Supplementary Material

Manipulating Fermentation Pathways in the Hyperthermophilic Archaeon *Pyroccous furious* for Ethanol Production up to 95°C Driven by Carbon Monoxide Oxidation

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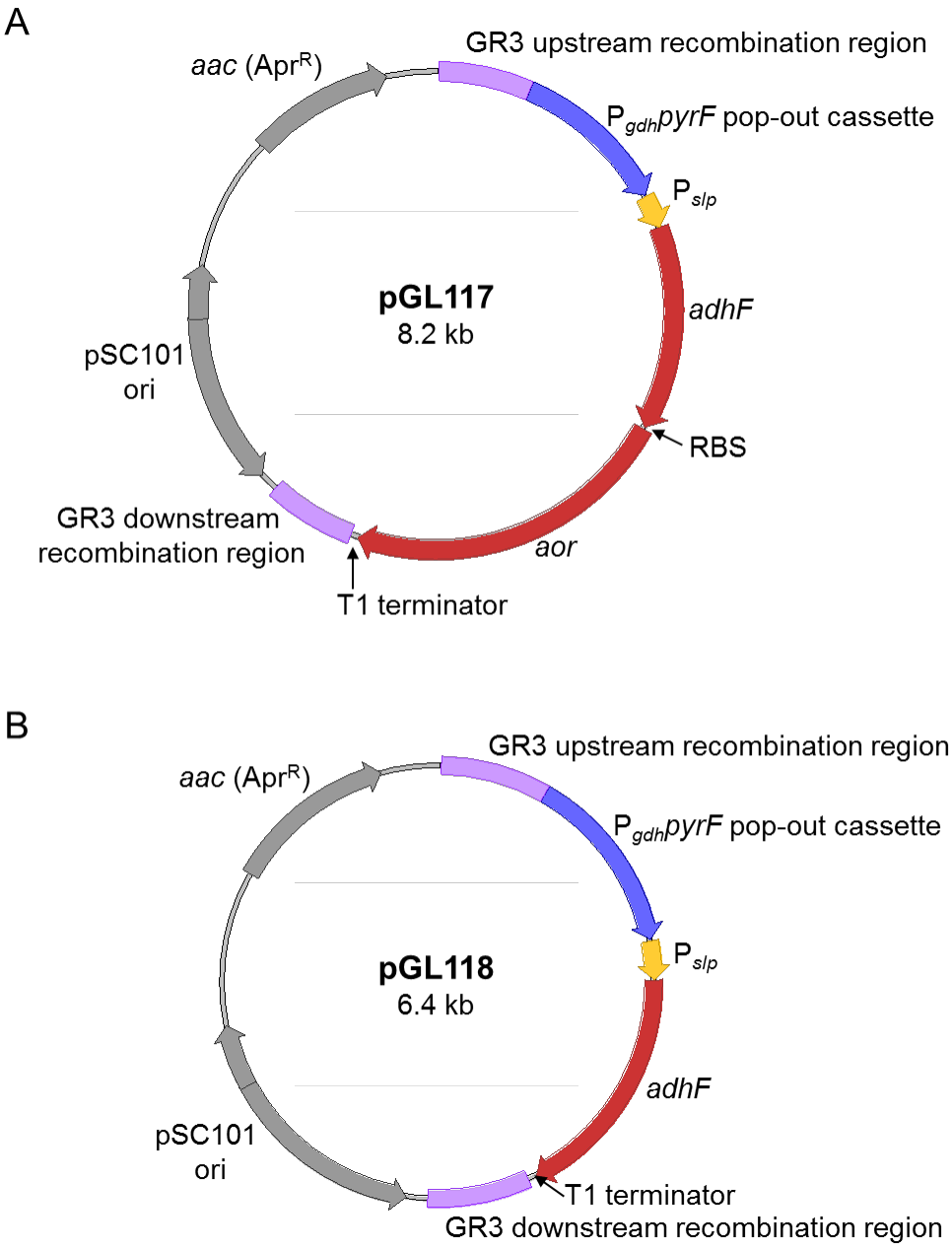
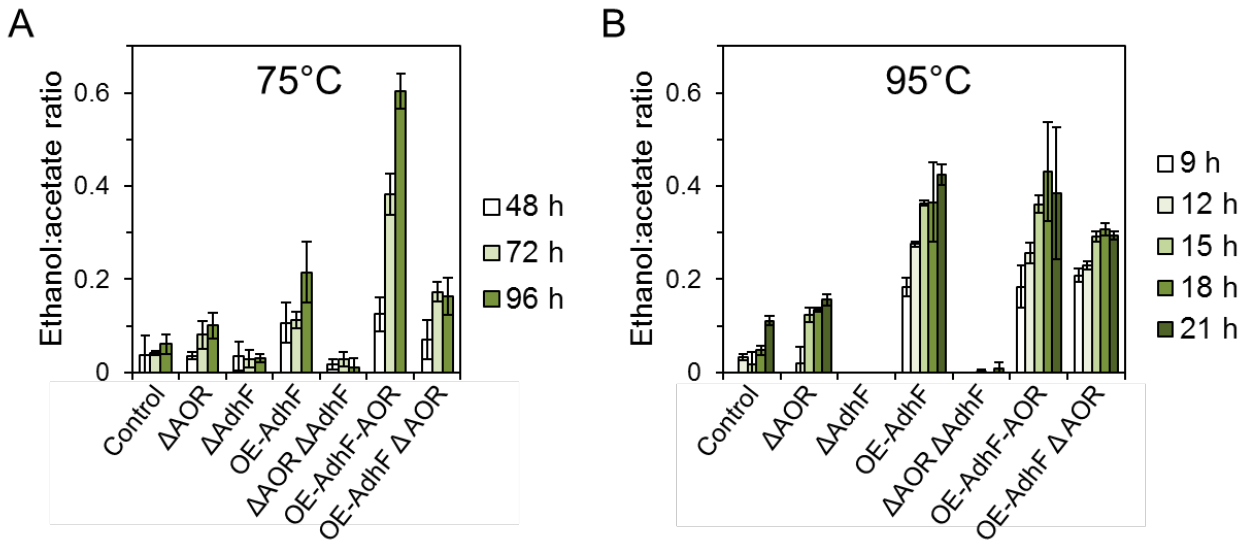
Supplementary Figures 1 – 7

