Supplementary Material for

Metabolic engineering of *Actinobacillus succinogenes* provides insights into succinic acid biosynthesis

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Figures

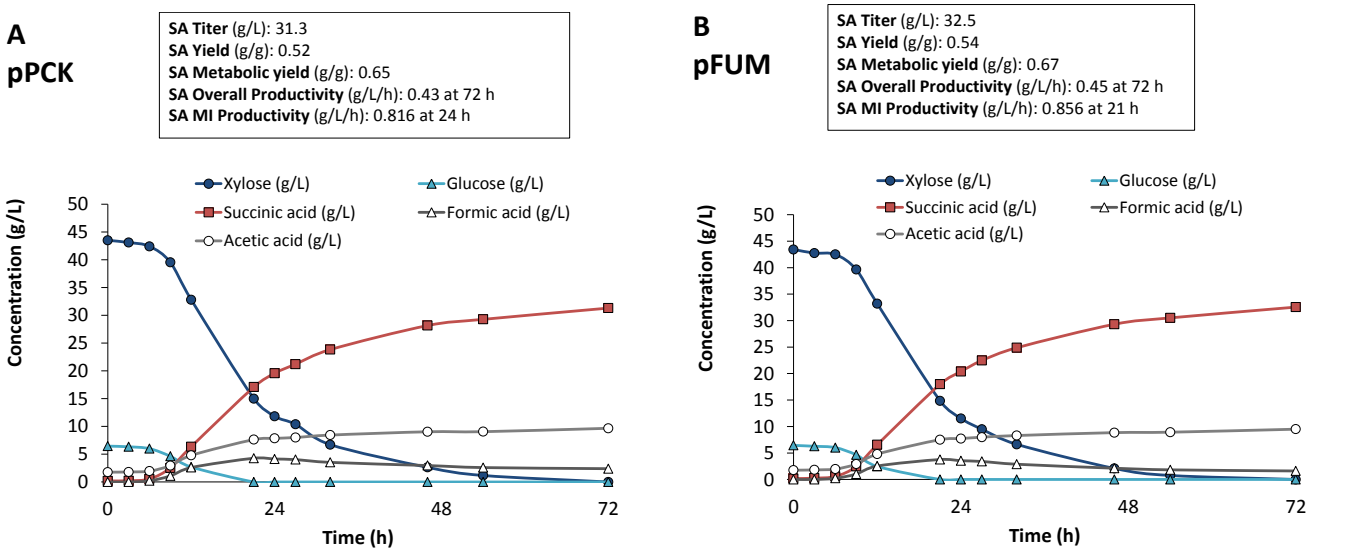

Fig. S1: Evaluation of different fermentation and metabolic parameters in (A) pPCK and (B) pFUM strains. Scatter plots present utilization of xylose and glucose, and acids production such as SA, acetic acid, and formic acid. In the boxes, SA titers, yields and metabolic yields, and overall and maximum instantaneous productivities are presented. SA "yield" is calculated as the coefficient of SA (g/L) and the sugars consumed (g/L) at the end of the fermention. SA "metabolic yield" is calculated as the yield but considering the dilution factor at the end of the fermentation.

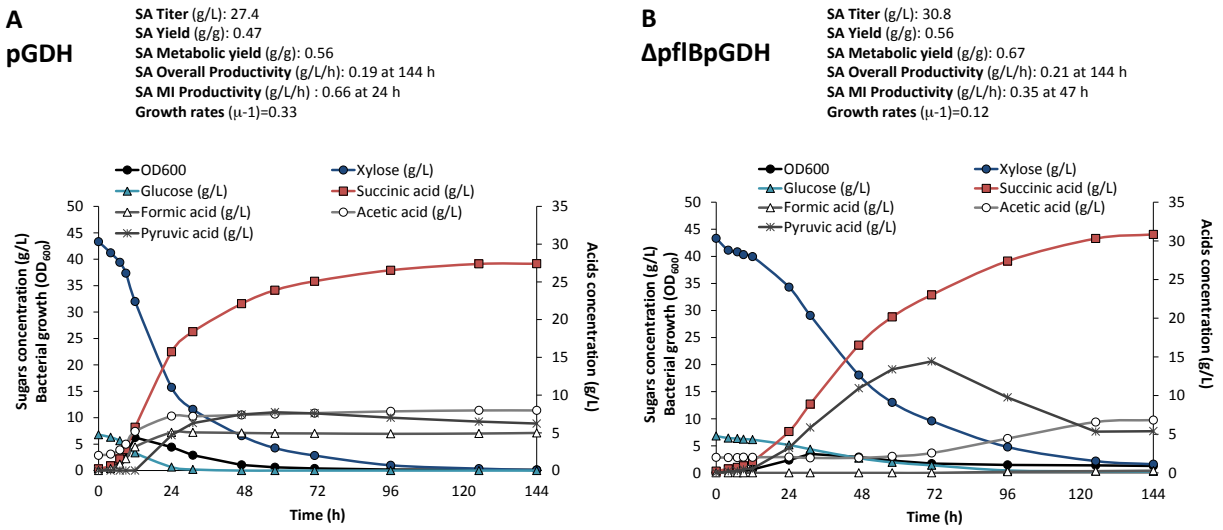

Fig. S2: Evaluation of different fermentation and metabolic parameters in (A) pPCK and (B) pFUM strains. Scatter plots present bacterial growth, utilization of xylose and glucose, and acids production such as SA, acetic acid, formic acid, and pyruvic acid. In the boxes, growth rates, SA titers, yields and metabolic yields, and overall and maximum instantaneous productivities are presented. SA "yield" is calculated as the coefficient of SA (g/L) and the sugars consumed (g/L) at the end of the fermention. SA "metabolic yield" is calculated as the yield but considering the dilution factor at the end of the fermentation.

