Appendix

Model-guided development of evolutionarily stable yeast chassis

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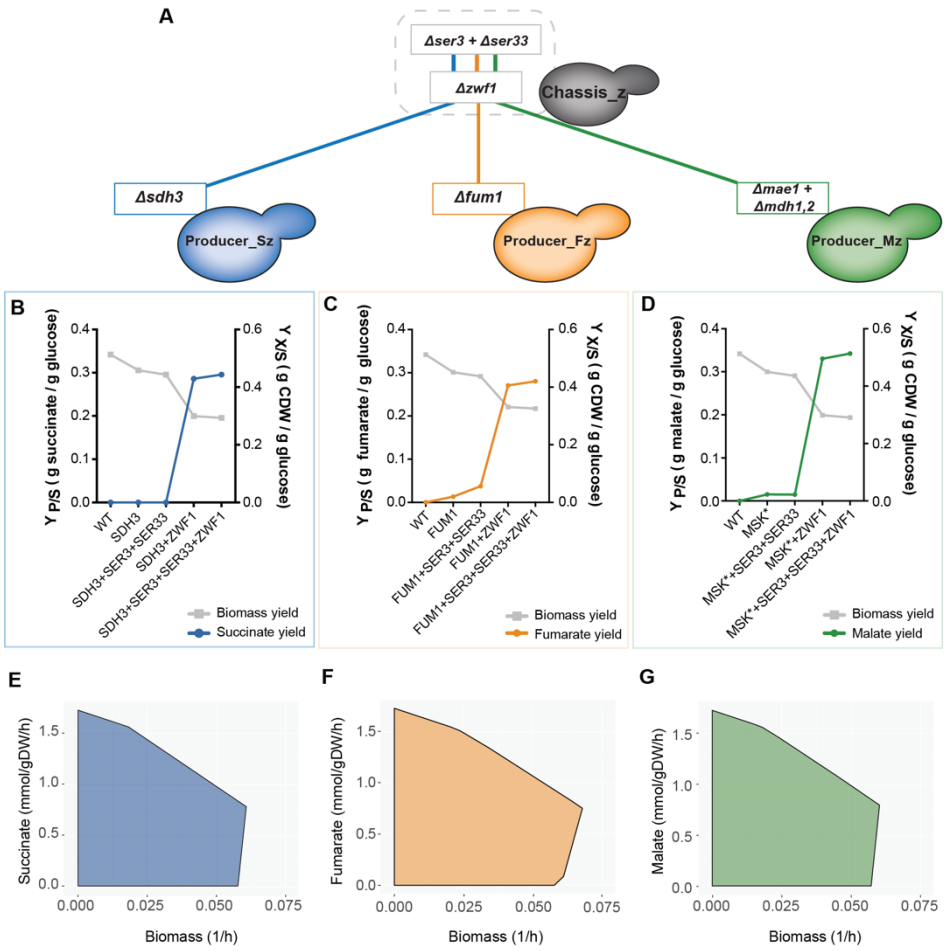


Appendix Figure S1. **Predicted effect of gene deletion targets for C4-dicarboxilic acids production.**

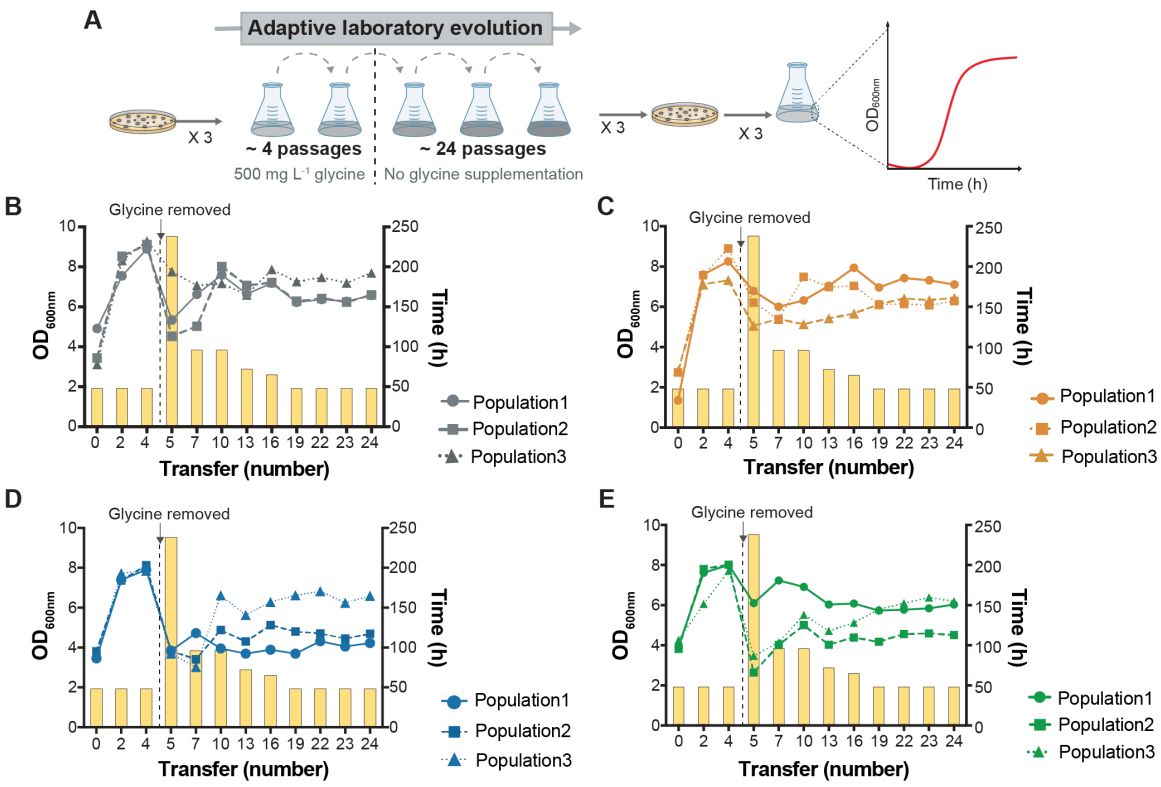
A. Illustrates the proposed strategy, simulated *in silico*, for chassis strain design (deletion of *ZWF1*, *SER3* and *SER33*) as well as the additional knockouts required for succinate (*SDH*-complex), fumarate (*FUM1*) and malate (*MDH1*, *MDH2* and *MDH3*) overproduction.

B, C, D. Panels Growth-coupling impact of the predicted knockouts on succinate (**B**), fumarate (**C**) and malate (**D**) production at product yield and biomass yield predicted using pFBA and iMM904. Only the combinations of knockouts selected for *in vivo* implementation are presented. WT stands for wild-type and MSK* stands for *MAE1+MDH1+MDH2* the "minimal set of knockouts" required for malate production.

E Predicted 'Production envelop' for succinate production in the *in vivo* implemented solution (*Δser3,33Δzwf1Δsdh3*).

