A comparative multidimensional LC-MS proteomic analysis reveals mechanisms for furan aldehyde detoxification in *Thermoanaerobacter pseudethanolicus* 39E

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**Abstract**

**Background:** Chemical and physical pretreatment of lignocellulosic biomass improves substrate reactivity for increased microbial biofuel production, but also restricts growth via the release of furan aldehydes, such as furfural and 5-hydroxymethylfurfural (5-HMF). The physiological effects of these inhibitors on thermophilic, fermentative bacteria are important to understand; especially as cellulolytic strains are being developed for consolidated bioprocessing (CBP) of lignocellulosic feedstocks. Identifying mechanisms for detoxification of aldehydes in naturally resistant strains, such as *Thermoanaerobacter* spp., may also enable improvements in candidate CBP microorganisms.

**Results:** *Thermoanaerobacter pseudethanolicus* 39E, an anaerobic, saccharolytic thermophile, was found to grow readily in the presence of 30 mM furfural and 20 mM 5-HMF and reduce these aldehydes to their respective alcohols in situ. The proteomes of *T. pseudethanolicus* 39E grown in the presence or absence of 15 mM furfural were compared to identify upregulated enzymes potentially responsible for the observed reduction. A total of 225 proteins were differentially regulated in response to the 15 mM furfural treatment with 152 upregulated versus 73 downregulated. Only 87 proteins exhibited a twofold or greater change in abundance in either direction. Of these, 54 were upregulated in the presence of furfural and 33 were downregulated. Two oxidoreductases were upregulated at least twofold by furfural and were targeted for further investigation. Teth39_1597 encodes a predicted butanol dehydrogenase (BdhA) and Teth39_1598, a predicted aldo/keto reductase (AKR). Both genes were cloned from *T. pseudethanolicus* 39E, with the respective enzymes overexpressed in *E. coli* and specific activities determined against a variety of aldehydes. Overexpressed BdhA showed significant activity with all aldehydes tested, including furfural and 5-HMF, using NADPH as the cofactor. Cell extracts with AKR also showed activity with NADPH, but only with four-carbon butyraldehyde and isobutyraldehyde.

**Conclusions:** *T. pseudethanolicus* 39E displays intrinsic tolerance to the common pretreatment inhibitors furfural and 5-HMF. Multidimensional proteomic analysis was used as an effective tool to identify putative mechanisms for detoxification of furfural and 5-HMF. *T. pseudethanolicus* was found to upregulate an NADPH-dependent alcohol dehydrogenase 6.8-fold in response to furfural. In vitro enzyme assays confirmed the reduction of furfural and 5-HMF to their respective alcohols.

**Keywords:** Thermophiles, Lignocellulosic, Biofuels, Proteomics, Inhibitor, Pretreatment, Furfural, 5-hydroxymethylfurfural, Butanol dehydrogenase
Background
Thermophilic bacteria, such as *Clostridium thermocellum* and *Caldicellulosiruptor* species have gained interest for their possible use as biocatalysts for converting lignocellulosic biomass into renewable fuels and chemicals [1-4]. The potential advantages of thermal bioprocessing include improved kinetics, reduced viscosities of concentrated slurries, lower oxygen solubility, and reduced process cooling requirements [2]. In addition, several bacterial phyla include thermophiles that are able to utilize plant cell walls directly through the action of complex (hemi)cellulase systems expressed either as free enzymes, cellulosomes, or multifunctional enzymes [5]. Relying on these native enzymes in a bioprocessing scheme could substantially reduce or even eliminate the need for exogenous enzymes for cellulase solubilization with a resulting improvement in process economics [3,6,7].

To render plant material more reactive to microbial or enzymatic digestion, physical and chemical pretreatment methods are generally applied, and improvement in pretreatment technologies remains a highly active field of research [8,9]. Pretreatment with dilute acid at high temperatures has the benefit of solubilizing the hemicellulose fraction of biomass, which produces fermentable C5 oligomers and monomers [9]. However, one disadvantage of dilute acid pretreatment is that the process is non-specific and, depending on its severity, generates a number of toxic by-products [10]. Inhibitory compounds generated by dilute acid pretreatment typically fall into four categories: organic acids (acetic acid, ferulic acid), ketones (acetovanillone), phenolics (coniferyl alcohol, catechol), and aldehydes (furfural, hydroxymethylfurfural, vanillin). Mixtures of inhibitors, especially those including the furan aldehyde furfural and 5-hydroxymethylfurfural (5-HMF). The addition of 10 mM 5-HMF was between 20 and 30 mM after both 12 and 30 to 40 mM after 24 h. The IC50 for 5-HMF was between 20 and 30 mM after both 12 and 24 h. As shown in Table 1, the determined values are comparable to or slightly higher than those of other thermophilic bacteria, while they are higher than reported values for *E. coli, S. cerevisiae*, and *Zymomonas mobilis*. Though direct comparisons are difficult due to differences in the growth conditions used in the various studies, these results suggest that *T. pseudethanolicus* 39E has a comparable if not higher tolerance to the furan aldehydes furfural and 5-HMF than other studied organisms.

**Furan aldehyde reduction and glucose fermentation**

In order to establish the mechanism of increased furan aldehyde tolerance, *T. pseudethanolicus* 39E was grown in the presence and absence of 15 mM furfural or 5-HMF and the furan aldehyde and respective furan...
alcohol concentrations were measured. As shown in Figure 2, concomitant with growth, furfural and 5-HMF concentrations decreased while 2,5-furandimethanol concentration increased, indicating that \textit{T. pseudethanolicus} 39E reduced 5-HMF to 2,5-furandimethanol. Furfural was most likely also reduced to furfuryl alcohol; however, quantitation of this compound is complicated by its polymerization at the growth temperature of \textit{T. pseudethanolicus} 39E.