Supplementary Table 1

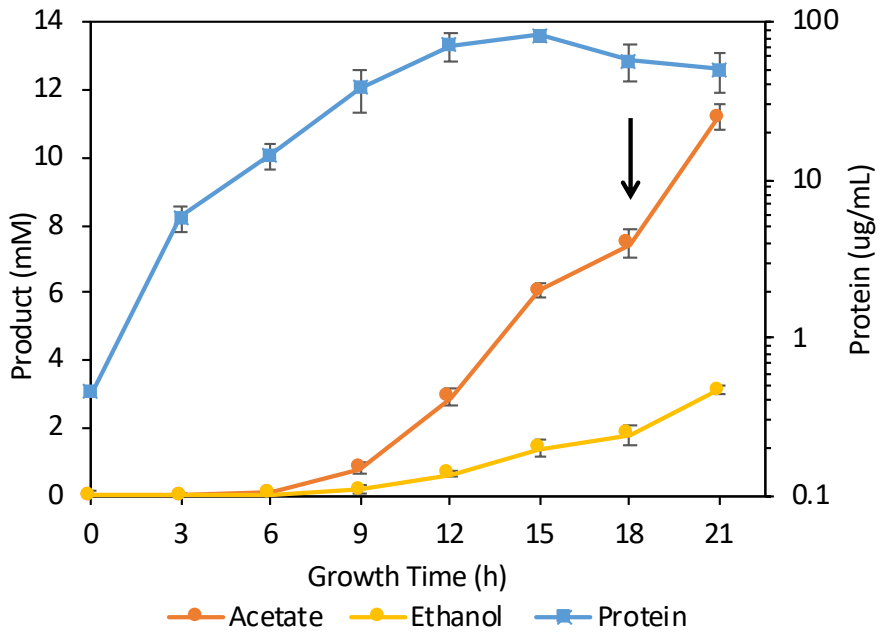


Supplementary Figure 1. Plasmid maps of pGL117 (A) and pGL118 (B) for homologous overexpression of *adhF* with and without *aor*, respectively. Color coding of plasmid elements is as follows: *E. coli* plasmid construction elements, grey (pSC101 origin of replication and apramycin resistance gene cassette); target recombination regions for *P. furiosus* genome integration, purple (genome region 3), *P. furiosus* selectable genetic marker, blue (P_{gdh}*pyrF* pop-out cassette); overexpression promoter, yellow (P_{slp}); overexpressed genes, red (*adhF*, *aor*). Terminator (T1) and ribosome binding site (RBS) elements are indicated with arrows.