Tables

Table S1: T-test (at 95% confidence level, non-pair, and homoscedastic) for titers, yields, and productivities between the wild-type (with at least 4 biological replicates) and engineered strains (biological replicates). Gray boxes highlight the significant differences among strains (*p* value <0.05). Precision in wild type was calculated as (100-(SD*100/AV)) being SD the standard deviation of the different replicates and AV the average value.

Fermentation parameter	Precision in wild-type*	*p*-value							
	≥ 4 biological replicates	AackA	ApflB	ΔpflBΔackA	pPMF	pMDH	ΔackApPMF	ΔpflBpPMF	pflBΔackApPMF
Titers	94.6%	0.008	0.630	0.029	0.035	0.034	0.987	35.3%	7.1%
Yield	95.9%	0.005	0.666	0.030	0.010	0.022	0.320	79.3%	0.015
Metabolic yield	96.0%	0.002	0.419	0.006	0.016	0.017	0.114	55.0%	0.008
Overall productivity	91.8% (96 h)	0.037	0.617	0.045	0.276	0.205	0.006	0.007	0.004
Max productivity	89.3% (12 h)**	0.002	0.030	0.002	0.446	0.944	0.001	0.001	0.001

* The error in wild-type reflects both the biological variation in the fermentation and the analytical methods.
** The larger variation in maximum productivity in wild-type might be due to the slightly different initial lags on bacterial growth which, at shorter periods of times, can present larger differences.

Table S2. Details of the carbon balance calculations. All values are reported in millimoles of carbon, error ranges are reported as mean ± standard deviation when multiple measurements were available. Carbon dioxide consumption and production was estimated based on known metabolic pathways in *A. succinogenes* and the measured fermentation products: each mole of succinate produced consumed one mole of CO_2, and CO_2 was produced from ethanol and acetate production after subtracting the formate produced. Carbon moles in cell-dry biomass were calculated from CHN elemental analyses as detailed in materials and methods, and percent closure was calculated as the carbon moles of products divided by that of sugar consumed.

Strain	Glucose	Xylose	Galactose	Arabinose
pPMF	-71.8 ± 0.17	-457 ± 4.4	-4.89 ± 0.63	-53.9 ± 3.4
pMDH	-73.2 ± 0.71	-460 ± 0.0	-0.553 ± 2.0	-48.3 ± 2.9
130z	-78.6 ± 13	-422 ± 48	-7.19 ± 1.4	-54.8 ± 9.1

Strain	Succinic	Lactic	Formic	Acetic	Ethanol	Pyruvic	CO_2	Biomass	% Closure
pPMF	429 ± 16	-3.42 ± 4.9e-3	5.90 ± 0.60	108 ± 3.1	-1.30 ± 1.8	0.00 ± 0.0	-59.1 ± 1.8	84.3 ± 9.7	96.2 ± 0.20
pMDH	422 ± 5.3	-2.94 ± 0.40	14.5 ± 4.0	120 ± 3.0	0.00 ± 0.0	0.00 ± 0.0	-60.1 ± 1.1	87.4 ± 2.7	99.9 ± 0.28
130z	392 ± 35	1.55 ± 1.4	27.8 ± 5.7	107 ± 15	0.775 ± 0.73	24.7 ± 26	-71.9 ± 9.9	95.9	93.5

Table S3. Details of the degree of reduction balance. Degree of reduction values, γ, quantify the number of electrons in a molecule with regards to a number of common reference compounds *(1)*. Each atom in a molecule contributes the degree of reduction, in which C=4, H=1, O=-2, N=-3. We perform a redox balance by calculating γ values for each compound and multiplying by the millimoles consumed and/or produced. Error ranges are reported as mean ± standard deviation when multiple measurements were available. Biomass degree of reduction assumes an elemental composition of $CH_2O_{0.5}N_{0.2}$. Percent closures are calculated as the sum of products (second table) divided by that of the substrates (first table).

Strain	Glucose (γ=24)	Xylose (γ=20)	Galactose (γ=24)	Arabinose (γ=20)
pPMF	-287 ± 0.69	-1830 ± 17	-19.5 ± 2.5	-216 ± 14
pMDH	-293 ± 2.8	-1840 ± 0.0	-2.21 ± 7.9	-193 ± 12
130z	-314 ± 53	-1690 ± 1.9e+2	-28.7 ± 5.6	-219 ± 36

Strain	Succinic (γ=14)	Lactic (γ=12)	Formic (γ=2)	Acetic (γ=8)	Ethanol (γ=12)	Pyruvic (γ=10)	Biomass (γ=4.4)	% Closure
pPMF	1500 ± 55	-6.84 ± .010	11.8 ± 1.2	432 ± 12	-7.81 ± 11	0.00 ± 0.0	371 ± 43	98.3 ± 0.37
pMDH	1480 ± 18	-5.89 ± 0.79	28.9 ± 7.9	479 ± 12	0.00 ± 0.0	0.00 ± 0.0	384 ± 12	102 ± 0.25
130z	1370 ± 120	3.10 ± 2.8	55.7 ± 11	428 ± 62	4.65 ± 4.4	82.2 ± 86	422	95.8

References

1. **Smolke C.** 2009. The metabolic pathway engineering handbook: tools and applications, vol 2. CRC press.