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SUPPLEMENTARY INFORMATION

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The industrial yeast *Pichia pastoris* is converted from a heterotroph into an autotroph capable of growth on CO_2

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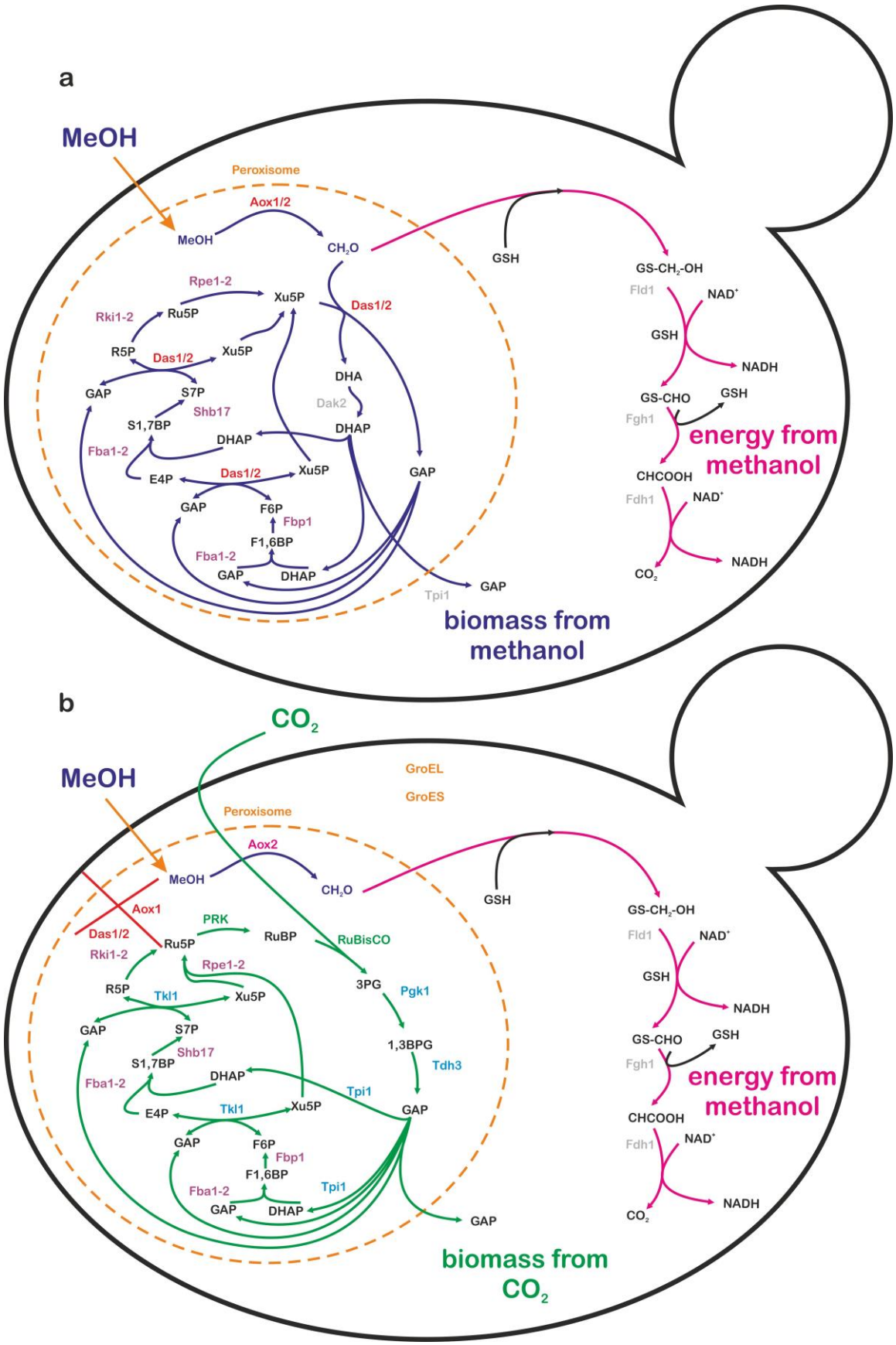
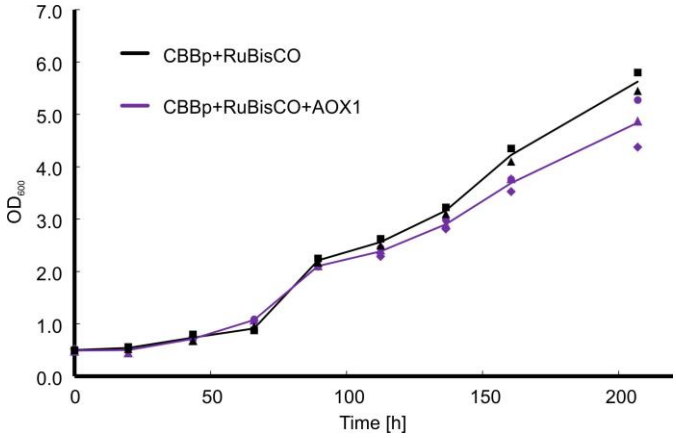

