

Figure S1 Fractional labeling of GC-MS fragments for *C. thermocellum* proteinogenic amino acids in CTFUD rich (red bar) and minimal medium (blue bar) by feeding 5 g/L 20% fully ^{13}C labeled and 80% fully unlabeled glucose. Dotted line represents the theoretical fractional labeling value of 0.2 when labeling steady state is reached.

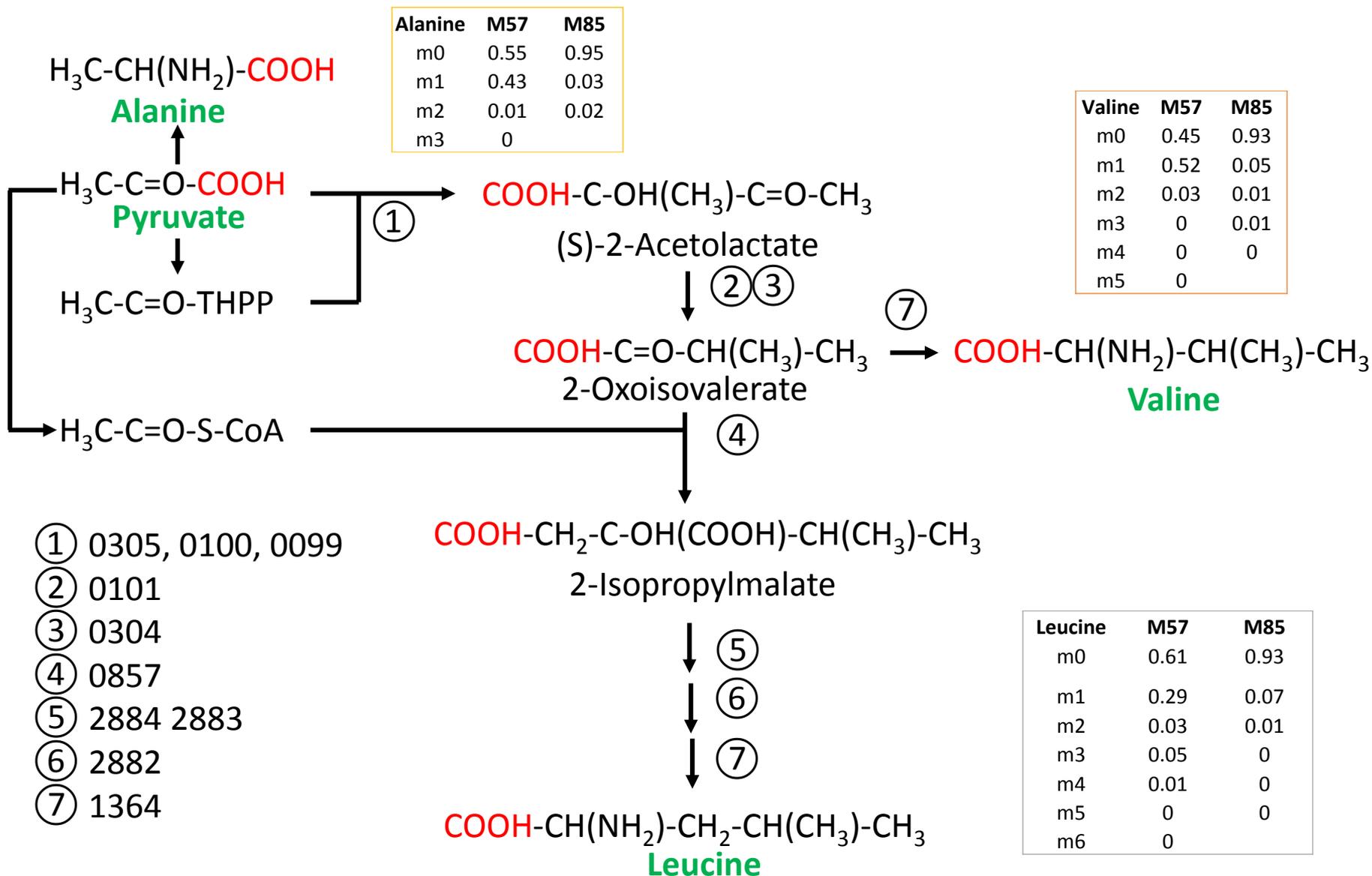


Figure S2 Isotope analysis using ^{13}C -bicarbonate confirms leucine and valine biosynthesis in *Clostridium thermocellum* DSM1313. ^{13}C -labeled positions are marked in red. ① acetolactate synthase (ALS), ② keto-acid reductoisomerase (KARI), ③ dihydroxy-acid dehydratase (DHAD), ④ 2-isopropylmalate synthase (IPMS), ⑤ 3-isopropylmalate dehydratase (IPMD), ⑥ 3-isopropylmalate dehydrogenase (IPMDH), ⑦ branched-chain aminotransferase (BCAT). These enzymes are encoded by corresponding genes in the genome of *C. thermocellum* DSM 1313. Their numbers are indicated. The mass distribution vectors for each amino acid in the pathway are presented. M57 and M85 represent two typical GC-MS fragments of the amino acids, respectively. M57 indicates the fragments lose a tert-butyl group from the derivatized reagent (Molecular weight 57). M85 indicates the one loses a tert-butyl group and a CO of the amino acid (Molecular Weight: 87). Therefore, M57 fragment reserves all carbons in the amino acid, while M85 loses the C1 carbon but reserves rest carbons in the amino acid.

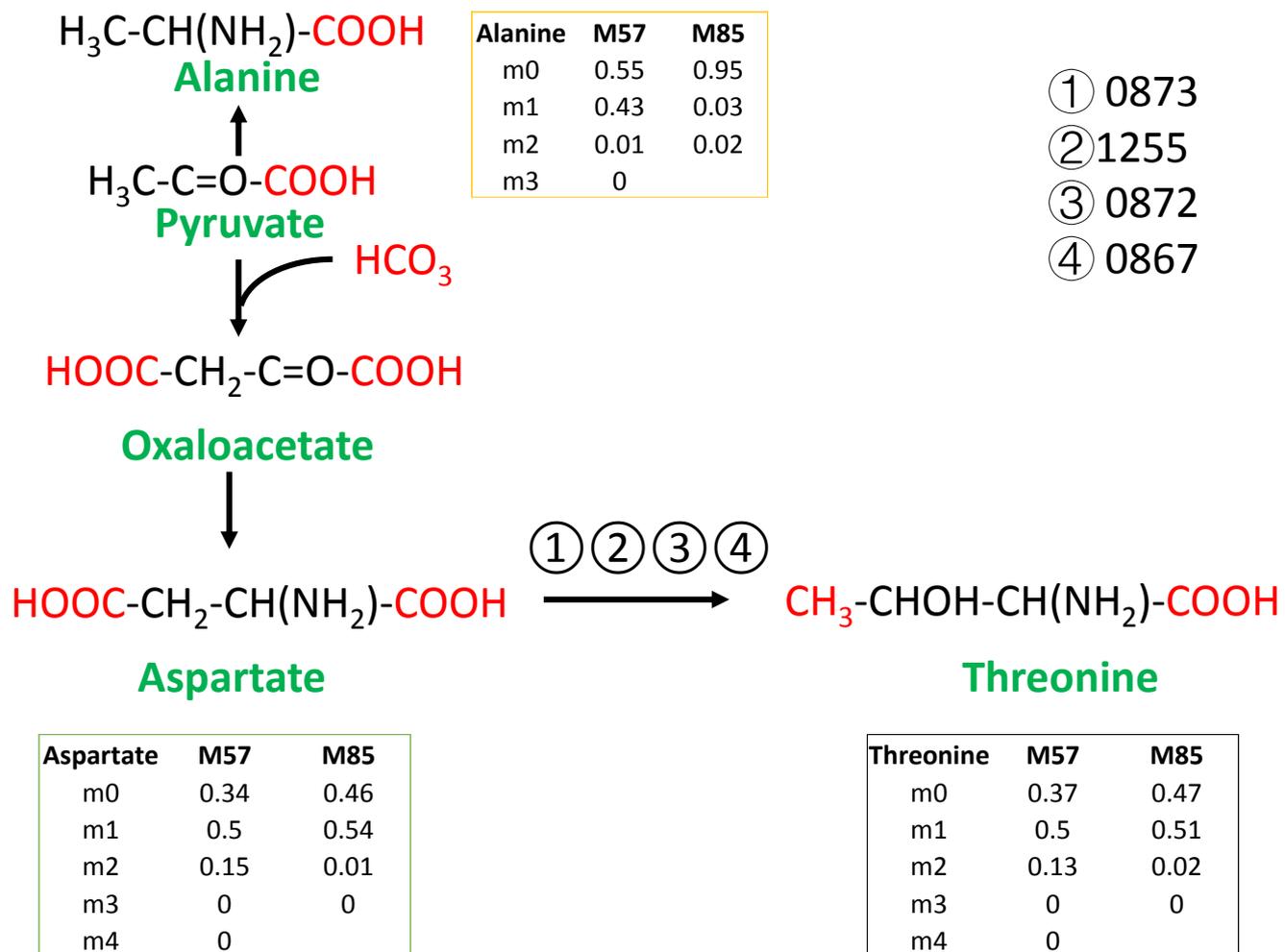
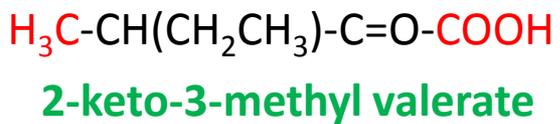
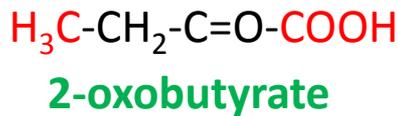