The addition of furan aldehydes affected end-product formation by \textit{T. pseudethanolicus} 39E, as determined by HPLC analysis (Figure 3). At 10 mM concentrations, more acetate and lactate are produced, while ethanol production remains constant. At furan aldehyde concentrations above 10 mM where growth is observed (15, 20, 30 mM furfural and 15, 20 mM 5-HMF), ethanol decrease and acetate increase are directly proportional, suggesting that acetyl coenzyme A is converted to acetate through phosphotransacetylase (PTA) and acetate kinase (AK) rather than serving as an electron acceptor for ethanol production via NAD(P)H-dependent bifunctional alcohol dehydrogenase (ADH) activity. \textit{T. pseudethanolicus} 39E possesses seven ADHs, but ethanol is primarily produced from NADPH-dependent AdhB \cite{34-36}. The oxidative branch of the pentose phosphate pathway is also present in 39E, which could supply NADPH \cite{34}. Reduction of furfural/5-HMF to their corresponding alcohols during growth competes with ethanol production for electrons delivered by NADPH. This is also suggested by stoichiometric shifts in end products, where added aldehydes resulted in about a 0.5 times decrease in corresponding molar ethanol concentrations (that is, the 30 mM furfural addition resulted in a decrease of 15 mM ethanol versus the control). This shift in ethanol/acetate concentrations is consistent with an electron balance.

| Organism | Furfural | 5-HMF | YE (% w/v) | Carbon (% w/v) | Time (h) | Temp (°C) | pH | Ref. |
|----------|-----------|-------|------------|----------------|----------|-----------|----|------|
| \textit{T. pseudethanolicus} 39E | 3 | 2 - 3 | 0.1 | 0.7 | 12 | 65 | 7.0 | T5 |
| \textit{T. pseudethanolicus} 39E | 3 - 4 | 2 - 3 | 0.1 | 0.7 | 24 | 65 | 7.0 | T5 |
| \textit{Bacillus coagulans} MXL-9 | 2.5 - 5 | 5 | 0.5$^a$ | 5-10 | 24 | 50 | 7.0 | \cite{29} |
| \textit{C. saccharolyticus} | 1 - 2 | 1 - 2 | 0.1 | 1 | 16, 40 | 72 | 7.0 | \cite{30} |
| \textit{Thermoanaerobacterium} strain AK17$^1$ | 2 | 3 | 0.2 | 0.4 | 120 | 60 | 6.0 | \cite{11} |
| \textit{Tm. thermosaccharolyticum} | 1.25 | 1 | 0.2 | 1 | 40 | 60 | 7.0 | \cite{31} |
| \textit{Thermotoga neapolitana} | 2 - 4 | 2 - 4 | 0.1 | 1 | 16, 40 | 80 | 7.0 | \cite{30} |
| \textit{E. coli} LY180 | 1 - 1.5 | nd | none | 5 | 48 | 37 | 6.5 | \cite{23} |
| \textit{E. coli} LY180 | < 0.4 | nd | none | 5 (xyl) | 48 | 37 | 6.5 | \cite{23} |
| \textit{E. coli} LY180 | 1.5 | nd | 0.1 | 5 (xyl) | 48 | 37 | 6.5 | \cite{23} |
| \textit{S. cerevisiae} CBS 1200 | 0.5 | < 1 | 0.3 | 2 | 24 | 26 | 5.8 | \cite{32} |
| \textit{S. cerevisiae} NSI 113 | 2 | nd | 0.3 | 1 | 48 | 30 | 5.3 | \cite{33} |
| \textit{Z. mobilis} ATCC 10988 | 2 | 5 | 0.3 | 2 | 24 | 30 | 5.6 | \cite{32} |

$^a$Concentration at which 50% inhibition of growth occurred with furfural and 5-hydroxymethylfurfural (5-HMF). Values determined in this study are highlighted in boldface. $^\ddagger$Medium also included 1% tryptone; $^*$measured as 50% inhibition of ethanol production; YE, yeast extract; nd, not determined; xyl, xylose; T5, This study.
of one NAD(P)H per furan aldehyde and two NAD(P)H per ethanol. The reason for increased lactate with added furan aldehyde, especially 5-HMF, is less clear. Further redox imbalances from the presence of furfural or 5-HMF may direct more NADH generated from glycolysis to be oxidized via lactate dehydrogenase (LdhA). Furfural addition to a growing culture of the related thermophilic bacterium *C. thermocellum* also resulted in increased lactate production and cessation of ethanol production [37], although the reason for these changes is unknown. The mesophilic ethanologen *S. cerevisiae* has also been shown to remove furan aldehydes by reduction to their respective alcohols at the expense of ethanol production [38]. This has been shown to involve an upregulation of central carbon metabolism, especially the NADPH-generating pentose phosphate pathway [39], and downregulation of enzymes involved in ethanol formation, thereby increasing the availability of reducing equivalents for aldehyde detoxification [40].

**Cell-wide proteomic response to furfural**

In order to identify potential enzymes involved in reducing furfural and 5-HMF and also to examine cell-wide physiological effects of furan aldehyde exposure, proteomic analysis was performed comparing *T. pseudethanolicus* grown with and without furfural. A concentration of 15 mM furfural was used to challenge the cells since this was the maximum amount that displayed a stimulatory effect during the growth experiments (Figure 1A). Triplicate
cultures were grown in parallel either in the presence or absence (control) of furfural and harvested at 8 h after inoculation, which corresponded to the time interval necessary for complete reduction of furfural to furfuryl alcohol (Figure 2A). Peptide samples were prepared and analyzed as described in the Methods section. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium [41] via the PRIDE partner repository with the dataset identifier PXD001446. The complete proteomics dataset and statistical analysis are also provided in Additional file 1: Table S1, which includes an in-table, color-coded heat map corresponding to Figure 4. In total, 1,294 proteins were identified across both conditions with roughly 300,000 spectra (SpC) assigned to constituent peptides. Of the 1,294 proteins, only 918 passed the 99% SpC cutoff and were moved to the ANOVA analysis. Using a P-value cutoff of 0.05, 225 proteins were found to be differentially expressed with 152 upregulated in furfural treated cells versus 73 downregulated. Culling this list even further, only 87 proteins exhibited a twofold or greater change in abundance in either direction. Of these, 54 were upregulated in the presence of furfural (Table 2) and 33 were downregulated (Table 3). Significantly regulated proteins were grouped into 11 clusters based on abundance pattern across all replicates (Figure 4). Many cellular functions were affected by furfural, with the most highly downregulated proteins involved in cell wall biosynthesis or sporulation. Hydrogenase-related proteins were also downregulated, along with several redox proteins predicted to use NAD(P)H. Upregulated proteins fell into 12 general cellular functions, with those regulated fivefold or higher falling into three categories: polar amino acid biosynthesis (arginine, cysteine), nucleotide metabolism, and redox proteins.