a
MeOH
Peroxisome
Aox1/2
MeOH
CH2O
GSH
GS-CH2-OH
Fld1
NAD+
GSH
NADH
GS-CHO
GSH
Fgh1
energy from methanol
CHCOOH
Fdh1
NAD+
CO2
NADH
Rpe1-2
Xu5P
Das1/2
Rki1-2
Ru5P
R5P
Das1/2
Xu5P
DHA
GAP
S7P
Dak2
Shb17
S1,7BP
DHAP
DHAP
Fba1-2
E4P
Das1/2
Xu5P
GAP
GAP
F6P
Fbp1
F1,6BP
Fba1-2
GAP
DHAP
Tpi1
GAP
biomass from methanol
b
CO2
MeOH
GroEL
GroES
Peroxisome
Aox2
MeOH
CH2O
GSH
GS-CH2-OH
Fld1
NAD+
GSH
NADH
GS-CHO
GSH
Fgh1
energy from methanol
CHCOOH
Fdh1
NAD+
CO2
NADH
Aox1
Das1/2
PRK
RuBP
RuBisCO
Ru5P
Rki1-2
Rpe1-2
R5P
Tkl1
Xu5P
3PG
Pgk1
GAP
S7P
1,3BPG
Shb17
S1,7BP
Tdh3
Fba1-2
DHAP
Tpi1
E4P
Tkl1
Xu5P
GAP
GAP
F6P
Fbp1
F1,6BP
Fba1-2
Tpi1
GAP
DHAP
GAP
biomass from CO2

Supplementary Figure 1

Strategy for engineering chemoorganoautotrophy in *Pichia pastoris*.

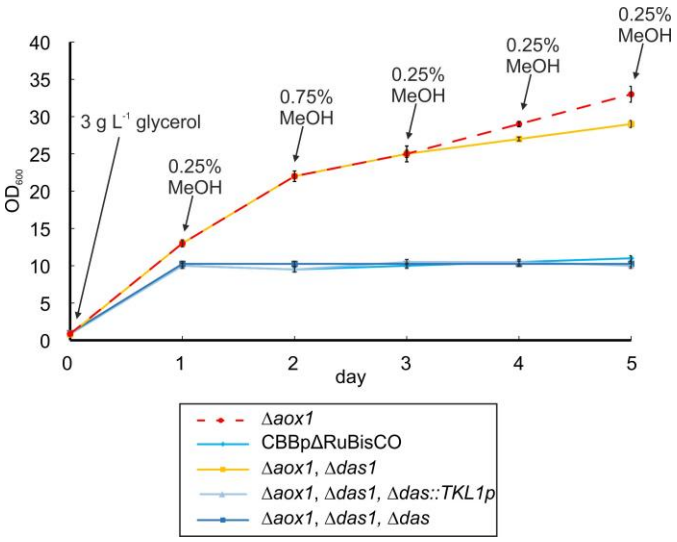
(**a**) **Wild type *P. pastoris*** is able to use methanol (MeOH) for biomass in the assimilatory branch of the MeOH utilization by fixation of formaldehyde (FA) to xylulose-5-phosphate (Xu5P) which is regenerated in the xylulose monophosphate (XuMP) cycle (purple pathway) and as an energy source in the dissimilatory branch (pink pathway) by oxidation to carbon dioxide (CO_2) under formation of NADH. (**b**) In an **engineered strain** MeOH assimilation is blocked by deletion of dihydroxyacetone synthase (*DAS1* and *DAS2*) and a CO_2 fixation pathway similar to a Calvin-Benson-Bassham (CBB) cycle is integrated (integration of *RuBisCO*, *PRK*, *TDH3*, *PGK1*, *TKL1*, *TPI1*, *groEL* and *groES*). RuBisCO carboxylates ribulose-1,5-bisphosphate which is regenerated in the synthetic CBB cycle; Additionally, the alcohol oxidase 1 gene *AOX1* was deleted to avoid accumulation of formaldehyde. The enzymatic steps shown in magenta are present in both pathways (a and b). Abbreviations: alcohol oxidase (Aox), dihydroxyacetone synthase (Das), fructose-1,6-bisphosphate aldolase (Fba1-2), fructose-1,6-bisphosphatase (Fbp1), sedoheptulose-1,7-bisphosphatase (Shb17), ribose-5-phosphate ketol-isomerase (Rki1-2), D-ribulose-5-phosphate 3-epimerase (Rpe1-2), formaldehyde dehydrogenase (Fld1), S-formylglutathione hydrolase (Fgh1), formate dehydrogenase (Fdh1), ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), dihydroxyacetone (DHA), glyceraldehyde-3-phosphate (GAP), 3-phosphoglycerate (3PGA), glutathione (GSH), ribose-5-phosphate (R5P), ribulose-1,5-bisphosphate (RuBP).



Supplementary Figure 2

Re-integration of *AOX1* does not lead to higher growth rates.