F Predicted 'Production envelop' for fumarate production in the *in vivo* implemented solution (*Δser3,33Δzwf1Δfum1*).

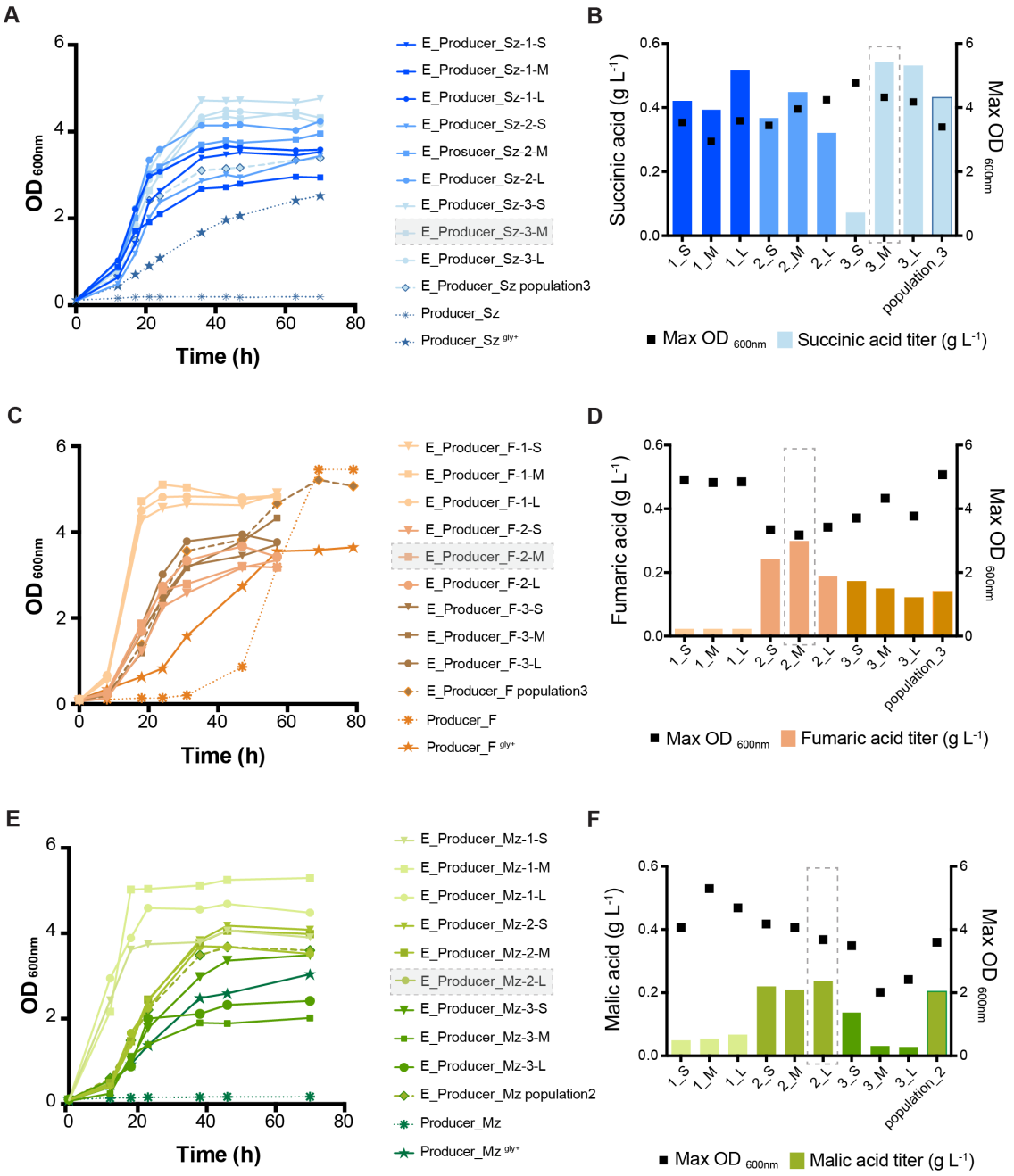
G Predicted 'Production envelop' for malate production in the *in vivo* implemented solution (*Δser3,33Δzwf1Δmae1Δmdh1,2*) with impaired fumarate drain.



Appendix Figure S2. Adaptive laboratory evolution experimental approach and fitness profile of the paralleled evolved populations during ALE.

A. Scheme of the ALE experimental set-up and isolation of final evolved strains.

B,C,D,E. Fitness profile of paralleled evolved populations. For each population, The OD_{600} and the cultured time (h) were recorded - for all transfers, before transferring it into fresh media. The three independent populations of all parental strains are represented by round, triangle and square symbols for Chassis_z (**B**), Producer_F (**C**), Producer_Sz (**D**) and Producer_Mz (**E**).



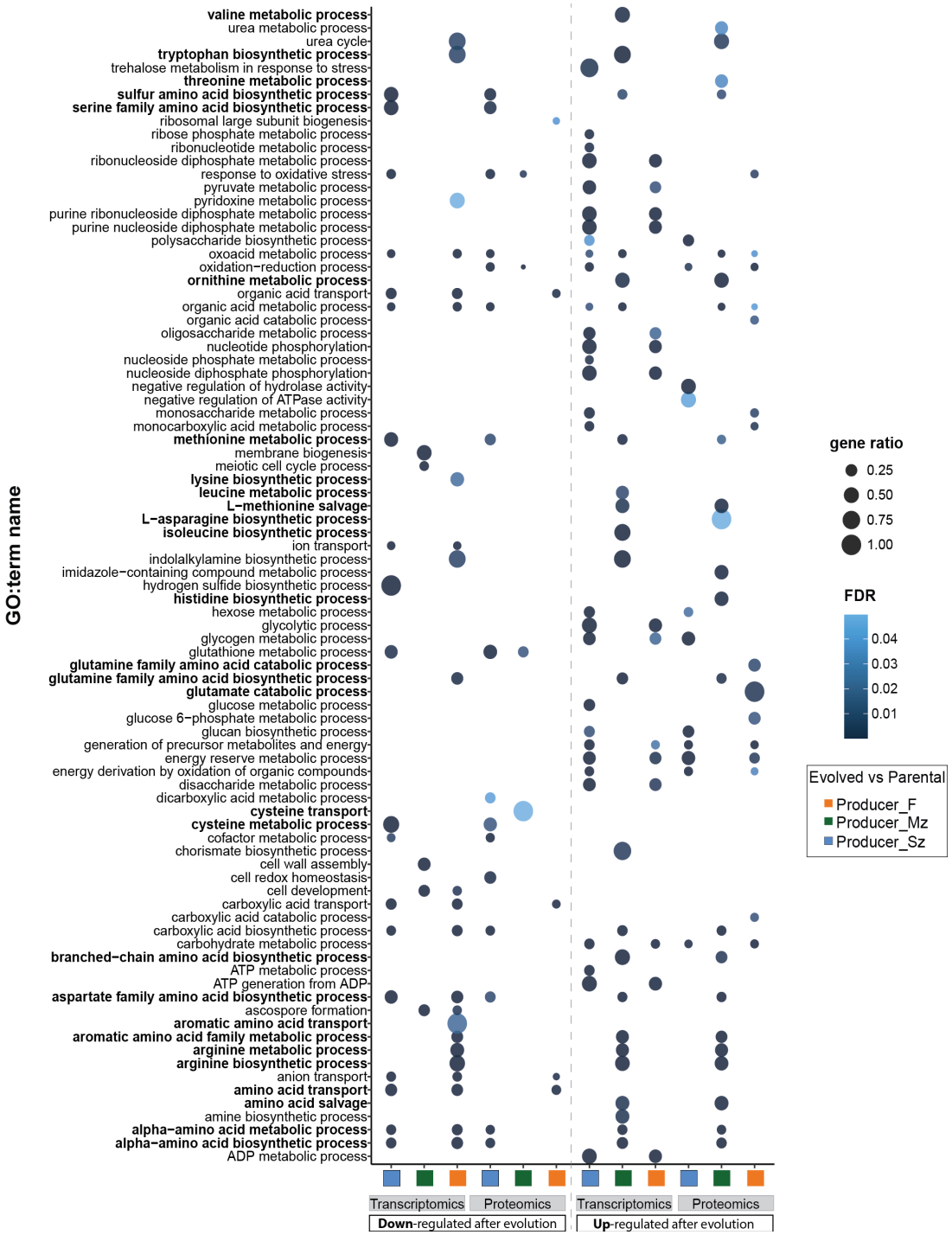
Appendix Figure S3. Screening of three isolated strains from evolved populations.