Figure S3 Aspartate and threonine biosynthesis in *Clostridium thermocellum* DSM1313. ^{13}C -labeled positions are marked in red. ① aspartate kinase, ② aspartate-semialdehyde dehydrogenase, ③ homoserine dehydrogenase, ④ threonine synthase.

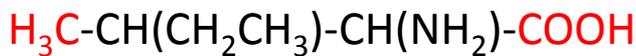
Threonine	M57	M85
m0	0.37	0.47
m1	0.5	0.51
m2	0.13	0.02
m3	0	0
m4	0	0



⑨ ↓

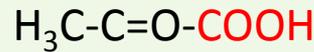


⑧ ↓

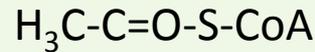


Isoleucine

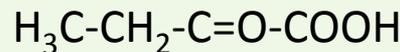
**Isoleucine biosynthesis I
(from threonine)**



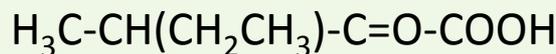
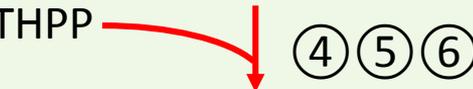
Pyruvate



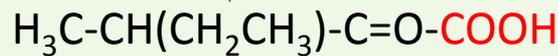
① ↓



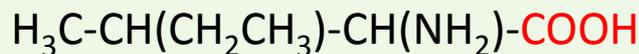
2-oxobutyrate



2-keto-3-methyl valerate



⑧ ↓



Isoleucine

**Isoleucine biosynthesis II
(from citramalate)**

- ① Citramalate synthase
- ② Isopropylmalate isomerase
- ③ Isopropylmalate dehydrogenase
- ④ 2-aceto-2-hydroxybutanoate synthase
- ⑤ Acetohydroxyacid isomeroreductase
- ⑥ Dihydroxyacid dehydratase
- ⑦ Branched chain keto acid dehydrogenase
- ⑧ Isoleucine transaminase
- ⑨ Threonine ammonia-lyase

Isoleucine	M15	M85
m0	0.50	0.92
m1	0.42	0.07
m2	0.03	0.01
m3	0.04	0.00
m4	0.00	0.00
m5	0.00	0.00
m6	0.00	0.00

✗ Unmatched labeling pattern

Figure S4 Proposed isoleucine biosynthesis from threonine and citramalate pathway. (Labeling experiment used ^{13}C -bicarbonate as the carbon source.) ^{13}C -labeled positions are marked in red. The confirmed citramalate pathway is boxed.