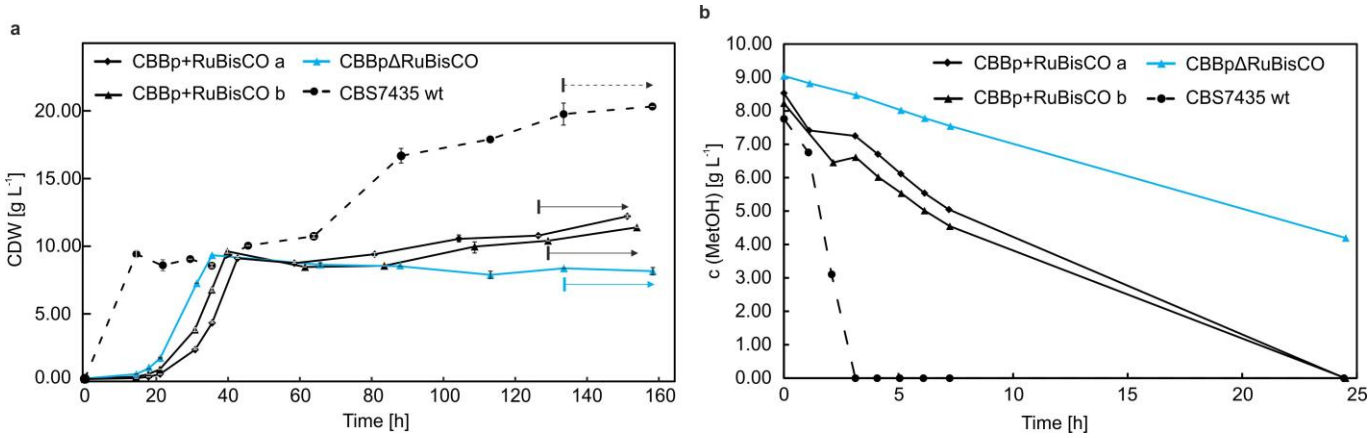
CBBp+RuBisCO (black line) and CBBp+RuBisCO (purple line) were cultivated under chemoorganoautotrophic conditions and the mean of at least three biological replicates (independent transformations) with single values (black and purple signs) is shown.



Supplementary Figure 3

Transketolase 1 (Tkl1) does not substitute for dihydroxyacetone synthase (Das1/2).

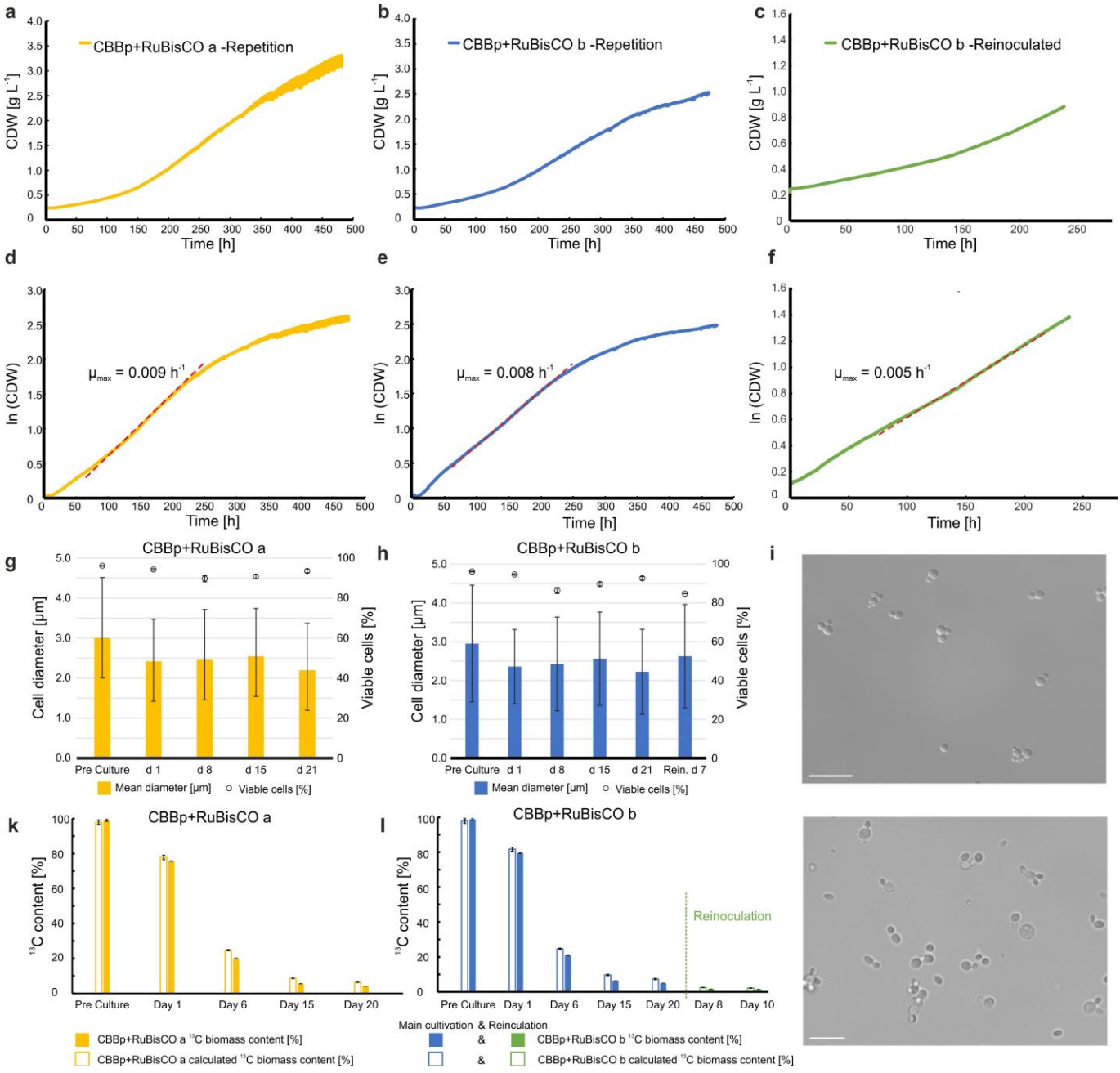
Cells were cultivated in 3 g L^{-1} glycerol containing minimal medium and then fed with methanol (MeOH) shots as indicated (first shot was for induction while glycerol was still present). **Strains**: **Δ*aox1*** (red dashed line), **Δ*aox1*,Δ*das1***: Δ*aox1::TDH3p*,*PGK1p* and Δ*das1* (yellow line), **Δ*aox1*,Δ*das1*,Δ*das2::TKL1p***: Δ*aox1*::*TDH3p*,*PGK1p*, Δ*das1* and Δ*das2*::*TKL1p* (dark blue line), **Δ*aox1*,Δ*das1*,Δ*das2***: Δ*aox1*::*TDH3p*,*PGK1p*, Δ*das1* and Δ*das2* (blue line) and **CBBΔRuBisCO**: Δ*aox1*::*TDH3p*,*PRKp*,*PGK1p*, Δ*das1* and Δ*das2*::*TPI1p*,*TKL1p*, Each clone was cultivated in duplicates (error bars indicate *s.e.m.*) and the Δ*aox1* control was cultivated as triplicate (error bars show *s.d.* of three independent cultivations, n=3).