A,B. Growth profile (**A**) and succinate production for evolved Producer_Sz isolated strains and Population 3 (**B**).

C,D. Growth profile (**C**) and fumarate production for evolved Producer_F isolated strains and Population 3 (**D**).

E,F. Growth profile (**E**) and malate production for evolved Producer_Mz isolated strains and Population 2 (**F**).

Data information: Target compounds titer was evaluated at the end of growth curve (Max OD_{600}). The OD_{600} (**A**, **C** and **E**) was measured during cultivation for the 9 isolated colonies, one population and the respective parental strain without or with glycine supplementation ($^{+gly}$). Populations (1,2 or 3) were plated in solid minimal media with 20 g L^{-1} of glucose and three isolated colonies (named S, M or L) were selected for characterization.



Appendix Figure S4. GO term analyses of differentially expressed genes (Transcriptomics) and protein abundances (Proteomics) between evolved and parental strains.

GO term enrichment analysis performed with gProfiler in RStudio. Dot colour (false discovery rate, FDR) and size (number of genes in query/number of genes associated to given GO:term, gene ratio). GO:term names highlighted in bold are related with amino acid metabolism.

Appendix Table S1. List of modifications to the original iMM904 model.

Modification	Details	Source
Added reaction r_4039	acetate_m + succinyl_CoA_m<---->succinate_m + acetyl_CoA_m	Yeast 7.11
Modified r_0718	NADP_m + S_malate_m----->NADPH_m + pyruvate_m + carbon_dioxide_m	Yeast 7.11
Modified r_0773	NADH_m + H_m + ubiquinone_6_m-----> NAD_m + ubiquinol_6_m	Yeast 7.11
Modified r_0226	4 H_c + 1 ADP_m + 1 phosphate_m -----> 3 H_m + 1 H2O_m + 1 ATP_m	Yeast 7.11
Modified r_2129	H_m <----- H_c	Yeast 7.11
Modified r_1110	ADP_c + ATP_m <----> ATP_c + ADP_m	Yeast 7.11
Modified r_0470	NAD_c + H2O_c + L_glutamate_c -----> H_c + NADH_c + ammonium_c + 2_oxoglutarate_c	Yeast 7.11
Removed r_0303	citrate_c -----> H2O + cis_aconitate_c	Yeast 7.11
Removed r_0338	ubiquinone_6_m + S_dihydroorotate_c -----> ubiquinol_6_m + orotate_c	Yeast 7.11
Removed r_0339	oxygen_c + S_dihydroorotate_c -----> hydrogen_peroxide_c + orotate_c	Yeast 7.11
Removed r_2127	NAD_c + S_dihydroorotate_c -----> NADH_c + orotate_c	Yeast 7.11
Modified r_1254	H_e + pyruvate_e <----> H_c + pyruvate_c	Yeast 7.11
Gene rule update r_0530	(YPL252C /YDR376W) or (YPL252C/ YDR376W/ YER141W)	Yeast 7.11
Updated biomass reaction r_4041	Add 1.0E-6 * chitin_c and 1.0E-6* heme_a_m to reactants	Yeast 7.11
Gene rule update r_0076	(YER037W or YGL224C)	Yeast 7.11
Gene rule update r_0078	(YER037W or YGL224C)	Yeast 7.11
Gene rule update r_1619	YER037W	Yeast 7.11
Removed r_0333	phosphate_c + 2_deoxyuridine_c <----> uracil_c + 2_deoxy_D_ribofuranose_1_phosphate_c	Yeast 7.11
Removed r_0944	phosphate_c + adenosine_c<----> alpha_D_ribose_1_phosphate_c + adenine_c	Yeast 7.11
Removed r_0945	phosphate_m + adenosine_m <----> adenine_m + alpha_D_ribose_1_phosphate_m	Yeast 7.11
Removed r_0946	phosphate_c + 2_deoxyadenosine_c <----> 2_deoxy_D_ribofuranose_1_phosphate_c + adenine_c	Yeast 7.11
Removed r_0947	phosphate_c + 2_deoxyguanosine_c <----> guanine_c + 2_deoxy_D_ribofuranose_1_phosphate_c	Yeast 7.11
Removed r_0948	phosphate_c + 2_deoxyinosine_c <----> hypoxanthine_c + 2_deoxy_D_ribofuranose_1_phosphate_c	Yeast 7.11
Removed r_0952	phosphate_c + xanthosine_c <----> alpha_D_ribose_1_phosphate_c + 9H_xanthine_c	Yeast 7.11
Removed r_1044	phosphate_c + thymidine_c <----> 2_deoxy_D_ribofuranose_1_phosphate_c + thymine_c	Yeast 7.11
Added reaction r_4045	H2O_c + uridine_c -----> uracil_c + D_ribose_c	Yeast 7.11
Gene rule update r_0888	(YMR105C or YKL127W)	Yeast 7.11
Gene rule update r_0907	(YMR278W or YMR105C) or YKL127W)	Yeast 7.11
Modified r_0110	H_c + coenzyme_A_c + acetate_c <----- H2O_c + acetyl_CoA_c	Pereira et al., 2016
Added reaction r_0234x	NAD_c + zymosterol_intermediate_1c_c -----> H_c + NADH_c + carbon_dioxide_c + zymosterol_intermediate_2_c	Pereira et al., 2016
Added reaction r_0939x	prephenate_c + NAD_c -----> NADH_c + 3_4_hydroxyphenylpyruvate_c + carbon_dioxide_c	Pereira et al., 2016
Modified r_1117	H_c + L_aspartate_c -----> H_m + L_aspartate_m	Pereira et al., 2016
Inactivated r_1840	3_hydroxy_3_methylglutaryl_CoA_c <-----> 3_hydroxy_3_methylglutaryl_CoA_m	No data supporting this reaction

Appendix Table S2. Contribution of each gene knockout for succinate overproduction.