Supplementary Figure 2. Ratios of ethanol to acetate in engineered *adhF* strains. Ethanol:acetate ratios are plotted for selected time points (hours of growth) from growth curves shown in Fig. 3 and Fig. 4, for growth temperatures of 75°C (A) and 95°C (B).

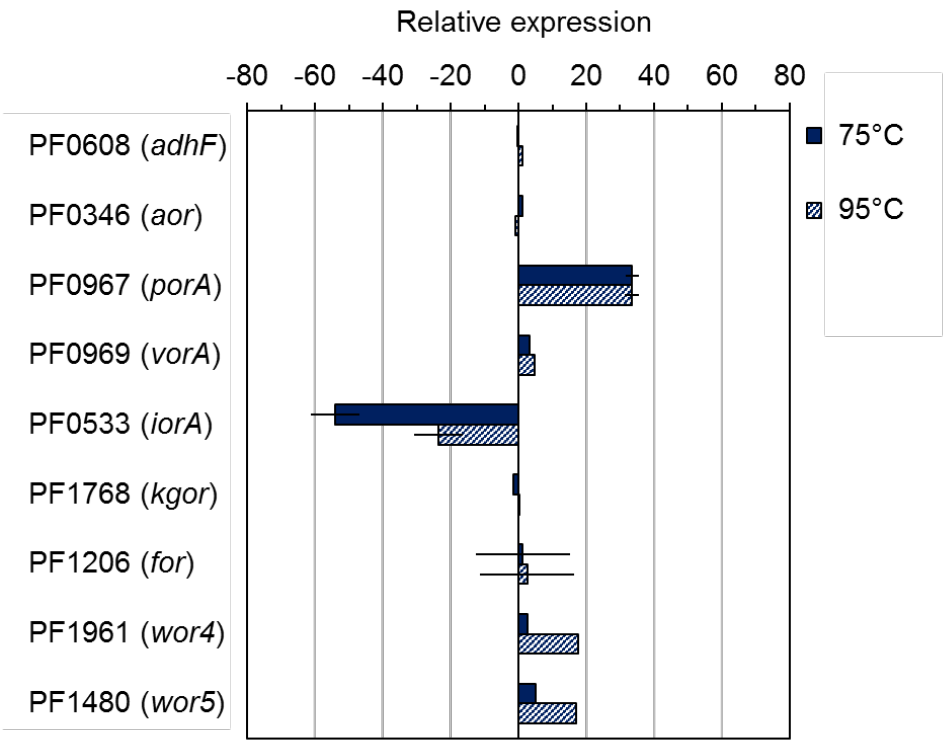
A



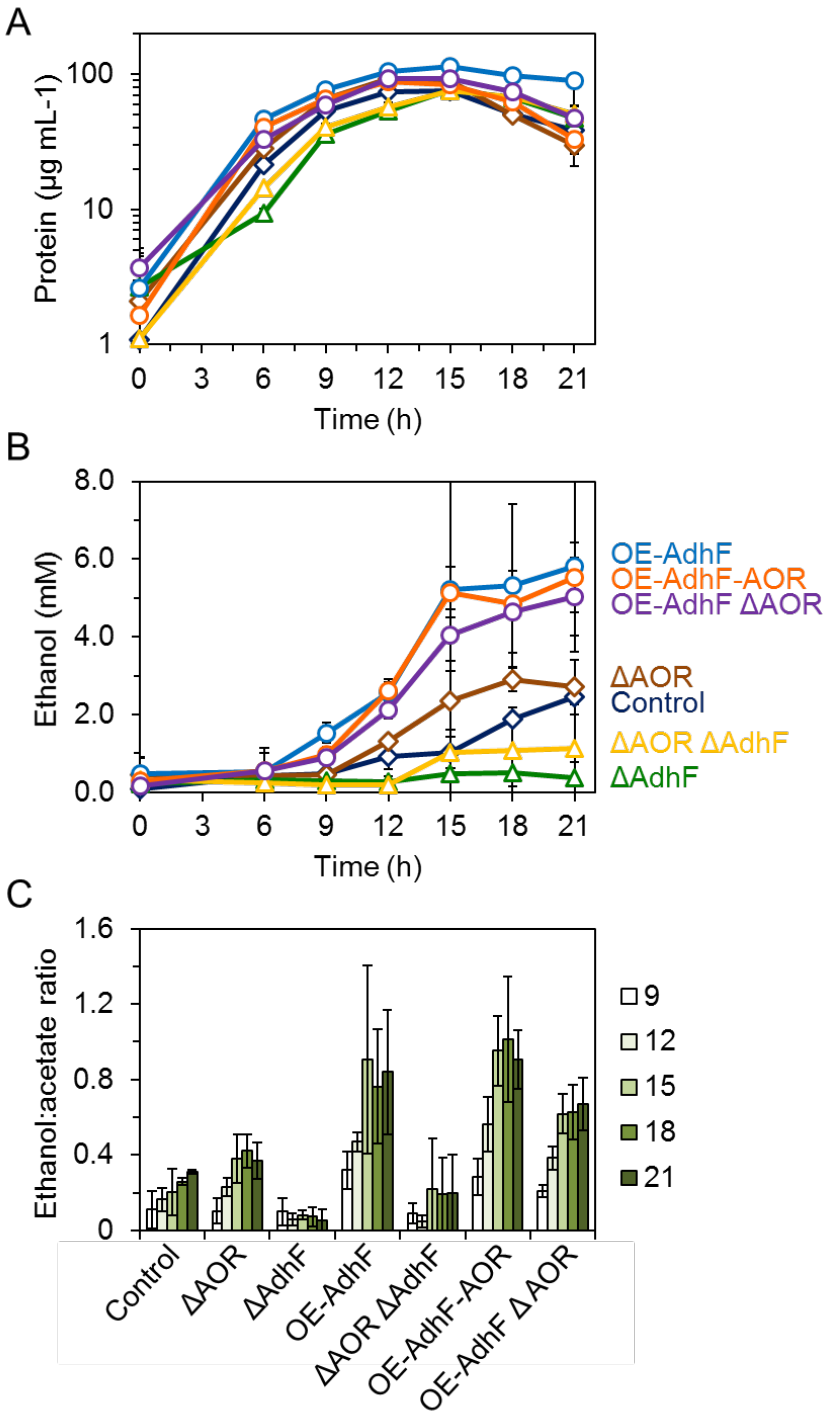
B

C6 at T_0 (mM)	C6 at 18 hr (mM)	C6 used (mM)	Acetate (mM)	Ethanol (mM)	CO_2 (mM)	Mol ethanol per mol C6	Mol acetate per mol C6
43.1	28.7	14.4	7.4	1.8	9.2	0.13	0.52

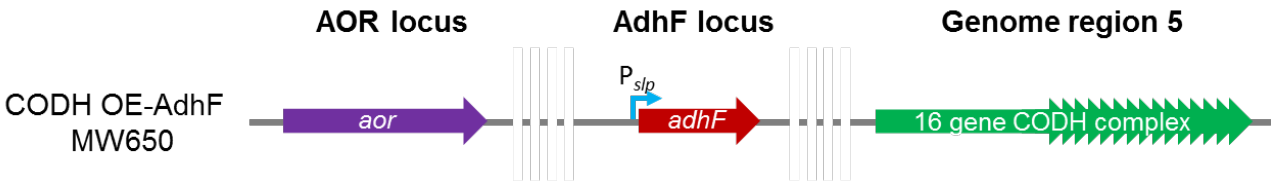
Supplementary Figure 3. Quantitation of the growth substrate (maltose) utilized and the products generated by the OE-AdhF strain. A) Growth curve showing the concentrations of protein (blue), acetate (orange) and ethanol (yellow). Samples were taken after 18 hr of growth (indicated by the arrow were used for mass balance analyses. B) Iniital and final (18 hr) concentrations of the disaccharide maltose (expressed per glucose unit), ethanol and acetate, and the corresponding molar yields of ethanol and acetate (per glucose).