Supplementary Figure 4

Engineered CBBp+RuBisCO grow in presence of methanol and CO_2.

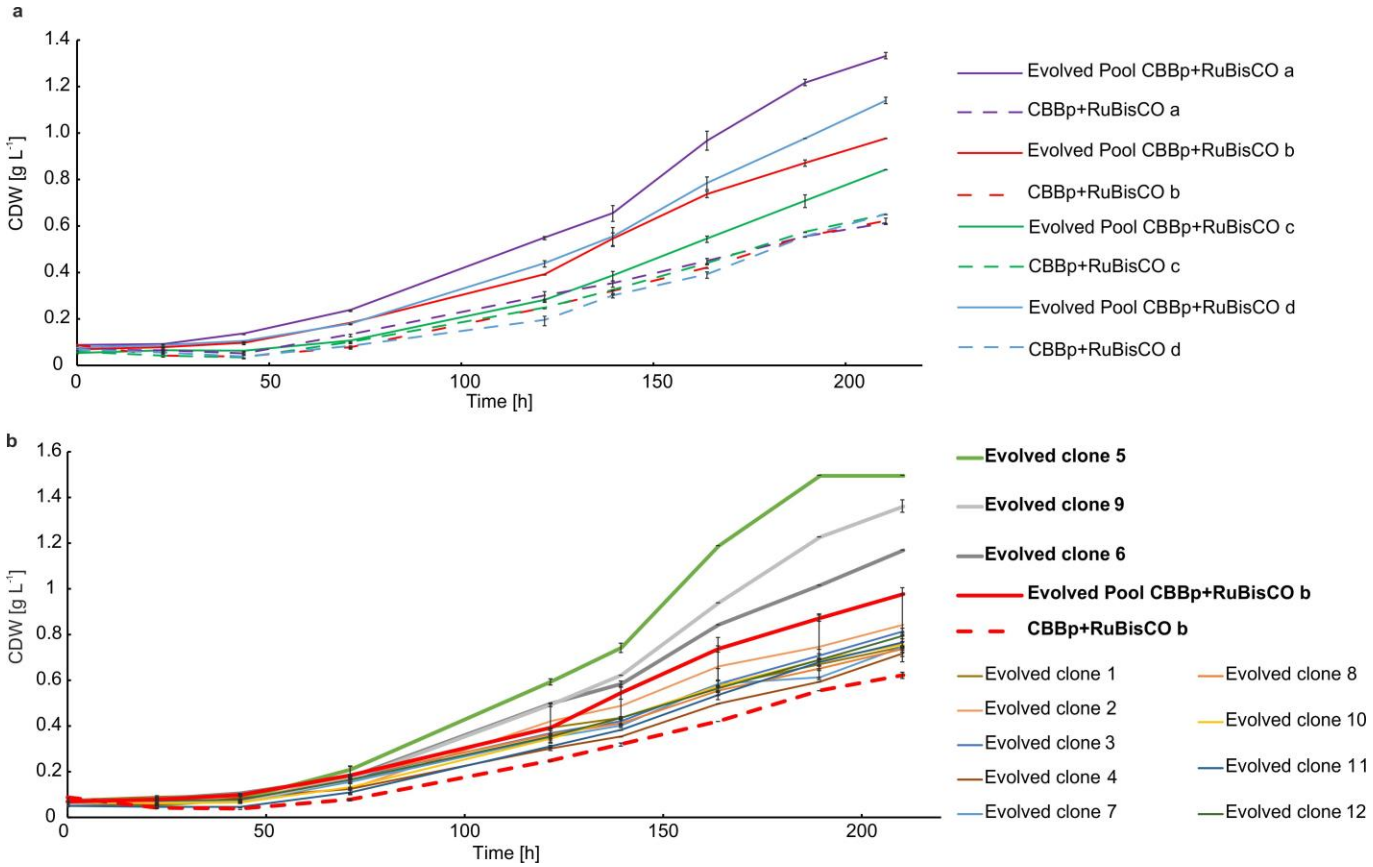
(**a**) **Engineered CBBp+RuBisCO (a and b) are able to grow in presence of methanol and CO_2 while CBBpΔRuBisCO are not.** CBS7435 *wt* cells grow well in presence of both substrates, since methanol can be utilized for biomass and energy generation. Cells were cultivated in batch phase (16.0 g L^{-1} glycerol) until 10 g L^{-1} cell dry weight (CDW) and then fed with 0.5 – 1.0% (v/v) methanol pulses and a constant inflow of 5% CO_2. CDW values are calculated from OD measurements (correlation: 1 OD_{600} unit = 0.191 g L^{-1} CDW) and standard error bars indicate *s.e.m.*; (**b**) **Methanol consumption.** Methanol uptake rates were determined during cultivation (time frame of 24 h indicated by arrows in (a) and showed the highest methanol utilization by CBS7435 *wt* cells followed by the engineered CBBp+RuBisCO (a and b – experiment repeated twice). The strain lacking RuBisCO (CBBpΔRuBisCO) showed slow methanol utilization.