**Energy production and carbohydrate metabolism**

Comparatively, the proteomic response of *T. pseudethanolicus* 39E to furfural showed similarities at the functional level to the responses of *C. thermocellum*, as well as *S. cerevisiae* and *E. coli*. While central carbon metabolism did not appear to be significantly impacted by furfural in *T. pseudethanolicus* 39E, upregulated carbohydrate-related proteins included beta-galactosidase, mannose-6-phosphate isomerase, and kojibiose phosphorylase, while dihydroyacetone kinase and two fructose-specific, phosphoenolpyruvate-dependent sugar phosphotransferase system transport proteins were downregulated. Genes involved in energy production and conversion, as well as carbohydrate transport and metabolism, were also regulated in *C. thermocellum* ATCC 27405 [37]. Acetate kinase and phosphoacetyltransferase were both downregulated, though this is likely a general stress response rather than furfural-specific. As in *T. pseudethanolicus* 39E, beta-galactosidase was upregulated in *C. thermocellum* ATCC 27405 upon furfural exposure, as were several glycosyl transferase family proteins, though the reason for this regulation is unclear. On the other hand, central carbon metabolism is significantly upregulated in both the *S. cerevisiae* and *E. coli* response to furfural. In anaerobic *S. cerevisiae* fermentations, an 8 g/L furfural treatment repressed the synthesis of enzymes involved in glucose catabolism and the tricarboxylic acid (TCA) cycle. Conversely, addition of 17 g/L furfural to an aerobic *S. cerevisiae* culture increased expression of proteins involved in glycolysis and the TCA cycle, while repressing expression of proteins involved in glycerol and ethanol production [42]. Analysis of a single-gene disruption library of *S. cerevisiae* BY4741 against growth with furfural identified several genes in the pentose phosphate pathway as important in furfural tolerance [39], especially *ZWFI*, whose overexpression allowed for growth with 50 mM furfural. In an ethanologenic strain of *E. coli* (LY180), a moderate furfural challenge (0.5 g/L) perturbed the expression of about 400 genes at least twofold, 15 min after exposure, with central carbon and energy metabolism being among the pathways regulated [22]. It is interesting to note that central carbon metabolism is significantly regulated in the mesophilic *S. cerevisiae* and *E. coli* and is much less affected in the thermophilic *C. thermocellum* and *T. pseudethanolicus*, though the reason for this difference remains unclear.

**Stress response**

With 15 mM furfural, *T. pseudethanolicus* 39E did not display a typical stress phenotype indicated by a reduced growth rate. Nevertheless, some functions associated with stress were differentially regulated. The expression of eight predicted transporters was affected by furfural, including upregulation of three metal transporters and one efflux transporter. A variety of nucleotide-related genes were also upregulated, including two de novo purine biosynthesis genes (Teth39_1713 and Teth39_1803) and two genes involved in nucleoside degradation (Teth39_1828 and Teth39_1832). Additionally, Teth39_1216, predicted to be involved in isoprenoid biosynthesis, and Teth39_0175, predicted to function in cell wall turnover, are downregulated in response to furfural. Similarly, a number of stress
responses are upregulated by furfural in *C. thermocellum* ATCC 27405, including many genes that are homologous to class I and class IV heat shock response genes in *Bacillus subtilis*, though these genes were also upregulated by heat [37] and ethanol treatment [43]. A number of

| Locus         | Fold change | Description                               | P-value |
|---------------|-------------|-------------------------------------------|---------|
| Amino acid biosynthesis |            |                                           |         |
| Teth39_0141  | 2.22        | Threonine synthase                        | 0.015   |
| Teth39_0223  | 4.31        | N-acetyl-gamma-glutamyl-phosphate reductase| 0.001   |
| Teth39_0224  | 6.61        | Arginine biosynthesis bifunctional protein ArgJ | 0.000   |
| Teth39_0225  | 2.50        | Acetylglutamate kinase                     | 0.010   |
| Teth39_0226  | 2.65        | Aminocitrate synthase and succinylamino transferase | 0.038   |
| Teth39_0227  | 7.69        | Carbamoyl phosphate synthase, small subunit | 0.010   |
| Teth39_0228  | 12.73       | Carbamoyl phosphate synthase, large subunit | 0.008   |
| Teth39_0229  | 5.74        | Argininosuccinate synthase                 | 0.000   |
| Teth39_0279  | 3.50        | Cysteine synthase A                        | 0.001   |
| Teth39_0280  | 6.68        | Cysteine desulfurase                       | 0.001   |
| Teth39_0281  | 9.01        | tRNA methyltransferase                     | 0.014   |
| Teth39_0559  | 2.56        | Diaminopimelate decarboxylase              | 0.001   |
| Teth39_0983  | 3.14        | Prephenate dehydratase                     | 0.002   |
| Teth39_1666  | 2.17        | Glutamine synthetase, catalytic region     | 0.003   |
| Teth39_1810  | 3.56        | Aspartate 1-decarboxylase                  | 0.033   |
| Carbohydrate metabolism | |                                      |         |
| Teth39_0611  | 2.28        | Beta-galactosidase                         | 0.011   |
| Teth39_0744  | 2.26        | Mannose-6-phosphate isomerase, class I     | 0.034   |
| Teth39_1512  | 3.34        | Kojibiose phospholysase                   | 0.043   |
| Cell division/sporulation/motility | |                                      |         |
| Teth39_1000  | 2.05        | SpolID/LytB domain                         | 0.009   |
| Teth39_1257  | 4.17        | Flagellar M-ring protein FLIF              | 0.043   |
| Teth39_1280  | 2.08        | Chromosome segregation protein SMC         | 0.008   |
| Chaperones    |             |                                           |         |
| Teth39_0115  | 2.15        | Chaperonin Cpn10                          | 0.013   |
| Teth39_1392  | 2.21        | Chaperone protein DnaJ                    | 0.005   |
| Energy related |            |                                           |         |
| Teth39_1820  | 2.04        | Homocitrate synthase                       | 0.003   |
| Teth39_2064  | 6.75        | 2-hydroxyacid dehydrogenase, NAD-binding   | 0.019   |
| Hypothetical proteins | |                                      |         |
| Teth39_0463  | 2.28        | Hypothetical protein                       | 0.012   |
| Teth39_0919  | 2.78        | Hypothetical protein                       | 0.023   |
| Iron-sulfur cluster metabolism | |                                      |         |
| Teth39_0116  | 2.14        | FeS assembly ATPase SuF/C                  | 0.020   |
| Teth39_0117  | 2.65        | FeS assembly protein SuF                   | 0.001   |
| Teth39_0118  | 2.97        | SuFBD protein                              | 0.000   |