Gene Deletion(s)	Inactivated reaction(s)	Biomass rate [1]	Succinate Flux [2]	BPCY [3]	CYIELD [4]	Product Yield ($Y_{P/S}$) [5]	Biomass Yield ($Y_{X/S}$) [6]
Wild-type	-	0.1062	0.00000	0.00000	0.00000	0.00000	0.51266
SDH3	R_SUCD1m; R_SUCD2_u6m; R_SUCD3_u6m	0.0950	0.00006	0.00000	0.00003	0.00002	0.45853
SDH3+SER3	R_SUCD1m; R_SUCD2_u6m; R_SUCD3_u6m	0.0950	0.00006	0.00000	0.00003	0.00002	0.45853
SDH3+SER33	R_SUCD1m; R_SUCD2_u6m; R_SUCD3_u6m	0.0950	0.00006	0.00000	0.00003	0.00002	0.45853
SDH3+MET2	R_SUCD1m; R_SUCD2_u6m; R_SUCD3_u6m; R_HSERTA	0.0950	0.00006	0.00000	0.00003	0.00002	0.45853
SDH3+ZWF1	R_SUCD1m; R_SUCD2_u6m; R_SUCD3_u6m; R_G6PDH2	0.0621	0.75267	0.04064	0.43633	0.28600	0.29973
SDH3+SER3+MET2	R_SUCD1m; R_SUCD2_u6m; R_SUCD3_u6m; R_HSERTA	0.0950	0.00006	0.00000	0.00003	0.00002	0.45853
SDH3+SER33+MET2	R_SUCD1m; R_SUCD2_u6m; R_SUCD3_u6m; R_HSERTA	0.0950	0.00006	0.00000	0.00003	0.00002	0.45853
SDH3+SER3+SER33	R_SUCD1m; R_SUCD2_u6m; R_SUCD3_u6m; R_PGCD	0.0918	0.00015	0.00001	0.00009	0.00006	0.44308
SDH3+ZWF1+SER3	R_SUCD1m; R_SUCD2_u6m; R_SUCD3_u6m; R_G6PDH2	0.0621	0.75267	0.04064	0.43633	0.28600	0.29973
SDH3+ZWF1+SER33	R_SUCD1m; R_SUCD2_u6m; R_SUCD3_u6m; R_G6PDH2	0.0621	0.75267	0.04064	0.43633	0.28600	0.29973
SDH3+ZWF1+MET2	R_SUCD1m; R_SUCD2_u6m; R_SUCD3_u6m; R_G6PDH2; R_HSERTA	0.0620	0.75307	0.04060	0.43656	0.28615	0.29925
SDH3+SER3+SER33+ MET2	R_SUCD1m; R_SUCD2_u6m; R_SUCD3_u6m; R_PGCD; R_HSERTA	0.0914	0.00015	0.00001	0.00009	0.00006	0.44115
SDH3+SER3+MET2+ ZWF1	R_SUCD1m; R_SUCD2_u6m; R_SUCD3_u6m; R_G6PDH2; R_HSERTA	0.0620	0.75307	0.04060	0.43656	0.28615	0.29925
SDH3+SER33+MET2+ ZWF1	R_SUCD1m; R_SUCD2_u6m; R_SUCD3_u6m; R_G6PDH2; R_HSERTA	0.0620	0.75307	0.04060	0.43656	0.28615	0.29925
SDH3+SER33+SER33+ ZWF1	R_SUCD1m; R_SUCD2_u6m; R_SUCD3_u6m; R_PGCD; R_G6PDH2	0.0608	0.77816	0.04114	0.45111	0.29569	0.29346
SDH3+SER3+SER33+ MET2+ZWF1	R_SUCD1m; R_SUCD2_u6m; R_SUCD3_u6m; R_PGCD; R_G6PDH2; R_HSERTA	0.0606	0.78075	0.04114	0.45261	0.29667	0.29249

[1] h^{-1}

[2] $mmol.gCDW^{-1}.h^{-1}$

[3] $mol\ succinate.\ mol\ glucose^{-1}.h^{-1}$

[4] $C\text{-}mol\ succinate.\ C\text{-}mol\ glucose^{-1}$

[5] $g\ succinate.\ g\ glucose^{-1}$

[6] g CDW. g glucose

Appendix Table S3. Contribution of each gene knockout for fumarate overproduction.

Gene Deletion(s)	Inactivated reaction(s)	Biomass rate [1]	Fumarate Flux [2]	BPCY [3]	CYIELD [4]	Product Yield ($Y_{P/S}$) [5]	Biomass Yield ($Y_{X/S}$) [6]
Wild-type	-	0.1062	0.00000	0.00000	0.00000	0.00000	0.51266
FUM1	R_FUM; R_FUMm	0.0936	0.03527	0.00287	0.02045	0.01317	0.45177
FUM1+SER3	R_FUM; R_FUMm	0.0936	0.03527	0.00287	0.02045	0.01317	0.45177
FUM1+SER33	R_FUM; R_FUMm	0.0936	0.03527	0.00287	0.02045	0.01317	0.45177
FUM1+MET2	R_FUM; R_FUMm; R_HSERTA	0.0935	0.03525	0.00287	0.02043	0.01317	0.45129
FUM1+ZWF1	R_FUM; R_FUMm; R_G6PDH2	0.0686	0.72404	0.04319	0.41973	0.27042	0.33111
FUM1+SER3+MET2	R_FUM; R_FUMm; R_HSERTA	0.0935	0.03525	0.00287	0.02043	0.01317	0.45129
FUM1+SER33+MET2	R_FUM; R_FUMm; R_HSERTA	0.0935	0.03525	0.00287	0.02043	0.01317	0.45129
FUM1+SER3+SER33	R_FUM; R_FUMm; R_PGCD	0.0906	0.10056	0.00792	0.05830	0.03756	0.43729
FUM1+ZWF1+SER3	R_FUM; R_FUMm; R_G6PDH2	0.0690	0.7175	0.04305	0.41594	0.26798	0.33304
FUM1+ZWF1+SER33	R_FUM; R_FUMm; R_G6PDH2	0.0690	0.7175	0.04305	0.41594	0.26798	0.33304
FUM1+ZWF1+MET2	R_FUM; R_FUMm; R_G6PDH2; R_HSERTA	0.0686	0.72444	0.04321	0.41997	0.27057	0.33111
FUM1+SER3+SER33+MET2	R_FUM; R_FUMm; R_PGCD; R_HSERTA	0.0902	0.10932	0.00857	0.06337	0.04083	0.43536
FUM1+SER3+MET2+ZWF1	R_FUM; R_FUMm; R_G6PDH2; R_HSERTA	0.0686	0.72444	0.04321	0.41997	0.27057	0.33111
FUM1+SER33+MET2+ZWF1	R_FUM; R_FUMm; R_G6PDH2; R_HSERTA	0.0686	0.72444	0.04321	0.41997	0.27057	0.33111
FUM1+SER33+SER33+ZWF1	R_FUM; R_FUMm; R_PGCD; R_G6PDH2	0.0675	0.75026	0.04404	0.43493	0.28021	0.32580
FUM1+SER3+SER33+MET2+ ZWF1	R_FUM; R_FUMm; R_PGCD; R_G6PDH2; R_HSERTA	0.0673	0.75292	0.04406	0.43648	0.28120	0.32483