Supplementary Figure 5

Labeling of engineered *P. pastoris* cells shows complete biomass formation from ^{12}C carbon dioxide.

(**a** - **c**) Bioreactor cultivation of ^{13}C labelled biomass from two CBBp+RuBisCO clones (**a** and **b** – individual transformations) and the reinoculated biomass from one clone (**c**) with $^{12}CO_2$ as a carbon source. The ^{13}C biomass content of the inoculum was enriched by cultivation on fully labelled ^{13}C glycerol. During cultivation, cells were fed with 5 % $^{12}CO_2$ and methanol. (**d** - **f**) Logarithmic CDW value indicates the specific growth rate for the strains in all three reactors. (**g** and **h**) Mean cell size (error bars show cell size *s.d.* of yeast population – 50000 events recorded) and cell viability (error bars indicate *s.e.m.*) during the cultivation of CBBp+RuBisCO a and b. (**i**) Characteristic bright field image from day 1 (upper panel) and day 21 (lower panel) of cultivation (bar = 10µm). (**k** and **l**) ^{13}C content of the biomass measured by EA-IRMS for CBBp+RuBisCO a and CBBp+RuBisCO b (**k**) and its reinoculation (**l**) empty bars indicate calculated values and full bars show measured values ± *s.e.m*.



Supplementary Figure 6

Adaptive Laboratory Evolution (ALE) leads to higher growth rates.

Cultivation of strains after ALE in serial batch cultivations for 27 to 29 generations; Cell Dry Weight (CDW) values are calculated from OD_{600} measurements (correlation: 1 OD_{600} unit = 0.191 g L^{-1} CDW, error bars indicate *s.e.m.*), (**a**) The pools (full lines) from evolved cells were compared for growth on CO_2 and methanol to each parental strain cultivated under the same conditions (dashed lines); (**b**) from one Pool (CBBp+RuBisCO b) 12 single colonies were further characterized under the same conditions showing better growth for 3 single colonies derived from the evolved population. This experiment was repeated twice with similar results.

Supplementary Tables

Supplementary Table 1: Strains used in this study

Strain name	Genotype
CBS 7435 Pichia pastoris	wt
CBBp+RuBisCO	Δ*aox1*::(*TDH3p*, *PRKp, PGK1p*) Δ*das1::(RuBisCOp*, *GroELc*, *GroESc*) Δ*das2*::(*TKL1p, TPI1p*)
CBBpΔRuBisCO	Δ*aox1*::(*TDH3p*, *PRKp, PGK1p*) Δ*das1* Δ*das2*::(*TKL1p, TPI1p*)
CBBc	Δ*aox1*::(*TDH3c*, *PRKc, PGK1c*) Δ*das1::(RuBisCOc*, *GroELc*, *GroESc*) Δ*das2*::(*TKL1c, TPI1c*)
Δ*aox1*, Δ*das1*	Δ*aox1*::(*TDH3p*, *PGK1p*) Δ*das1*
Δ*aox1*, Δ*das1*, Δ*das2:TKL1p*	Δ*aox1*::(*TDH3p, PGK1p*) Δ*das1* Δ*das2*::(*TKL1p*)
Δ*aox1*, Δ*das1*, Δ*das2*	Δ*aox1*::(*TDH3p, PGK1p*) Δ*das1* Δ*das2*
CBBp+RuBisCO+AOX1	Δ*aox1*::(*TDH3p*, *PRKp, PGK1p*) Δ*das1::(RuBisCOp*, *GroELc*, *GroESc*) Δ*das2*::(*TKL1p, TPI1p*) -*AOX1*tt-P_{AOX1}-AOX1-*RPS2*tt-*G418*-*AOX1*tt-3’