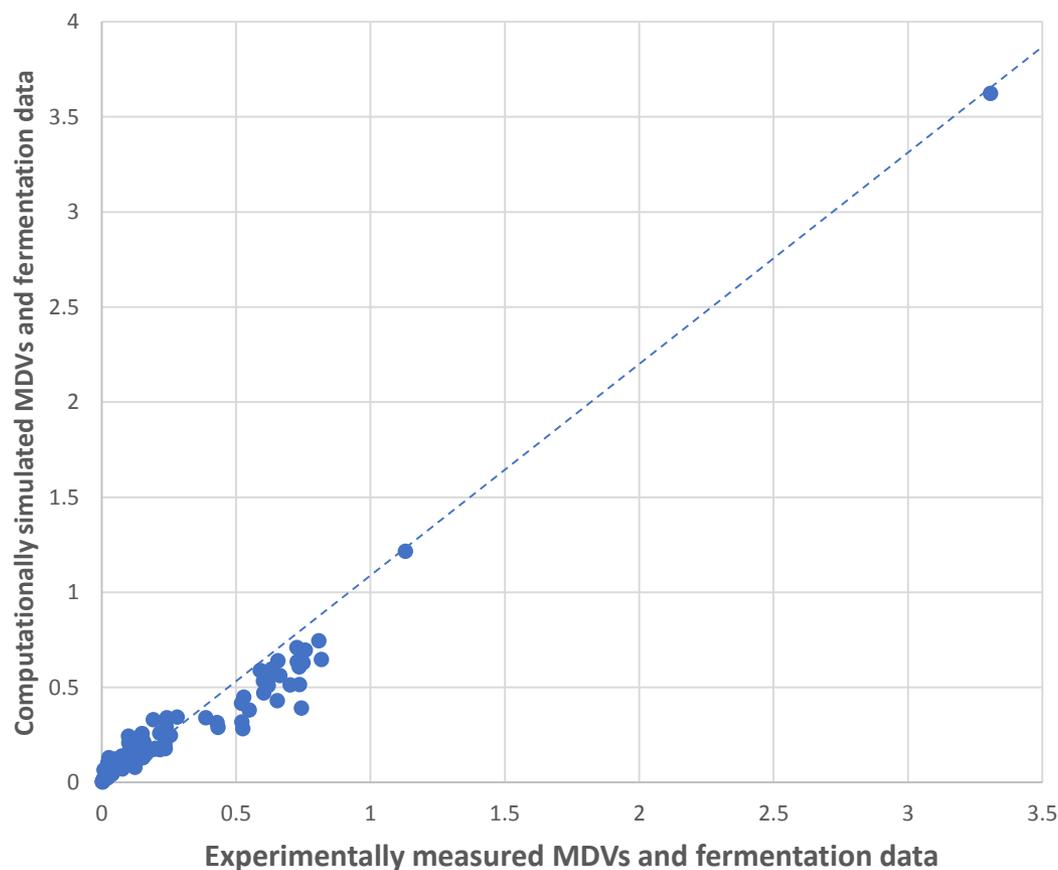


Figure S5. ^{13}C -MFA model fitting for *Clostridium thermocellum* metabolism. The data points include the mass isotopomer distributions (MID) of GC-MS fragments for amino acids including ALA232, ALA260, Gly218, Gly246, Val260, Val288, LEU274, ILE200, ILE274, SER288, SER362, SER390, THR376, THR404, PHE234, PHE302, PHE308, PHE336, ASP302, ASP316, ASP390, ASP418, GLU330, GLU404, GLU432, TYR302 and extracellular fluxes for sugar, lactate, acetate etc. (Amino acids are represented with their 3-letter abbreviations and the m/z of the unlabeled fragments are represented by following numbers.) The fit between simulated and measured data is good with $R^2 = 0.95$.