[1] h^{-1}

[2] $mmol.gCDW^{-1}.h^{-1}$

[3] mol fumarate. $mol\ glucose^{-1}.h^{-1}$

[4] C-mol fumarate. $C\text{-}mol\ glucose^{-1}$

[5] g fumarate. $g\ glucose^{-1}$

[6] g CDW. $g\ glucose^{-1}$

Appendix Table S4. Contribution of each gene knockout for malate overproduction.

Gene Deletion(s)	Inactivated reaction(s)	Biomass rate[1]	Malate Flux[2]	BPCY [3]	CYIELD [4]	Product Yield ($Y_{P/S}$) [5]	Biomass Yield ($Y_{X/S}$) [6]
Wild-type	-	0.1062	0.00000	0.0000	0.0000	0.00000	0.51266
MSK (*MDH1+MDH2+ MAE1*)	R_ME2m; R_ME1m; R_MDHm; R_MDH	0.0932	0.03507	0.0028	0.0203	0.01513	0.44984
MSK+*SER3*	R_ME2m; R_ME1m; R_MDHm; R_MDH	0.0932	0.03507	0.0028	0.0203	0.01513	0.44984
MSK+*SER33*	R_ME2m; R_ME1m; R_MDHm; R_MDH	0.0932	0.03507	0.0028	0.0203	0.01513	0.44984
MSK+*MET2*	R_ME2m; R_ME1m; R_MDHm; R_MDH; R_HSERTA	0.0931	0.03505	0.0028	0.0203	0.01512	0.44936
MSK+*IRC7*	R_ME2m; R_ME1m; R_MDHm; R_MDH; R_THRAi; R_THRA2i	0.0932	0.03507	0.0028	0.0203	0.01513	0.44984
MSK+*ZWF1*	R_ME2m; R_ME1m; R_MDHm; R_MDH; R_G6PDH2	0.0619	0.76549	0.0412	0.4438	0.33028	0.29877
MSK+*MET2+SER3*	R_ME2m; R_ME1m; R_MDHm; R_MDH; R_HSERTA	0.0931	0.03505	0.0028	0.0203	0.01512	0.44936
MSK+*MET2+SER33*	R_ME2m; R_ME1m; R_MDHm; R_MDH; R_HSERTA	0.0931	0.03505	0.0028	0.0203	0.01512	0.44936
MSK+*SER3+GLY1*	R_ME2m; R_ME1m; R_MDHm; R_MDH; R_THRAi; R_THRA2i	0.0932	0.03985	0.0032	0.0231	0.01719	0.44984
MSK+*SER33+IRC7*	R_ME2m; R_ME1m; R_MDHm; R_MDH; R_THRAi; R_THRA2i	0.0932	0.03507	0.0028	0.0203	0.01513	0.44984
MSK+*MET2+IRC7*	R_ME2m; R_ME1m; R_MDHm; R_MDH; R_HSERTA; R_THRAi; R_THRA2i	0.0932	0.03507	0.0028	0.0203	0.01513	0.44984
MSK+*MET2+ZWF1*	R_ME2m; R_ME1m; R_MDHm; R_MDH; R_HSERTA; R_G6PDH2	0.0619	0.76587	0.0412	0.4440	0.33045	0.29877
MSK+*ZWF1+SER3*	R_ME2m; R_ME1m; R_MDHm; R_MDH; R_G6PDH2	0.0619	0.76549	0.0412	0.4438	0.33028	0.29877
MSK+*ZWF1+SER33*	R_ME2m; R_ME1m; R_MDHm; R_MDH; R_G6PDH2	0.0619	0.76549	0.0412	0.4438	0.33028	0.29877
MSK+*SER3+SER33*	R_ME2m; R_ME1m; R_MDHm; R_MDH; R_PGCD	0.0903	0.03445	0.0027	0.0200	0.01486	0.43584
MSK+*SER3+SER33+MET2*	R_ME2m; R_ME1m; R_MDHm; R_MDH; R_PGCD; R_HSERTA	0.0899	0.03431	0.0027	0.0199	0.01480	0.43391
MSK+*SER33+SER3+ZWF1*	R_ME2m; R_ME1m; R_MDHm; R_MDH; R_PGCD; R_G6PDH2	0.0602	0.79314	0.0415	0.4598	0.34221	0.29056
MSK+*SER33+SER3+IRC7*	R_ME2m; R_ME1m; R_MDHm; R_MDH; R_PGCD; R_THRAi; R_THRA2i	0.0903	0.03445	0.0027	0.0200	0.01486	0.43584
MSK+*SER3+MET2+IRC7*	R_ME2m; R_ME1m; R_MDHm; R_MDH; R_HSERTA; R_THRAi; R_THRA2i	0.0927	0.04426	0.0036	0.0257	0.01910	0.44743
MSK+*SER33+MET2+IRC7*	R_ME2m; R_ME1m; R_MDHm; R_MDH; R_HSERTA; R_THRAi; R_THRA2i	0.0927	0.04426	0.0036	0.0257	0.01910	0.44743
MSK+*MET2+ZWF1+IRC7*	R_ME2m; R_ME1m; R_MDHm; R_MDH; R_HSERTA; R_G6PDH2; R_THRAi; R_THRA2i	0.0616	0.7708	0.0413	0.4468	0.33258	0.29732
MSK+*SER33+SER3+ IRC7+MET2*	R_ME2m; R_ME1m; R_MDHm; R_MDH; R_PGCD; R_THRAi; R_THRA2i; R_HSERTA	0.0895	0.04321	0.0034	0.0250	0.01864	0.43198
MSK+*SER33+SER33+ IRC7+ZWF1*	R_ME2m; R_ME1m; R_MDHm; R_MDH; R_PGCD; R_THRAi; R_THRA2i; R_G6PDH2	0.0602	0.79314	0.0415	0.4598	0.34221	0.29056
MSK+*SER33+SER33+ IRC7+MET2+ZWF1*	R_ME2m; R_ME1m; R_MDHm; R_MDH; R_PGCD; R_THRAi; R_THRA2i; R_HSERTA; R_G6PDH2	0.0597	0.8005	0.0416	0.4641	0.34539	0.28815

[1] h^{-1}

[2] $mmol.gCDW^{-1}.h^{-1}$

[3] mol fumarate. $mol\ glucose^{-1}.h^{-1}$

[4] C-mol fumarate. $C\text{-}mol\ glucose^{-1}$

[5] g fumarate. $g\ glucose^{-1}$

[6] g CDW. $g\ glucose^{-1}$

Appendix Table S5. Impact of each gene deletion in the selected solutions using LMOMA and the iMM904 model.