Supplementary Table 2: Fractional ^{13}C labeling of free amino acids determined by GC-TOFMS

	CBBpΔRuBisCO			**CBBp+RuBisCO I**			**CBBp+RuBisCO II**			**CBBp+RuBisCO III**				
Strain	**CBBpΔRuBisCO**			**CBBp+RuBisCO**			**CBBp+RuBisCO**			**CBBp+RuBisCO**				
C-source	$^{12}CO_2$ / $^{13}CH_3OH$			$^{12}CO_2$ / $^{13}CH_3OH$			$^{12}CO_2$ / $^{13}CH_3OH$			$^{12}CO_2$ / $^{12}CH_3OH$				
	Fractional Label [%]													
Time [h]	**45 h**	**85 h**	**158 h**	**45 h**	**85 h**	**158 h**	**45 h**	**85 h**	**158 h**	**45 h**	**85 h**	**158 h**	**Instr. prec.†**	**Biol. prec. ‡**
Amino Acid													**[%]**	**[%]**
Ala	98	93	86	98	16	10	98	17	10	97	11	2	1	1
Asp	98	87	76	98	21	13	98	22	12	98	17	6	1	3
Glu	98	94	94	98	28	15	98	29	14	99	19	7	4	1
Ile	94	96	93	100	71	53	98	72	51	97	69	45	8	4
Leu	91	91	91	93	71	53	91	72	53	94	68	45	1	1
Phe	99	98	98	98	63	41	98	64	42	96	59	33	<1	1
Pro	98	95	94	98	64	46	98	65	46	98	61	36	2	1
Thr	98	94	93	98	51	29	98	51	30	98	47	22	3	<1
Val	97	96	96	98	56	35	97	56	35	98	52	26	1	1
Gln	97	94	92	98	33	17	98	34	17	98	22	6	<1	4
Gly	95	93	91	94	37	16	92	37	16	86	32	7	1	1
Ser	95	94	94	96	50	27	93	51	26	89	36	16	1	1

Precision was calculated as standard deviation and is listed in the table as instrumental precision (†), obtained for n=3 standards and as (‡) biological precision, obtained for 3 biological replicates (CBBp+RuBisCO I and II, 45, 85, 158 h), both under repeatability conditions of measurement. The standard deviation was calculated as the square root of the within-group variance according to the Eurachem Guide "The Fitness for Purpose of Analytical Methods" (2nd edition), with the grouping factor p being the evaluated isotopologues of the respective compound, and n being the number of observations (n=3 for instrumental precision, n=2 for biological precision). A correction factor for the estimation of standard deviation calculated from small numbers of observations (n) was applied in order to avoid underestimation 1.

Supplementary Table 3: EA-IRMS results from the labeling experiment (s.e.m. is included as percentage obtained from 3 technical replicates)*

	CBBpΔRuBisCO		**CBBp+RuBisCO I**		**CBBp+RuBisCO II**		**CBBp+RuBisCO III**	
Strain	**CBBpΔRuBisCO**		**CBBp+RuBisCO**		**CBBp+RuBisCO**		**CBBp+RuBisCO**	
C-source	$^{12}CO_2$ / $^{13}CH_3OH$		$^{12}CO_2$ / $^{13}CH_3OH$		$^{12}CO_2$ / $^{13}CH_3OH$		$^{12}CO_2$ / $^{12}CH_3OH$	
Time [h]	$^{13}C^{cal}$	$^{13}C^{m}$	$^{13}C^{cal}$	$^{13}C^{m}$	$^{13}C^{cal}$	$^{13}C^{m}$	$^{13}C^{cal}$	$^{13}C^{m}$
45 Batch end	96.3±0.1%	95±0.5%	96.5±0.2%	97±0.1%	96.5±0.2%	97±0.3%	96.4±0.0%	97±0.1%
85	96.3±1.5%	95±0.8%	80.3±0.3%	76±0.9%	76.3±3.0%	77±0.6%	68.8±2.2%	72±0.2%
133	96.3±0.1%	95±0.3%	57.4±0.6%	57±0.6%	56.8±2.6%	58±0.1%	52.8±1.9%	50±0.4%
158	96.3±2.3%	96±0.5%	50.3±0.6%	52±0.3%	50.0±2.4%	48±0.2%	45.4±2.3%	43±0.4%

*The reported *s.e.m.* is calculated by Gaussian error propagation based on the repetition of technical replicates (n=3) combined with the estimated error of the two-point referencing to the bicarbonate standards.