Table 2 Proteins upregulated twofold or more by 15 mM furfural (P ≤0.05) (Continued)

| Locus         | Fold change | Description                                      | P-value |
|---------------|-------------|--------------------------------------------------|---------|
| Teth39_0120  | 2.66        | SUF system FeS assembly protein, NifU family      | 0.001   |
| Nucleotide related |            |                                                  |         |
| Teth39_0775  | 2.04        | MutS2 family protein                             | 0.005   |
| Teth39_1049  | 2.07        | tRNA methyltransferase                           | 0.018   |
| Teth39_1323  | 2.46        | DNA-directed RNA polymerase, omega subunit       | 0.005   |
| Teth39_1713  | 2.12        | Phosphoribosylformylglycinamidine synthase II     | 0.006   |
| Teth39_1803  | 36.39       | Phosphoribosylaminoimidazolylcarboxylase, ATPase subunit | 0.000   |
| Redox proteins |            |                                                  |         |
| Teth39_0646  | 3.61        | FAD-dependent pyridine nucleotide disulfide oxidoreductase | 0.005   |
| Teth39_0720  | 3.77        | 4Fe-4S ferredoxin, iron-sulfur binding domain protein | 0.044   |
| Teth39_1597  | 6.84        | Iron-containing alcohol dehydrogenase            | 0.000   |
| Teth39_1598  | 6.32        | Aldo/keto reductase                              | 0.000   |
| Ribosomal proteins |        |                                                  |         |
| Teth39_0365  | 2.00        | Ribosomal protein L7/L12                        | 0.020   |
| Teth39_1753  | 2.01        | RNA binding S1 domain protein                    | 0.001   |
| Teth39_2275  | 2.33        | Ribosomal protein S6                            | 0.006   |
| Transports   |             |                                                  |         |
| Teth39_0231  | 2.85        | Calcium translocating P-type ATPase, PMCA-type   | 0.001   |
| Teth39_0278  | 2.82        | Heavy metal translocating P-type ATPase          | 0.036   |
| Teth39_0282  | 4.65        | Copper translocating P-type ATPase              | 0.004   |
| Teth39_1033  | 2.26        | Efflux transporter, RND family, MFP subunit      | 0.015   |
| Teth39_1765  | 4.62        | Extracellular solute-binding protein, family 3   | 0.001   |
| Teth39_2232  | 2.43        | Type IV secretory pathway VirB4 components-like protein | 0.011   |
| Vitamin related |            |                                                  |         |
| Teth39_0307  | 3.10        | Biotin/lipoil attachment domain-containing protein | 0.007   |
| Teth39_1205  | 2.03        | Riboflavin biosynthesis protein RibF             | 0.001   |
| Teth39_1559  | 2.00        | SNO glutamine amidotransferase                   | 0.001   |

Sorted by general cellular function. Proteins in boldface are regulated greater than fivefold. Descriptions are from the National Center for Biotechnology Information.
uncharacterized transporters were also regulated, as well as genes involved in transcription, RNA processing and modification, chromatin structure and dynamics, and DNA replication, recombination, and repair. In *S. cerevisiae*, stress responses upregulated by furfural include osmotic and salt stress, DNA damage, and pH stress [40]. *S. cerevisiae* also responds to furan aldehydes by regulating cell adaptation and survival processes, especially with respect to drug resistance, transport, and cell membrane composition [38]. In *E. coli* LY180, transport functions, as well as cell structure, DNA, and lipid synthesis functions are also regulated by furfural [22]. A general stress response to furfural thus appears to include upregulation of transport functions and nucleotide metabolism.

### Amino acid metabolism

In *T. pseudethanolicus* 39E grown with 15 mM furfural, both cysteine and arginine biosynthetic genes were upregulated (cysteine synthase A, Teth39_0279; cysteine desulfurase, Teth39_0280; arginine biosynthesis, Teth39_0223-0229). Amino acid metabolism is also affected by furfural stress in *C. thermocellum*, *S. cerevisiae*, and *E. coli*. In *C. thermocellum* ATCC 27405, arginine biosynthetic genes are upregulated upon furfural addition [37]. While sulfur amino acid biosynthesis is not directly regulated, genes involved in sulfate transport and sulfur assimilation are upregulated by furfural. In *S. cerevisiae*, proteins involved in sulfur amino acid biosynthesis are downregulated upon exposure to 8 g/L furfural under anaerobic conditions [40]. In *E. coli* LY180, 0.5 g/L furfural also repressed genes involved in arginine biosynthesis, but induced expression of sulfur-containing amino acid biosynthetic genes [22]. This is due to a decrease in NADPH availability, which is instead used by the aldehyde reductases YqhD and DkgA.