Target compounds	Gene Deletion(s)	Biomass rate[1]	Target Flux[2]	BPCY[3]	CYIELD[4]
Succinate	SDH-complex *	0.00792	0.96589	0.00665	0.55994
	SDH-c+*ZWF1*	0.00000	1.01621	0.00000	0.58911
	SDH-c+*SER3*+*SER33*	0.00424	1.00873	0.00372	0.58477
	SDH-c+*SER3*+*SER33*+*ZWF1*	0.00000	1.09639	0.00000	0.63559
Fumarate	FUM1	0.03327	0.87633	0.02535	0.50802
	FUM1+*ZWF1*	0.02894	0.92834	0.02336	0.53817
	FUM1+*SER3*+*SER33*	0.03458	0.86465	0.02600	0.50125
	FUM1+*SER3*+*SER33*+*ZWF1*	0.02684	0.95174	0.02221	0.55173
Malate	MSK **	0.01910	0.93368	0.01551	0.54126
	MSK+*ZWF1*	0.01050	0.12954	0.00118	0.07510
	MSK+*SER3*+*SER33*	0.01687	0.83475	0.01225	0.48391
	MSK+*SER3*+*SER33*+*ZWF1*	0.00788	0.98084	0.00672	0.56860
	Wild-type	0.10622	0.00000	0.00000	0.00000

[1] h^{-1}

[2] $mmol.gCDW^{-1}.h^{-1}$

[3] $mol\ target\ product.\ mol\ glucose^{-1}.h^{-1}$

[4] $C\text{-}mol\ target\ product.\ C\text{-}mol\ glucose^{-1}$

* SDH-c = SDH-complex

* MSK = MDH1+MDH2+MAE1

Appendix Table S6. Analysis of the *in-silico* productivity values using different *S. cerevisiae* GSMMs and simulation methods. Simulations were performed in Optflux v3.2.8, using CPLEX ILOG solver, with a glucose uptake rate of 1.15 mmol.gCDW.h^{-1}.

				CYIELD[1]		BPCY[2]	
Target compounds	**Gene deletions**		**GSMM**	**pFBA**	**LMOMA**	**pFBA**	**LMOMA**
Fumarate	*SER 3*		iMM904*	0.4349	0.5517	0.044	0.0222
	SER 33	*FUM1*	iND750	0.4210	0.5331	0.0452	0.0209
	ZWF1		Yeast 6*	0.4657	0.584	0.0348	0.0069
Succinate	*SER 3*		iMM904*	0.4511	0.6356	0.0411	0.0000
	SER 33	*SDH2*	iND750	0.0603	0.5206	0.006	0.0000
	ZWF1		Yeast 6*	0.0411	0.6021	0.003	0.0000
Malate	*SER3*	*MAE1*	iMM904*	0.4598	0.5686	0.0415	0.0067
	SER33	*MDH1*	iND750	0.421	0.0452	0.0236	0.0009
	ZWF1	*MDH2*	Yeast 6*	0.0098	0.1885	0.0007	0.0000

[1] C-mol target product. C-mol glucose^{-1}.h^{-1}

[2] mol target product. mol glucose^{-1}.h^{-1}

* Modified versions (see Supplementary Table 1)

Appendix Table S7. *Saccharomyces cerevisiae* strains used in this study.

Strain name [a]	Genotype	Details	Source
CEN.PK119	*MATa/MATalfa URA3/ura3-52 MAL2-* 8c/ *MAL2-* 8c SUC2/ SUC2	(diploid wild-type)	P. Kötter[b]
WT (CEN.PK113-7D)	*MATa URA3 MAL2-* 8^c *SUC2*	(wild-type, reference strain)	P. Kötter[b]
Chassis	MATa *URA3 MAL2-* 8c *SUC2* ser3Δ ser33Δ	ser3(1,1240)::loxP-**kanMX4**-loxP ser33(1,1090)::loxP-**natMX4**-loxP	This study
Chassis_z	*MATa URA3 MAL2-* 8^c *SUC2 ser3Δ ser33Δ zwf1Δ*	ser33(1,1090)::loxP-**natMX4**-loxP ser3(1,1240)::loxP-**kanMX4**-loxP zwf1(41,1475)::loxP-**kanMX4**-loxP	This study
S1	MATa URA3 MAL2- 8^c SUC2 *sdh3Δ*	sdh3(1,540)::loxP-**hphMX4**-loxP	This study
Producer_S	MATa URA3 MAL2-8^c SUC2 *ser3Δ ser33Δ sdh3Δ*	ser3(1,1240)::loxP-**kanMX4**-loxP ser33(1,1090)::loxP-**natMX4**-loxP sdh3(1,540)::loxP-**hphMX4**-loxP	This study
Producer_Sz	MATa URA3 MAL2- 8c SUC2 *ser3Δ ser33Δ sdh3Δ zwf1Δ*	ser33(1,1090)::loxP-**natMX4**-loxP sdh3(1,540)::loxP-**hphMX4**-loxP ser3(1,1240)::loxP-**kanMX4**-loxP zwf1(41,1475)::loxP-**kanMX4**-loxP	This study
M1	MATa URA3 MAL2- 8c SUC2 *mdh1Δ mdh2Δ mae1Δ*	mdh1(1,900)::loxP-**natMX4**-loxP mdh2(41,1259)::loxP-**hphMX4**-loxP mae1(41,1269)::loxP-**KanMX**-loxP	This study
Producer_M	MATa URA3 MAL2- 8c SUC2 *ser3Δ ser33Δ mdh1Δ mdh2Δ mae1Δ*	ser3(1,1240)::loxP-**kanMX4**-loxP ser33(1,1090)::loxP-**natMX4**-loxP mdh1(1,900)::loxP-**natMX4**-loxP mdh2(41,1094)::loxP-**hphMX4**-loxP mae1(41,1269)::loxP-**hphMX4**-loxP	This study
Producer_Mz	*MATa URA3 MAL2-* 8^c *SUC2 ser3Δ ser33Δ zwf1Δ mdh1Δ mdh2Δ mae1Δ*	zwf1(41,1475)::loxP-**kanMX4**-loxP ser3(1,1240)::loxP-**kanMX4**-loxP ser33(1,1090)::loxP-**natMX4**-loxP mdh1(1,900)::loxP-**natMX4**-loxP mdh2(41,1094)::loxP-**hphMX4**-loxP mae1(41,1269)::loxP-**hphMX4**-loxP	This study
F1	MATa URA3 MAL2- 8c SUC2 *fum1Δ*	fum1(1,1050)::loxP-**hphMX4**-loxP	This study
Producer_F	MATa URA3 MAL2- 8c SUC2 *ser3Δ ser33Δ fum1Δ*	ser33(1,1090)::loxP-**natMX4**-loxP ser3(1,1240)::loxP-**kanMX4**-loxP fum1(1,1050)::loxP-**hphMX4**-loxP	This study
Producer_Fz	MATa URA3 MAL2- 8c SUC2 *ser3Δ ser33Δ zwf1Δ fum1Δ*	fum1(1,1050)::loxP-**hphMX4**-loxP zwf1(41,1475)::loxP-**hphMX4**-loxP ser3(1,1240)::loxP-**kanMX4**-loxP ser33(1,1090)::loxP-**natMX4**-loxP	This study
Producer_FzG	MATa *ura3-52* MAL2- 8c SUC2 *ser3Δ ser33Δ zwf1Δ fum1Δ gdh1Δ::GDH2*	fum1(1,1050)::loxP-**hphMX4**-loxP zwf1(41,1475)::loxP-**hphMX4**-loxP ser3(1,1240)::loxP-**kanMX4**-loxP ser33(1,1090)::loxP-**natMX4**-loxP gdh1::GDH2_**URA3MX4**	This study
E_Chassis_z	*MATa URA3 MAL2-* 8^c *SUC2 ser3Δ ser33Δ zwf1Δ*	Evolved isolate from Chassis_z,	This study
E_Producer_Sz	MATa URA3 MAL2- 8c SUC2 *ser3Δ ser33Δ sdh3Δ zwf1Δ*	Evolved isolate from Producer_Sz, (isolate SZ-3-M)	This study
E_Producer_F	MATa URA3 MAL2- 8c SUC2 *ser3Δ ser33Δ sdh3Δ zwf1Δ*	Evolved isolate from Producer_F, (isolate F-2-M)	This study
E_Producer_Mz	MATa URA3 MAL2- 8c SUC2 *ser3Δ ser33Δ sdh3Δ zwf1Δ*	Evolved isolate from Producer_Mz, (isolate MZ-2-L)	This study
E_Producer_Fz	MATa URA3 MAL2- 8c SUC2 *ser3Δ ser33Δ sdh3Δ zwf1Δ*	Evolved isolate from Producer_Fz, (isolate 2)	This study
E_Producer_FzG	MATa URA3 MAL2- 8c SUC2 *ser3Δ ser33Δ sdh3Δ zwf1Δ*	Evolved isolate from Producer_FzG, (isolate 3)	This study