Supplementary Table 4: Fractional ^{13}C labeling of free metabolites determined by GC-TOFMS and LC-MS/MS

	CBBpΔRuBisCO			**CBBp+RuBisCO I**			**CBBp+RuBisCO II**			**CBBp+RuBisCO III**				
Strain	**CBBpΔRuBisCO**			**CBBp+RuBisCO**			**CBBp+RuBisCO**			**CBBp+RuBisCO**				
C-source	$^{12}CO_2$ / $^{13}CH_3OH$			$^{12}CO_2$ / $^{13}CH_3OH$			$^{12}CO_2$ / $^{13}CH_3OH$			$^{12}CO_2$ / $^{12}CH_3OH$				
	Fractional Label [%]													
Time [h]	**45 h**	**85 h**	**158 h**	**45 h**	**85 h**	**158 h**	**45 h**	**85 h**	**158 h**	**45 h**	**85 h**	**158 h**	**Instr. prec.†**	**biol. prec.‡**
Metabolite													**[%]**	**[%]**
2PGA	100	100	100	98	13	14	n/a	18	9	100	9	4	5	8
3PGA	99	100	90	98	12	13	100	17	9	96	8	3	5	7
DHAP	100	100	100	100	17	8	100	11	10	100	6	2	2	4
GAP	100	98	100	100	18	10	91	14	13	100	9	5	2	4
R5P	95	96	92	96	27	5	100	27	5	100	15	3	5	4
RuBP	98	97	94	98	7	12	98	14	6	98	6	3	4	12
F6P	97	100	n/a	99	12	13	98	14	10	99	12	7	8	5
Glc	93	84	67	92	41	32	93	71	15	93	37	12	4	11
M6P	99	100	n/a	99	15	14	99	16	11	98	13	6	5	6

Precision was calculated as standard deviation and is listed in the table as instrumental precision (†), obtained for n=3 standards and as (‡) biological precision, obtained for 3 biological replicates (CBBp+RuBisCO I and II, 45, 85, 158 h), both under repeatability conditions of measurement. The standard deviation was calculated as the square root of the within-group variance according to the Eurachem Guide "The Fitness for Purpose of Analytical Methods" (2nd edition), with the grouping factor p being the evaluated isotopologues of the respective compound, and n being the number of observations (n=3 for instrumental precision, n=2 for biological precision). A correction factor for the estimation of standard deviation calculated from small numbers of observations (n) was applied in order to avoid underestimation 1.

Supplementary Table 5: Comparison with enginnered and native autotrophic systems

Strain	**Specific growth rate μ [h^{-1}]**	**genotype**	**Energy source**	**Cultivation System**	**Reference**
Methylobacterium extorquens AM1	0.003*	*engineered*	methanol	stirred reactor	[2]
Eubacterium aggregans	0.005	*wt*	hydrogen	syngas - anaerobic flasks	[3]
Chlorella ellipsoidea	0.007	*wt*	light	bubble column	[4]
Chlorella sp. NCTU-2	0.008	*wt*	light	bubble column	[5]
Chlorella sp. NCTU-2	0.009	*wt*	light	centric tube	[5]
Chlorella sp. NCTU-2	0.011	*wt*	light	porous centric tube	[5]
Pichia pastoris	0.018	*engineered*	methanol	stirred reactor / shake flask	This Study
Neochloris conjuncta	0.019	*wt*	light	bubble column	[6]
Acetobacterium fimetarium	0.020	*wt*	hydrogen	syngas - anaerobic flasks	[3]
Neochloris texensis	0.022	*wt*	light	bubble column	[6]
Neochloris terrestris	0.028	*wt*	light	bubble column	[6]
Botryococcus braunii	0.030	*wt*	light	bubble column	[6]
Nannochloropsis salina	0.037	*wt*	light	combined light photobioractor	[7]
Scenedesmus obliquus.	0.044	*wt*	light	bubble column	[6]

Strain	**Specific growth rate μ [h^{-1}]**	**genotype**	**Energy source**	**Cultivation System**	**Reference**
Blautia hydrogenotrophica	0.066	*wt*	hydrogen	syngas - anaerobic flasks	[3]
Sporomusa acidovorans	0.071	*wt*	hydrogen	syngas - anaerobic flasks	[3]
Acetobacterium wieringae	0.081	*wt*	hydrogen	syngas - anaerobic flasks	[3]
Chlorella sorokiniana	0.10	*wt*	light	foam-bed photo-bioreactor	[8]
Clostridium magnum	0.150	*wt*	hydrogen	syngas - anaerobic flasks	[3]
Sporomusa ovata	0.150	*wt*	hydrogen	syngas - anaerobic flasks	[3]
Ralstonia eutropha	0.17	*wt*	formate	stirred reactor	[9]
Synechococcus sp. PCC 7002	0.20	*wt*	light	mixed photo-bioreactor	[10]
Ralstonia eutropha H16	0.22	*wt*	hydrogen	stirred reactor	[9]
Terrisporobacter mayombei	0.24	*wt*	hydrogen	syngas - anaerobic flasks	[3]
Synechococcus UTEX 2973	0.330	*wt*	light	mixed photo-bioreactor	[11]
Ignicoccus hospitalis	0.35 – 0.7	*wt*	hydrogen	anaerobic bioreactor	[12]

*limited to one cell doubling

Supplementary Table 6: Illumina sequencing data of evolved strains showing increased growth compared to the parental strain (Evolved clone 5, 6, 9 from evolved Pool CBBp+RuBisCO b, compare Supplementary Figure 6). Sequence variants are background corrected against the parental strain CBBp+RuBisCO b and listed according to their frequency of occurrence.