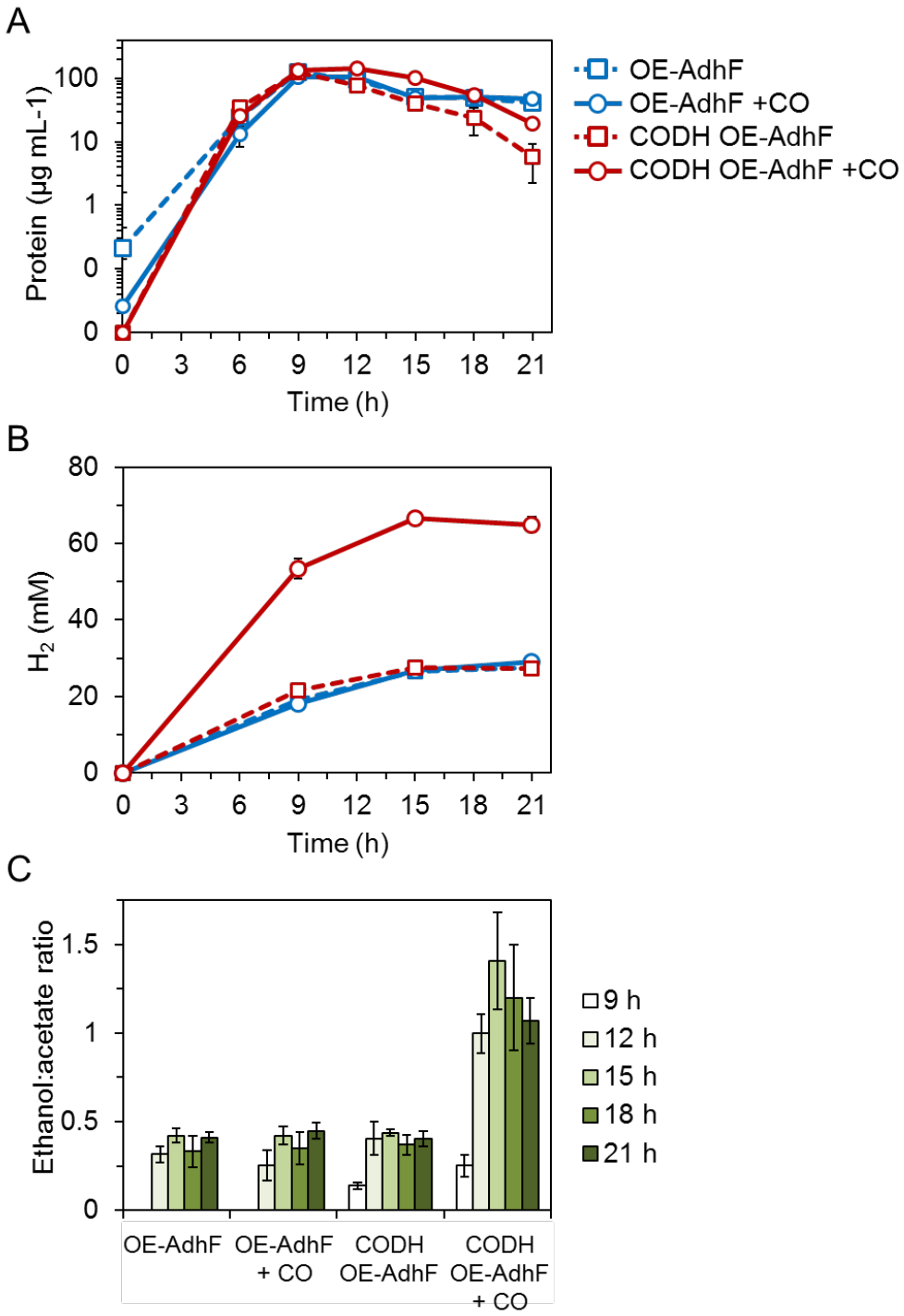
Supplementary Figure 4. Expression levels of *adhF* and various oxidoreductase genes. *P. furiosus* Control strain (MW004) was grown in minimal maltose medium at 75°C and 95°C to mid-log phase. Expression of the constitutive gene PF0983 encoding the DNA polymerase sliding clamp was used as an internal standard to calculate relative expression values. For enzymes that are encoded by more than one gene, a single representative genes was chosen. Gene symbols represent the following enzymes: *adhF*, alcohol dehydrogenase F; *aor*, aldehyde oxidoreductase; *porA*, pyruvate ferredoxin oxidoreductase subunit alpha, *vorA*, 2-ketoisovalerate ferredoxin oxidoreductase subunit alpha; *iorA*, indolepyruvate ferredoxin oxidoreductase subunit alpha; *kgor*, 2-ketoglutarate ferredoxin oxidoreductase subunit alpha; *for*, formaldehyde ferredoxin oxidoreductase; *wor4*, tungsten-containing oxidoreductase 4; *wor5*, tungsten-containing oxidoreductase 5.