### Table 3 Proteins downregulated twofold or more by 15 mM furfural (*P* ≤ 0.05)

| Locus      | Fold change | Description                                      | P-value |
|------------|-------------|--------------------------------------------------|---------|
| Amino acid metabolism |             |                                                  |         |
| Teth39_0216 | -3.10       | Glutamate synthase, homotetrameric                | 0.000   |
| Teth39_0217 | -2.47       | Oxidoreductase FAD/NAD(P)-binding domain          | 0.004   |
| Teth39_0487 | -2.15       | Alanine racemase                                  | 0.038   |
| Teth39_1661 | -3.77       | Glutamine amidotransferase, class II              | 0.016   |
| Teth39_2007 | -2.58       | Aromatic amino acid beta-eliminating lyase        | 0.004   |
| Cell division/sporulation/motility |             |                                                  |         |
| Teth39_0175 | -52.70      | Peptidoglycan-binding LysM                        | 0.000   |
| Teth39_0252 | -30.04      | YabP family protein                              | 0.000   |
| Teth39_1446 | -2.42       | Cell division topological specificity factor MinE | 0.016   |
| Teth39_1772 | -2.33       | Flagellar protein FlaG protein                    | 0.011   |
| Teth39_1783 | -2.51       | Flagellar hook-associated protein 3              | 0.009   |
| Energy related |            |                                                  |         |
| Teth39_0466 | -2.27       | Thiamine pyrophosphate enzyme domain              | 0.038   |
| Hydrogenase related |         |                                                  |         |
| Teth39_0221 | -2.17       | Hydrogenase with PAS/PAC sensor                   | 0.005   |
| Teth39_1459 | -24.50      | Histidine kinase                                  | 0.000   |
| Hypothetical proteins |         |                                                  |         |
| Teth39_0794 | -5.93       | Hypothetical protein                             | 0.041   |
| Teth39_0842 | -36.68      | Hypothetical protein                             | 0.000   |
| Nucleotide related |         |                                                  |         |
| Teth39_1137 | -2.51       | Metal-dependent phosphohydrolase                  | 0.002   |
| Teth39_2157 | -9.29       | SirA family protein                               | 0.000   |
| Redox proteins |            |                                                  |         |
| Teth39_0445 | -2.19       | Thioredoxin reductase                             | 0.002   |
| Teth39_1916 | -2.73       | Oxidoreductase FAD/NAD(P)-binding domain          | 0.001   |
| Teth39_1917 | -4.88       | 4Fe-4S ferredoxin, iron-sulfur binding domain     | 0.000   |
| Teth39_2155 | -4.30       | FAD-dependent pyridine nucleotide-disulfide oxidoreductase | 0.000 |
| Transcriptional regulator |         |                                                  |         |
| Teth39_0150 | -2.56       | Transcriptional regulator, DeoR family            | 0.011   |
| Teth39_0757 | -3.07       | Putative cold-shock DNA-binding domain protein    | 0.029   |
| Teth39_1109 | -2.27       | Sporulation transcriptional activator Spo0A      | 0.006   |
| Teth39_1292 | -2.06       | Hypothetical protein                             | 0.019   |
| Teth39_1796 | -2.17       | Two-component transcriptional regulator, winged helix family | 0.015 |

| Locus      | Fold change | Description                                      | P-value |
|------------|-------------|--------------------------------------------------|---------|
| Transporters |             |                                                  |         |
| Teth39_0333 | -2.98       | PTS system, fructose subfamily, IIC subunit       | 0.001   |
| Teth39_0334 | -2.90       | PTS system, fructose-specific, III subunit        | 0.019   |
| Vitamin related |         |                                                  |         |
| Teth39_0787 | -4.71       | Lipoic acid synthetase                            | 0.033   |
| Teth39_0542 | -2.43       | Dihydroxyacetone kinase, DhaK subunit            | 0.006   |
| Teth39_1065 | -2.21       | HAD superfamily (subfamily IIIA) phosphatase, TIGR01668 | 0.024 |
| Teth39_1216 | -2.04       | 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase | 0.011 |

Sorted by general cellular function. Proteins in boldface are regulated greater than fivefold. Descriptions are from the National Center for Biotechnology Information.

Table 3 Proteins downregulated twofold or more by 15 mM furfural (*P* ≤ 0.05) (Continued)

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|------------|-------------|-----------------------------------|---------|
| Transporters |             |                                   |         |
| Teth39_0333 | -2.98       | PTS system, fructose subfamily, IIC subunit |       |
| Teth39_0334 | -2.90       | PTS system, fructose-specific, III subunit |   |
| Vitamin related |         |                                   |         |
| Teth39_0787 | -4.71       | Lipoic acid synthetase             | 0.033   |
| Teth39_0542 | -2.43       | Dihydroxyacetone kinase, DhaK subunit | 0.006   |
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for furfural reduction [22]. Upregulation of cysteine biosynthetic pathways in *T. pseudethanolicus* 39E suggests that, as in *E. coli*, increased expression of NAD(P)H-dependent aldehyde reductases (described below) may decrease NADPH availability for sulfur amino acid biosynthesis.