[a] Strain name used in the results section, where the letter "S" for succinate, "F" for fumarate, "M" for malate, "z" indicates that *ZWF1* gene is deleted in the chassis strain genotype and "E_" stands for evolved strain.
[b] Institut für Mikrobiologie, der Johan Wolfgang Goethe-Universität, Frankfurt am Main, Germany (EUROSCARF).

Appendix Table S8. List of primers used in this study

Name	Sequence 5'→ 3'
Primers for deletion-cassette amplification	
SDH3-F1	ACCAAAAATTGCCAATCACAAGCTCTAAGAATAATAAACG**CAGCTGAAGCTTCGTACGC**
SDH3-R1	AAGTACCGAGAACGGCGGTGAAACCAATGAGAGCGTAACC**GCATAGGCCACTAGTGGATCTG**
FUM1-F1	TCCATAAAGTCTAACTATTAAACGGATAAGAGATACAATC**CAGCTGAAGCTTCGTACGC**
FUM1-R1	AGATTTAGTCAACAACTCATGAATACGAGGCTCATTGGCT**GCATAGGCCACTAGTGGATCTG**
SER3-F1	ATACAGAACTCTATAAAGAACCACAGAAAAATCGACAGCA**CAGCTGAAGCTTCGTACGC**
SER3-R1	ATTGCTTTTCGATGTTATGGTTCGATAAAATATCATTGA**GCATAGGCCACTAGTGGATCTG**
SER33-F1	AAAAGTAACAAACACTGATTTCGGGTATTTCCTCCCTAAC**CAGCTGAAGCTTCGTACGC**
SER33-R1	ACAGAGTTACCTTCATTGATGTATTTGGACAATGCAGTAG**GCATAGGCCACTAGTGGATCTG**
ZWF1-S1	ATGAGTGAAGGCCCCGTCAAATTCGAAAAAAAATACCGTCA**CAGCTGAAGCTTCGTACGC**
ZWF1-S2	ATTATCCTTCGTATCTTCTGGCTTAGTCACGGGCCAAGCG**GCATAGGCCACTAGTGGATCTG**
MAE1-S1	ATGCTTAGAACCAGACTATCCGTTTCCGTTGCTGCTAGAT**CAGCTGAAGCTTCGTACGC**
MAE1-S2	GGGCGGGAGTGGAGTTAGCCTCGTAAGATTGTAGAATTAA**GCATAGGCCACTAGTGGATCTG**
MDH1-F1	AAGAAAAAAAAACAAAAGGAAAAGGAAGGATACCATATACA**CAGCTGAAGCTTCGTACGC**
MDH1-R1	GTAGCATTTCTTCTTCTTCTGAAGATAACTCACCTATTGG**GCATAGGCCACTAGTGGATCTG**
MDH2-S1	ATGCCTCACTCAGTTACACCATCCATAGAACAAGATTCGT**CAGCTGAAGCTTCGTACGC**
MDH2-S2	TTAAGATGATGCAGATCTCGATGCAACGAATTCCAAGCCC**GCATAGGCCACTAGTGGATCTG**
Diagnostic primers for deletion confirmation	
SDH3-A1	GCTGTATACCAACAGCCTTC
SDH3-A4	ATGACCGCCTATGTTTGC
FUM1-A1	TCCTTAAACCCTTCCGAATC
FUM1-A4	GTATGCCTATGCTCCTCTTC
SER3-A1	TTAAGCAGTTAGGCTGGACC
SER3-A4	AGAATTCGGGTTTGCGTTCC
SER33-A1	TCGTTTATACTGGCTGACCC
SER33-A4	AGCTCGACAGATTATCGTCC
ZWF1-A1	ATCTGGTGCGTAAACTGACC
ZWF1-A4	ATGGAGGGCAAAGGGACAG
MAE1-A1	CATACAACCAAGTATAGACGG
MAE1-A6	GTTGGTGATGGTGTCCAGG
MDH1-A5	GTGCGCTGCAGGGTGCTAC
MDH1-A8	CATTCAAAGCACGCATAGTAC
MDH2-A1	GCCCTCTTCTGGCGCCTG
MDH2-A4	CGTATTGCAGCGAGGGTTCC
K1-A	GGATGTATGGGCTAAATGTACG
K2-A	CATCATCTGCCCAGATGCG
MATa	ACTCCACTTCAAGTAAGAGTTTG
MATal	GCACGGAATATGGGACTACTTCG
MATa/al	AGTCACATCAAGATCGTTTATGG