Supplementary Figure 5. Rich medium improves growth and ethanol production of *adhF* overexpression strains. Growth (A.) and ethanol production (B.) of Control (dark blue, diamond), ΔAOR (brown, diamond), ΔadhF (green, triangle) and ΔAOR ΔAdhF (yellow, triangle) strains grown in rich medium without shaking at 95°C. C. Ethanol:acetate ratios for each strain for 9, 12, 15, 18 and 21 h time points during growth shown in A.



Supplementary Figure 6. Diagram of CODH OE-AdhF strain that contains the native *aor* locus, P_{slp} inserted upstream of the *adhF* gene, and the CODH operon inserted at genome region 5.



Supplementary Figure 7. Additional data for experiment shown in Figure 7: Addition of CODH with CO improves ethanol production by AdhF in shaking cultures. Growth (A.) and hydrogen production (B.) of OE-AdhF (blue) and CODH-OE-AdhF (red) strains grown in rich medium with shaking at 90°C, without CO (dashed lines, squares) or with CO (solid lines, circles) in the headspace. C. Ethanol:acetate ratios for each strain during growth for 9, 12, 15, 18 and 21 h time points during growth shown in A.

	p-Value	Effect_size	COM1 75C	COM1 95C	p-Value	Effect_size	COM1	COM1 +CODH
POR	0.00E+00	-3.13	30.64	13.47	0.00E+00	-1.61	30.80	19.63
GAPOR	1.40E-03	-0.04	49.98	49.72	1.01E-155	-0.59	50.21	48.04
NFN1	9.43E-19	-0.21	3.60	2.80	1.99E-01	0.04	3.29	3.41
MBH	1.40E-01	0.01	78.19	77.95	0.00E+00	-1.25	79.25	67.49
Acetate transport	6.15E-26	-0.26	22.22	19.57	0.00E+00	-1.51	22.48	10.69
Ethanol transport	1.62E-11	-0.17	6.37	5.12	4.07E-38	0.33	5.62	7.64
SHI	1.25E-03	0.16	9.75	9.89	9.63E-48	0.43	8.33	11.21
SHII	2.07E-14	-0.20	2.72	1.95	6.97E-02	0.14	1.88	1.90
ATP synthase	1.15E-15	0.25	16.61	17.12	0.00E+00	61.49	61.49	204.22
POR_acetaldehyde	0.00E+00	7.29	2.37	16.39	1.08E-99	0.48	1.97	4.08
AOR	0.00E+00	-2.59	3.46	-10.50	5.99E-01	-0.02	2.90	2.86
MalABC	7.61E-127	-0.39	14.32	14.04	6.30E-88	-0.32	14.32	14.06
Biomass_Pfu	9.83E-49	0.38	0.15	0.18	3.01E-46	0.35	0.18	0.20

Supplementary Table 1. Statistical comparison of reaction flux distributions from random simulations of the P. furiosus COM1 model at 75°C and 95°C (left) or with and without CODH added (right). CODH expression was simulated without any temperature-dependent constraints. P-values were reported from the Mann-Whitney U-test. The effect size metric is Cohen's d, which was calculated as the difference in means of the two distributions divided by the standard deviation. Effect sizes are referred to as small (d=0.2), medium (d=0.5), or large (d=0.8) based on benchmarks suggested by Lachenbruch and Cohen (1989).

Reference

Lachenbruch P. A. and Cohen J. 1989. Statistical power analysis for the behavioral sciences. J. Am. Stat. Assoc. 84:1096.