**Redox metabolism**

Six alcohol dehydrogenases (ADHs) identified in *T. pseudethanolicus* 39E were differentially regulated. The three functionally characterized ADHs [36,44], AdhA (Teth39_0220), AdhB (Teth39_0218), and AdhE (Teth39_0206), were all downregulated (*P* < 0.05); however, none more than twofold (AdhA 1.27-fold, AdhB 1.81-fold, and AdhE 1.59-fold). Of the other three identified alcohol dehydrogenases in *T. pseudethanolicus* 39E (Teth39_0878, Teth39_1597, Teth39_1979), only Teth39_1597 was significantly upregulated (6.8-fold; *P* < 0.001). Another oxidoreductase upregulated by *T. pseudethanolicus* 39E in response to furfural is Teth39_1598 (6.3-fold; *P* < 0.001). These genes potentially encode enzymes involved in reducing furfural and 5-HMF and will be discussed in more detail below. In *C. thermocellum* ATCC 27405, the bifunctional alcohol/aldehyde dehydrogenase Cthe_0423 and the redox regulator Rex (Cthe_0422) were downregulated upon furfural addition [37]. No other alcohol dehydrogenase or aldehyde reductase was differentially regulated; however, a putative carbon monoxide dehydrogenase (Cthe_0281) was upregulated by furfural and may play a role in redox balance in *C. thermocellum* ATCC 27405 [37]. In *S. cerevisiae*, NAD(P)H-dependent aldehyde reductases or alcohol dehydrogenases (ADH) have also been shown to affect furan aldehyde tolerance [45] and be regulated by furan aldehydes [40]. Transcriptomic expression analysis of known reductase and dehydrogenase genes showed that *ADH2* was highly expressed in hydrolysate-tolerant *S. cerevisiae* strain TMB3000 compared to the wild-type CBS8066 and was also induced by 5-HMF [45]. In a proteomic analysis of the response of *S. cerevisiae* to 17 g/L furfural, six ADHs showed differential regulation, with Adh1p, Adh5p, and Adh6p upregulated, Adh2p and Sfa1p downregulated, and Adh4p unregulated by furfural [42]. Adh6p and Adh7p have furfural and 5-HMF reductase activity, with the former using both NADH and NADPH and the latter only NADH [46]. In *E. coli* LY180, energy functional groups are also highly regulated [22]. As in *S. cerevisiae*, NADPH-dependent aldehyde reductases with furfural reducing capacity are upregulated, namely *yqhD* and *dkgA*.

**Enzyme cloning and activity measurements**

Teth39_1597 [GenBank GeneID:5874751] shares 36% identity/54% similarity with *yqhD* from *E. coli* LY180 [22]. Teth39_1597 belongs to the Fe-dependent alcohol dehydrogenase superfamily (pfam00465) with predicted butanol dehydrogenase activity (BDH, cd08178). The gene product appears to be a close homolog of Bdha in *Thermoanaerobacter mathrani* (88% identity/94% similarity), which has been experimentally verified to have BDH activity [47]. Thus, Teth39_1597 is considered to be a butanol dehydrogenase and will be referred to as Teth39 bdha. Teth39_1598 [GenBank GeneID:5874752] has 27% identity/41% similarity to *E. coli* DkgA, another enzyme shown to have NADPH-dependent furfural reductase activity [22]. Teth39_1598 is a predicted aldo/keto oxidoreductase and will be referred to as Teth39 akr.

Since both Teth39 bdha and Teth39 akr were significantly upregulated in response to furfural and are homologs to similarly upregulated *E. coli* genes *yqhD* and *dkgA*, further biochemical characterization was performed to determine their cofactor and substrate specificities. The coding regions for Teth39 bdha and Teth39 akr were PCR amplified from *T. pseudethanolicus* 39E genomic DNA and cloned into pET-30a behind a T7-lac promoter and N-terminal 6xHis- and S-tags. Overexpression plasmids, as well as the pET-30a plasmid alone, were transformed into *E. coli* BL21 (DE3) and Teth39 Bdha and Teth39 AKR were overexpressed (Figure 5). Whole cell lysates were prepared and assayed for aldehyde reductase activity aerobically at 60°C with acetaldehyde, furfural, and 5-HMF. Furfural was also assayed under anaerobic conditions. Teth39 Bdha and Teth39 AKR activities were compared to the vector-only control (Table 4). Neither enzyme showed any activity with NADH as cofactor. Teth39 AKR had minimal activity with both furan aldehydes using NADPH as the cofactor, but not above the vector control. In contrast, Teth39 Bdha showed activity above the vector control using NADPH as the cofactor with both furan aldehydes. The specific activity was 4.97 ± 0.17 U with furfural and 10.06 ± 0.80 U with 5-HMF.

Based on these results, overexpression of Teth39 bdha may increase microbial tolerance to the furan aldehydes furfural and 5-HMF produced during biomass pretreatment, especially in a thermophilic, cellulolytic microbe, such as *Caldicellulosiruptor* sp. or *Clostridium thermocellum*. This approach has proven successful in *S. cerevisiae*, where overexpression of *ADH6* or *ADH7* allowed growth with 40 mM 5-HMF, where none was seen in a control [46]. However, the increased expression of Teth39 bdha in *T. pseudethanolicus* 39E may have influenced other aspects of its furfural proteomic response. For example, Teth39 Bdha is predicted to contain iron-sulfur clusters, and iron-sulfur cluster biosynthetic genes (Teth39_0116-0120) are also upregulated by furfural. Additionally, other oxidoreductases predicted to use NADPH as a cofactor are...
downregulated (Teth39_0216, Teth39_0217, Teth39_1916), perhaps to increase NADPH availability for furfural reduction. Thus, while overexpression of Teth39 BdhA alone may increase furan aldehyde tolerance, coexpression of other genes may be required to achieve the phenotype overall.

Teth39 BdhA and Teth39 AKR also exhibited butyraldehyde and isobutyraldehyde reduction activity (Table 4), presumably forming butanol and isobutanol, with the Teth39 BdhA activity 2.7-fold higher than that of Teth39 AKR with isobutyraldehyde and 44.2-fold higher with butyraldehyde. Expression of Teth39 bdhA might therefore alternatively be used to biologically produce these higher energy fuel compounds in thermophilic microbes.

**Conclusions**

Inhibitors, such as furfural and 5-HMF, are generated from common pretreatment methods used for improving the reactivity of lignocellulosic biomass toward enzymatic solubilization. The physiological response and tolerance to these inhibitors must be understood in order to develop improved microorganisms such as *C. thermocellum* or a *Caldicellulosiruptor* sp. for thermophilic consolidated bioprocessing of biomass. We measured rapid growth in the presence of 10 to 15 mM furan aldehydes and their detoxification in situ by a thermophilic anaerobe, *T. pseudethanolicus* 39E. Physiological effects resulting from higher furfural and 5-HMF concentrations included decreased ethanol yield with increases in acetate and lactate production from glucose. A bottom-up proteomics approach was applied to screen for potential enzymes or pathways directly involved in furan aldehyde detoxification. While a number of cellular functions were impacted, including a decrease in expression of ADHs involved in ethanol production, an ADH encoded by Teth39_1597 was upregulated nearly sevenfold in the presence of 15 mM furfural. The enzyme, a putative butanol dehydrogenase, was cloned and overexpressed in *E. coli* and displayed NADPH-dependent activity against furfural and 5-HMF, suggesting a direct role in detoxifying furan aldehyde inhibitors in situ.