Appendix Table S9. Metabolites, retention time and ion fragments used for quantification with GC-MS

Metabolite	Metabolite_derivative	Retention Time (minutes)	Quantifying Ion Fragment (m/z)
Lactic acid	Lactic acid-2TMS	2.765	191.1
Pyruvic acid	Pyruvic acid-meto-TMS	3.742	174
Glycerol	Glycerol-3TMS	5.069	218.1
Glycine	Glycine-3TMS	6.112	174.1
Serine	Serine-3TMS	7.16	204.1
Fumaric acid	Fumaric acid-2TMS	7.562	245
Succinic acid	Succinic acid-2TMS	7.649	247.1
Glyceric acid	Glyceric acid-3TMS	8.081	189.1
Malic acid	Malic acid-3TMS	9.616	335
2-hydroxyglutaric acid	2-Hydroxyglutaric acid-3TMS	10.823	247.2
Ribitol (*internal standard*)	Ribitol-5TMS	10.892	319.1
Putrescine	Putrescine-4TMS	11.021	174.1
5-oxoproline	5-Oxoproline-2TMS	11.566	156.1
2-ketoglutaric acid	2-Ketoglutaric acid-meto-2TMS	12.01	198.1
Ornithine	Ornithine-4TMS	12.595	142.1
Glucose	Glucose-meto-5TMS (peak 1)	13.237	319.1
Inositol	Inositol-6TMS (peak 1)	14.623	305.1
Palmitic acid	Palmitic acid-TMS	15.605	313.3
Stearic acid	Stearic acid-TMS	17.338	341.3
Oleic acid	Oleic acid-TMS	17.355	339.3

Appendix Table S10. Production titers, yields and rates of the engineered strains, before and after evolution, from shake flask cultivations[a] and batch fermentations[b] .

Target product	Strain	Growth rate μ_{max} (h^{-1})	Max Titer (g L^{-1})	Max Yield P/S (g. g-glc^{-1})	Max Yield P/X (g. g biomass^{-1})	Productivity (g $L.h^{-1}$)
	WT	0.332	0.018	0.0009	0.008	0.006
	Producer_Sz $^{+gly}$	**0.024**	**0.472**	**0.0455**	**0.614**	**0.012**
Succinate[a]	**E_Producer_Sz**	**0.147**	**0.688**	**0.0344**	**0.504**	**0.101**
	Chassis_z	0.122	0.033	0.0017	0.004	0.004
	E_Chassis_z	0.325	0.009	0.0005	0.017	0.003
	WT	0.34	0.020	0.004	0.025	0.07
Succinate[b] *batch fermentation*	**Producer_Sz** $^{+gly}$	**0.17**	**0.93**	**0.019**	**0.23**	**0.16**
	E_Producer_Sz	**0.17**	**1.12**	**0.022**	**0.28**	**0.19**
	WT	0.332	ND	ND	ND	ND
	Producer_F $^{+gly}$	**0.039**	**0.253**	**0.023**	**0.317**	**0.010**
Fumarate[a]	**E_Producer_F**	**0.182**	**0.353**	**0.063**	**0.296**	**0.064**
	Chassis_z	0.122	0.004[§]	0.0002[§]	0.002[§]	0.000[§]
	E_Chassis_z	0.325	0.002[§]	0.0001[§]	0.001[§]	0.001[§]
	WT	0.332	0.009	0.0004	0.004	0.003
	Producer_Mz $^{+gly}$	**0.088**	**0.468**	**0.0234**	**0.589**	**0.041**
Malate[a]	**E_Producer_Mz**	**0.127**	**0.255**	**0.0128**	**0.188**	**0.032**
	Chassis_z	0.122	0.033	0.0016	0.016	0.004
	E_Chassis_z	0.325	0.004[§]	0.0002[§]	0.002[§]	0.001[§]

[a] Values are presented as means of biological triplicates grown in shake flasks in defined minimal medium (20 g L^{-1} glucose). Standard deviation (s.d) for each sample was below 15%. [b] Values are presented as means of biological triplicates grown in controlled environment (batch fermenters) of samples taken in stationary phase. Glycine was supplemented with 0.5 g L^{-1} , indicated by the suffix "+ gly". ND – compound not-detected; § - Not-quantified: Metabolite detected but AUC below quantification curve.

Appendix Table S11. Glucose consumption and secreted metabolite titers of WT and engineered strains, before and after evolution, from shake flask cultivations.

Strains	Consumed	Secreted metabolites						
	Glucose [a]	**Ethanol** [b]	**Pyruvate** [a]	**Acetate** [b]	**Lactate** [a]	**Succinate** [a]	**Fumarate** [a]	**Malate** [a]
WT	19.984±0.001	8.009±0.001	0.048±0.000	2.202±0.000	0.014±0.004	0.018±0.005	0.002±0.000	0.008±0.001
Chassis_z	19.984±0.000	8.934±0.001	0.048±0.001	0.845±0.001	0.012±0.002	0.033±0.008	0.004±0.000	0.033±0.007
E_Chassis_z	19.984±0.000	7.410±0.001	0.049±0.000	3.503±0.002	0.027±0.005	0.009±0.000	0.002±0.000	0.004±0.000
Producer_Sz*	10.529±1.194	3.560±0.458	0.163±0.016	0.780±0.083	0.011±0.001	**0.472±0.060**	0.003±0.000	0.009±0.003
E_ Producer_Sz	19.984±0.000	6.510±0.046	0.087±0.057	3.360±0.114	0.016±0.001	**0.688±0.088**	0.002±0.000	0.005±0.001
Producer_F*	9.297±0.471	4.160±0.386	0.256±0.019	0.463±0.015	0.023±0.004	0.189±0.022	**0.253±0.005**	0.004±0.000
E_Producer_F	18.510±0.000	7.171±0.004	0.168±0.029	0.290±0.017	0.022±0.005	0.108±0.013	**0.353±0.006**	0.004±0.000
Producer_Mz*	14.666±2.315	3.558±0.001	0.163±0.061	0.778±0.001	0.014±0.002	0.031±0.002	0.039±0.005	**0.468±0.020**
E_Producer_Mz	19.984±0.000	6.510±0.000	0.052±0.001	3.356±0.01	0.016±0.006	0.019±0.004	0.016±0.003	**0.255±0.045**

Values are presented as average of 3 biological replicates grown in shake flasks in defined minimal medium (20 g L^{-1} glucose) ± Standard deviation (s.d).

[a] Metabolite concentration was determined by GS-MS.

[b] Metabolite concentration was determined by UPLC.

The growth medium of parental strains was supplemented with glycine (0.5 g L^{-1}), indicated by '*'.