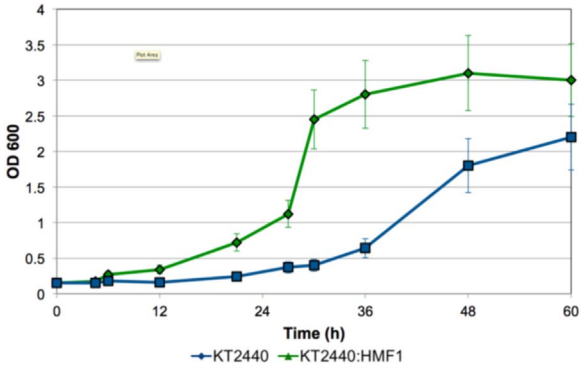

Fig. 5. Heterologous expression of the *hmf* catabolic gene cluster confers enhanced growth capacity on enzymatically hydrolyzed, pretreated corn stover. Wild type (blue) and engineered (green) strains of *P. putida* KT2440 were cultivated in 50% lignocellulosic hydrolysate supplemented with 5 mM NH_4Cl with 2 g/L furfural and 1 g/L HMF. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion and conclusions

Biological lignocellulose conversion strategies targeting the production of fuels and platform chemicals often encounter hurdles in the form of toxic and inhibitory sugar intermediates, notably including the sugar-derived aldehydes, furfural and HMF. Prior metabolic efforts targeting enhancement of aldehyde tolerance have been successful in *E. coli* (Jarboe et al., 2011a; Miller et al., 2010; Wang et al., 2013). Development of biocatalysts capable of conversion and utilization of such feedstock intermediates opens the door for enhanced biological upgrading strategies exhibiting higher carbon yields and improved process efficiency. Furthermore, such approaches enable more flexible biochemical and/or thermochemical feedstock pretreatment processes, wherein considerations associated with minimization of inhibitory intermediate formation might be mitigated. Additionally, such biocatalysts offer substrate detoxification potential in an array of hybrid bioprocess configurations.

Here, we have built upon prior studies to engineer a *P. putida* KT2440 biocatalyst with genome-integrated HMF and furfural conversion and assimilation capacity. The engineered microbe displays

growth on HMF and furfural as sole carbon and energy sources, as well as enhanced detoxification and growth on lignocellulosic hydrolysates. However, despite the enhanced cultivation capacity of the engineered strain, aldehyde conversion intermediate accumulation (namely, 2-furoic acid) and growth inhibition is still observed at higher furfural and HMF concentrations. Additional strain adaptation and metabolic engineering efforts offer a potential means to mitigate such growth defects (Koopman et al., 2010b).

These data underscore the potential for *P. putida* KT2440 as a biocatalyst for biochemical, thermochemical, and hybrid processes targeting conversion of biomass substrates. Indeed, in addition to biomass-derived sugars and sugar-derived aldehydes presented here, recent reports have identified the organism's capacity for conversion and utilization of an array of biomass-derived substrates, including lignin-derived aromatic compounds (Linger et al., 2014), levoglucosan (Linger et al., 2016), and phenol (Nurk et al., 1991; Vardon et al., 2015). Additionally, chromosomal integration of these HMF and furfural utilization genes affords the benefit of facile industrial deployment, with no antibiotic supplementation required to maintain the phenotype. Future efforts will continue to target incorporation of additional metabolic capabilities into this organism, ultimately targeting the generation of a biocatalyst with broad biomass conversion capacity.

Acknowledgements

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.meteno.2017.02.001.

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