**Methods**

**Growth experiments**

*Thermoanaerobacter pseudethanolicus* 39E (DSMZ 2355) was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. The anaerobic growth medium was prepared using a modified Hungate technique [48] and consisted of 4.5 mM KCl, 4.7 mM NH₄Cl, 2.5 mM MgSO₄·7H₂O, 1.0 mM NaCl, 0.7 mM CaCl₂·2H₂O, 0.25 mg/mL resazurin, 2.8 mM cysteine-HCl, 6.0 mM NaHCO₃, 1 mM potassium phosphate buffer (pH 6.8), 10 mM 3-(N-morpholino)propanesulfonic acid (pH 6.8), 1x Wolfe’s trace minerals [49], 1x Wolfe’s vitamin supplement [49], 0.1% (w/v) yeast extract (Fisher Scientific, Pittsburgh, PA), and 40 mM glucose. Furan aldehydes were added from degassed concentrated stock solutions. Cultures were grown at 65°C from a 1% inoculum in Balch tubes (10 mL) or 125-mL serum bottles

### Table 4 Specific activity (μmol/min/mg protein) of putative *T. pseudethanolicus* 39E aldehyde reductases

|                  | Vector control | Teth39 BdhA | Teth39 AKR |
|------------------|----------------|-------------|------------|
| Furfural         | 0.33 ± 0.13    | 4.97 ± 0.17 | 0.27 ± 0.04 |
| 5-Hydroxymethylfurfural | 0.44 ± 0.16  | 10.06 ± 0.80| 0.39 ± 0.07 |
| Acetaldehyde     | 0.02 ± 0.15    | 4.54 ± 0.76 | 0.02 ± 0.01 |
| Butyraldehyde    | 0.09 ± 0.05    | 14.58 ± 3.57| 0.33 ± 0.04 |
| Isobutyraldehyde | −0.10 ± 0.21   | 0.63 ± 0.29 | 0.23 ± 0.05 |

Whole cell lysate with pET-30a only (vector control) or expressing Teth39 BdhA (Teth39_1597) or Teth39 AKR (Teth39_1598) was assayed aerobically at 60°C using 0.2 mM NAD(P)H as the electron donor and 20 mM substrate. NAD(P)H oxidation activity was measured via loss of absorbance at 340 nm. Values in boldface are statistically significant. No activity was detected with either Teth39 BdhA or Teth39 AKR above vector control using NADH.

**Figure 5** Overexpression of Teth39_1597 and Teth39_1598 in *Escherichia coli*. Teth39_1597 (48.4 kDa) and Teth39_1598 (42.1 kDa) were cloned from *T. pseudethanolicus* into pET-30a and overexpressed from a T7 promoter after induction with IPTG. Overexpression was determined in whole cell extract by Coomassie-stained SDS-PAGE.
(50 mL). Cell growth was monitored by optical density at 600 nm, either directly in the Balch tube using a Spectronic 200 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) or as 200-µL samples transferred to a 96-well plate and read on a Synergy Mx plate reader (BioTek, Winooski, VT). All growth experiments were performed in triplicate.

Small molecule measurement
Glucose, lactate, acetate, and ethanol were measured via HPLC as previously described using an HPX-87H column (Bio-Rad Laboratories, Hercules, CA) at 60°C with detection via refractive index and 5 mM H2SO4 as the mobile phase [50]. Furfural and 5-HMF were measured spectrophotometrically (DU 800, Beckman Coulter, Brea, CA) at 304 and 323 nm, respectively, and concentrations were determined using standard curves generated in growth medium. 2,5-furandimethanol was measured using gas chromatography-mass spectrometry (GC-MS) following trimethylsilylation, with an Agilent 5975C standard quadrupole GC-MS using electron impact ionization (970 eV), as described previously [51].

Proteomic analysis: sample preparation
Cell pellets (10 to 50 mg) from cultures grown for 8 h (early stationary phase) with and without 15 mM furfural were resuspended in 1 mL lysis buffer (4% SDS, 100 mM Tris-HCl, pH 8.0 and 20 mM CaCl2 and digested with sequencing-grade trypsin (Promega, San Luis Obispo, CA) at a 1:75 (w/w) enzyme/protein ratio (16 h, RT). The samples were again diluted 1:1 (v/v) and digested with a second aliquot of trypsin (1:75; w/w) for an additional 4 h. Following digestion, each sample was adjusted to 200 mM NaCl and 0.1% formic acid and filtered through a 10-kDa cutoff spin column filter (Vivaspin 2, GE Healthcare, Pittsburgh, PA). The peptide-enriched flow-through was then quantified by the bicinchoninic acid assay.

Proteomic analysis: measurement of peptides by two-dimensional liquid chromatography-tandem mass spectrometry (LC-MS/MS)
For each sample, 100 µg of peptides were bomb-loaded onto a biphasic MudPIT back column [52] packed with about 5 cm of strong cation exchange (SCX) resin for charge-based separation of peptides followed by about 3 cm C18 reversed phase (RP) for online desalting (Luna and Aqua respectively, Phenomenex, Torrance, CA). Once loaded, the sample columns were washed offline with solvent A (5% acetonitrile, 95% HPLC-grade water, 0.1% formic acid) for 15 min, followed by a gradient to 100% solvent B (70% acetonitrile, 30% HPLC-grade water, 0.1% formic acid) over 30 min. The washed samples were then placed in-line with an in-house pulled nanospray emitter (100-µm inner diameter) packed with 15 cm of C18 RP material and analyzed via 24-h MudPIT two-dimensional LC-MS/MS (eleven salt pulses: 5, 7.5, 10, 12.5, 15, 17.5, 20, 25, 35, 50, 100% of 500 mM ammonium acetate followed by a 100-min gradient to 50% solvent B) with an LTQ XL mass spectrometer (Thermo Fisher Scientific) operating in data-dependent mode. A total of three biological replicate measurements were obtained for each sample.

