# Materials and methods

## Metabolite quantification

Dataset 2 from Tian et al. 2017 (Tian et al., 2017) was used. Briefly, a single 200 ml culture was grown to an OD600 of 0.1, the culture was split in half. Starting at 2 hours (post split), ethanol was added to one culture at a rate of about 9 g/L/h. No ethanol was added to the other culture. At 3 timepoints (T=2.0h, 3.8h and 5.9h), each culture was sampled twice for intracellular metabolites using previously described protocols (Olson et al., 2016; Rabinowitz and Kimball, 2007; Tian et al., 2017), and once for extracellular metabolites (supplemental table BBB).

The raw data was re-processed with El-Maven 0.5.0 and quantified using external standards (supplemental figure AAA). Since the response was not linear over the full range of the standards (0.1 µM to 100 µM), quantification was performed by piecewise linear interpolation (supplemental figure DDD).

Based on 4 measurements of our standard curves, our quantification error for

One significant change that we observed from the absolute quantification is that the energy charge (ATP or GTP, calculated using Equation 1) values which had previously been calculated to be around 0.9, were actually much lower, in the range of 0 to 0.15, which is much lower than expected (Rabinowitz and Kimball, 2007), and were excluded from analysis.

(Equation 1)

In addition, NADH was not observed for the no ethanol control cultures. Since NADH and NAD+ form a cofactor pair, both metabolites were excluded from analysis.

Since metabolites are typically diluted during the quenching and extraction process, to determine the *intracellular* concentration of metabolites, the measured concentration was adjusted using Equation 2.

(Equation 2)

The intracellular volume is assumed to be 3.9 µL for 1 ml of a culture at an OD600 density of 1 (Volkmer and Heinemann, 2011). The concentration factor was calculated separately for each extraction, based on the measured OD600 and sample volume. The target cell density (OD600 x ml) was 2.0, but varied from 1.6 to 2.1 (supplemental table CCC).

For ethanol, the intracellular concentration was assumed to be the same as the extracellular concentration, since the cytoplasmic membrane is not thought to be a barrier to ethanol diffusion.

## Thermodynamic model constraints

For performing the max-min driving-force (MDF) analysis, maximum and minimum metabolite concentration have to be established for non-measured metabolites. Noor et al. (Noor et al., 2014) proposed a range of 1 µM to 10 mM, based on largely on the work of Bennet et al. (Bennett et al., 2009). Based on our measurements, we decided to keep the lower default concentration at 1 µM, but raise the upper default concentration from 10 mM to 20 mM.

Although Noor et al. fixed the ratios of several cofactor pairs, we have relaxed these constraints, since the values have not been experimentally determined for *C. thermocellum*. Several cofactors were excluded from analysis due to measurement problems (see Metabolite quantification section). To avoid biasing the results by including only a few measured cofactors, we opted to ignore measured values for all cofactors (ATP, ADP, AMP, GTP, GDP, NAD+. NADH, NADP+ and NADPH). Concentration ranges for cofactors were set to allow ratios between 1:100 and 100:1 for the pairs (ATP/ADP, ATP/AMP, GTP/GDP, NADH/NAD+, NADPH/NADP+, Fd(red)/Fd(ox)). Default bounds for each metabolite is described in Supplementary table EEE.

## Stoichiometric model

Based on XXX. Previous stoichiometric models of C. thermocellum have ignored cofactor balance. To reintroduce some of that complexity back into our model, we…

# Results and discussion

## Description of metabolite dataset

Dataset 2 from Tian et al. 2017 (Tian et al., 2017) was used. This dataset represents intracellular metabolites collected from WT *C. thermocellum* growing with and without the presence of added ethanol. The maximum concentration of added ethanol is 40 g/L, which is thought to be a minimum titer for commercial viability (Dien et al., 2003).