Proteomic analysis: MS data analysis and evaluation
Acquired MS/MS spectra were assigned to specific peptide sequences using the SEQUEST search algorithm [53] with a FASTA proteome database specific to T. pseudethalonicus. The database contained common contaminant protein entries as well as reversed decoy sequences to assess protein-level false discovery rates. SEQUEST-scored peptide sequence data were filtered and assembled into protein loci using DTASelect [54] with the following conservative criteria: XCorr: +1 = 1.8, +2 = 2.5, +3 = 3.5, DeltCN 0.08, and two peptides per protein identification with at least one required to be unique.

Prior to the semiquantitative analysis, spectral counts were rebalanced to properly distribute non-unique/shared peptides between their potential parent proteins, as previously described [55]. To represent proteins that were sporadically identified across runs (that is, blank/zero values in a portion of the six sample runs), a fraction of a spectral count (0.33) was added to the entire dataset. This distributional shift maintains the originally measured spectral count differential but allows for blank/zero values to be considered in the ensuing statistical analysis [56]. These adjusted values were then converted to normalized spectral counts (nSpC), an extension of the widely recognized normalized spectral abundance factor (NSAF) [57] that is calculated by multiplying the NSAF values by an arbitrary number representative of the number of spectra collected for each run. In this case, the number 50,000 was used for facile data interpretation. Once calculated, an SpC cutoff was applied to all proteins identified in the
dataset so that 99% of the total raw SpC assigned to each (summed across all replicates and conditions) remained.

These remaining proteins were log2 transformed, and statistically assessed by ANOVA with JMP Genomics ver. 4.1 (SAS Institute, Cary, NC) to identify proteins in the furfural treated samples that were significantly (P ≤ 0.05) up- or downregulated relative to the control. These differentially expressed proteins were then hierarchically clustered based on their abundance patterns across all replicates and conditions using the “Fast Ward” algorithm. To remove differences based on raw magnitude differences in nSpC, each protein’s abundance was standardized to represent the number of standard deviations away from the row mean.

Aldehyde reductase cloning and overexpression
*T. pseudethanolicus* genomic DNA was isolated using the Wizard Genomic DNA Purification kit (Promega, Madison, WI). Teth39_1597 and Teth39_1598 were PCR amplified using Phusion Polymerase (New England Biolabs, Ipswich, MA), cloned into pET-30a (EMD Millipore, Billerica, MA) behind 6xHis- and S-tags, and the final constructs were sequence verified (University of Tennessee, Knoxville, Molecular Biology Resource Facility). Expression plasmids were transformed in BL21 (DE3) *Escherichia coli* according to the manufacturer’s protocol (Invitrogen, Grand Island, NY). Cells were grown in 50 mL 2×YT medium at 37°C to OD₆₀₀ of 0.8-1.0, then induced with 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG) and switched to 30°C for 16 h. Cells were harvested at 4°C (3,000 x g, 30 min), washed in 50 mL 100 mM sodium phosphate buffer, pH 7 (buffer A), and resuspended in 5 mL buffer A. The cell suspension (450 μL) was added to 0.1 mm zirconia beads (300 μL) and vortexed 4 x 60 s with 30 s on ice in between. The samples were centrifuged (14,000 x g, 2 min), and the resulting supernatant was used for enzyme assays.

**Enzyme assays**
The *in vitro* aldehyde reductase activity was measured as previously described [23] in 100 mM sodium phosphate buffer (pH 7) with 0.2 mM NAD(P)H and 20 mM substrate. The assay mix was added to a sealed 2-mL quartz cuvette and equilibrated to 60°C. Assays were read at 340 nm (DU 800) for 150 s to establish a baseline slope before whole cell lysate was added (1 to 5 μL). The cuvettes were inverted once to mix and read an additional 450 s. The decrease in absorbance over time was calculated and the baseline slope was subtracted. The NAD(P)H concentration was determined using the extinction coefficient (NADH: 6,220 M⁻¹ cm⁻¹, NADPH: 6,270 M⁻¹ cm⁻¹), and the specific activity was calculated as the change in μmol NAD(P)H/min/mg of whole cell lysate protein. Protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. The specific activity was measured for the pET-30a vector (control) and overexpressed Teth39_1597 and Teth39_1598 with acetaldehyde, butyraldehyde, isobutyraldehyde, furfural, and 5-HMF.

### Additional file

**Additional file 1: Table S1.** Description of data. Proteomics data file for 225 proteins that were differentially regulated in response to a 15 mM furfural treatment. Color-coded hierarchical cluster analysis, cluster number, cluster order, average nSpC per condition, fold change, and P-value are provided for all replicates. The raw output for all detected proteins and statistical analyses are also included.

**Abbreviations**
5-HMF: 5-hydroxymethylfurfural; ADH: alcohol dehydrogenase; AKR: Aldo/keto reductase; ANOVA: analysis of variance; BDH: butanol dehydrogenase; CBP: consolidated bioprocessing; GC-MS: gas chromatography-mass spectrometry; HPLC: high performance liquid chromatography; IPTG: isopropyl-β-D-thiogalactopyranoside; LC-MS: liquid chromatography-mass spectrometry; nSpC: normalized spectral counts; OD: optical density; RP: reverse phase; SpC: spectral counts; TCA: tricarboxylic acid.

**Competing interests**
The authors declare that they have no competing interests.

**Authors’ contributions**
SMC, SDH-B, and JGE conceived and designed the study; SMC, SDH-B, and JGE performed growth experiments; SMC measured furan aldehydes; SMC and JGE performed HPLC analyses; SDH-B prepared samples for proteomic analysis; RJG and RLH performed proteomic experiments and statistical analyses; NLE and TJT performed GC-MS quantifications; SMC performed cloning and enzyme activity assays; SMC, SDH-B, and JGE analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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