## Thermodynamic consistency of dataset

Based on measurements of extracellular metabolites (Supplementary table FFF) we know that cellobiose was converted to ethanol during all 3 timepoints for the no-ethanol control, which implies that all reactions must have a positive MDF. For the samples with added ethanol, we cannot directly measure the ethanol flux, since the added ethanol is much larger than the produced ethanol, but based on the consumption of cellobiose and the presence of other fermentation products (formate and acetate), we suspect that there was flux from cellobiose to ethanol at the first two timepoints (and thus MDF should be positive). For the 3rd timepoint, flux was close to zero. The expected MDF values are summarized in Table GGG.

**Table GGG. Expected MDF values for ethanol addition experiment**

|  |  |  |
| --- | --- | --- |
| Timepoint | Added ethanol | Control |
| T0 | + | + |
| T1 | + | + |
| T2 | 0 | + |

We attempted to fit all of the measured metabolite concentrations (excluding cofactors) to our thermodynamic model. For each measured metabolite, we included an uncertainty factor of 2 (see materials and methods for description). This resulted in negative MDF values for both the added ethanol and control cultures at all timepoints, indicating that the dataset is not thermodynamically consistent. To identify which metabolites were causing the inconsistency, we tested them one-by-one (Table HHH).

**Table HHH. MDF values for each metabolite tested one-by-one from each fermentation condition.**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **ID** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
|  | **graphName** | **A1\_1 et=5.0** | **A1\_2 et=5.0** | **A2\_1 et=20.0** | **A2\_2 et=20.0** | **A3\_1 et=40.0** | **A3\_2 et=40.0** | **B1\_1 et=0.2** | **B1\_2 et=0.2** | **B2\_1 et=0.47** | **B2\_2 et=0.47** | **B3\_1 et=0.58** | **B3\_2 et=0.58** |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| **C00010** | **coa** | 2.3 | 2.2 | 2.4 | 2.5 | 2.5 | 2.5 | 2.2 | 2.2 | 2.4 | 2.4 | 2.2 | 2.1 |
| **C00469** | **etoh** | 2.7 | 2.7 | 2.3 | 2.3 | 2.1 | 2.1 | 3.5 | 3.5 | 3.4 | 3.4 | 3.4 | 3.4 |
| **C00197** | **3pg** | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 |
| **C00024** | **accoa** | 0.6 | 0.3 | -1.1 | 2.1 | -1.2 | -1.2 | -0.5 | -0.2 | -0.8 | -1.2 | -0.1 | 0.1 |
| **C00111** | **dhap** | -5.5 | -4.7 | -2.3 | -2.0 | -2.0 | -1.7 | -4.8 | -4.3 | -4.6 | -5.8 | -3.6 | -5.2 |
| **C00085** | **f6p** | 1.8 | 1.8 | 1.9 | 1.9 | 2.1 | 2.0 | 1.9 | 1.9 | 1.9 | 1.9 | 1.8 | 1.1 |
| **C00354** | **fdp** | -1.5 | -1.2 | -0.3 | -0.4 | -0.3 | 0.0 | -0.9 | -0.8 | -1.1 | -1.0 | -1.0 | -0.9 |
| **C00092** | **g6p** | 1.5 | 1.4 | 1.5 | 1.5 | 1.5 | 1.6 | 1.5 | 1.5 | 1.6 | 1.5 | 1.7 | 1.8 |
| **C00103** | **g1p** | 1.6 | 1.8 | 2.1 | 2.1 | 2.1 | 2.1 | 1.9 | 1.9 | 2.0 | 2.1 | 2.1 | 2.1 |
| **C00031** | **glc-d** | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 |
| **C00074** | **pep** | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 |
| **C00022** | **pyr** | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 |
| **C00149** | **mal-l** | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 | 2.1 |

From this, we can see that there are potential problems with 3 metabolites: acetyl-coA (accoa), dihydroxyacetonephosphate (dhap), and fructose-1,6-bisphosphate (fdp).