Strain: Clone 5 from Evolved Pool CBBp+RuBisCO b

Chromosome	Region	Type	Reference	Mutation	Locus	Coding region change	Amino acid change	Proximity to other loci (±1000 bp)	Mutation in other isolate	Coverage	Frequency
cbs7435_chr4	241433	SNV	C	G	PRK	PRKp:c.5C>G	PRKp:p.Ala2Gly		Colony 9	36	100.00
cbs7435_chr1	400619	Deletion	T	-				5´of CSH1 and MBA1	Colony 6	10	80.00
cbs7435_chr3	2256237	SNV	T	G	rRNA PP7435_Chr3-1238					140	72.14
cbs7435_chr2	613695^613696	Insertion	-	G				5´of ARL1 and 3´of TYR1		17	70.59
cbs7435_chr3	2256252..2256253	MNV	GT	AC	rRNA PP7435_Chr3-1238					128	69.53
cbs7435_chr1	2842639^2842640	Insertion	-	TC	rRNA PP7435_Chr1-1572					31	67.74

Strain: Clone 6 from Evolved Pool CBBp+RuBisCO b

Chromosome	Region	Type	Reference	Mutation	Locus	Coding region change	Amino acid change	Proximity to other loci (±1000 bp)	Mutation in other isolate	Coverage	Frequency
cbs7435_chr1	1737865	SNV	C	T	NMA1	NMA1:c.1073C>T	NMA1:p.Thr358Ile			37	100.00
cbs7435_chr4	177160	Deletion	A	-				3´of PP7435_Chr4-0092 and 5´of PP7435_Chr4-0093		12	100.00
cbs7435_chr1	400619	Deletion	T	-				5´of CSH1 and MBA1	Colony 5	13	76.92
cbs7435_chr2	1370792..1370793	MNV	AC	TT				5´of LIA1 and ADD66		13	69.23
cbs7435_chr4	1329822^1329823	Insertion	-	A				´TRF5 and 5´of ILV2		15	66.67

Strain: Clone 9 from Evolved Pool CBBp+RuBisCO b

Chromosome	Region	Type	Reference	Mutation	Locus	Coding region change	Amino acid change	Proximity to other loci (±1000 bp)	Mutation in other isolate	Coverage	Frequency
cbs7435_chr4	241441	SNV	A	G	PRK	PRKp:c.13A>G	PRKp:p.Thr5Ala		Colony 5	24	100.00
cbs7435_chr1	2586535^2586536	Insertion	-	T				5´of SSH1 and TAE1		20	90.00
cbs7435_chr2	297433^297434	Insertion	-	A				3´ of PP7435_Chr2-0159 and PP7435_Chr2-2749		12	66.67

Supplementary Table 7: Genes used in this study including their sources

Gene Name	UniProt	Source	EC Number	Full Name	PTS added
RuBisCO*p/c*	Q60028	*Thiobacillus denitrificans* (ATCC 25259)	4.1.1.39	Ribulose-bisphosphate carboxylase	YES/NO*
PRKp/c	P09559.1	*Spinacia oleracea*	2.7.1.19	Phosphoribulokinase	YES/NO*
PGK1p/c	A0A1B7SCV2	*Ogataea polymorpha* (CBS 4732)	2.7.2.3	Phosphoglycerate kinase	YES/NO*
TDH3p/c	A0A1B7SCG5	*Ogataea polymorpha* (CBS 4732)	1.2.1.12	Glyceraldehyde-3-phosphate dehydrogenase	YES/NO*
TPI1p/c	W1Q838	*Ogataea parapolymorpha* (CBS11895)	5.3.1.1	Triosephosphate isomerase	YES/NO*
TKL1p/c	W1QKQ2	*Ogataea parapolymorpha* (CBS11895)	2.2.1.1	Transketolase	YES/NO*
groEL	B1XDP7	*Escherichia coli (DH10B)*	N/A	molecular chaperone GroEL	NO
groES	B1XDP6	*Escherichia coli (DH10B)*	N/A	molecular chaperone GroES	NO

* In case of peroxisomal targeting, a C-terminal peroxisome targeting signal 1 (PTS1) encoding for SKL (Methods Mol. Biol. 898, 161–9, 2012) was added by amplification with primers carrying the additional nucleotides

Supplementary Table 8: Promoters and terminators used in this study

Gene Name	P_{XXX}	Methanol Induced	location ID	T_{XXX}	location ID	locus
PGK1p/c	P_{ALD4}	Yes	PP7435_chr2 (1466285…1467148)	T_{AOX1}	PP7435_chr4 (240891…241840)	*AOX1*
TDH3p/c	P_{AOX1}	Yes	PP7435_chr4 (237941…238898)	T_{IDP1}	PP7435_chr1 (1012481…1012975)	*AOX1*
TPI1p/c	P_{SHB17}	Yes	PP7435_chr2 (340617…341606)	T_{DAS2}	PP7435_chr3 (629173…630076)	*DAS2*
TKL1p/c	P_{DAS2}	Yes	PP7435_chr3 (632201…633100)	T_{RPS2}	PP7435_chr1 (2506918…2507385)	*DAS2*
RuBisCO*p/c*	P_{DAS1}	Yes	PP7435_chr3 (634140…634688)	T_{RPS3}	PP7435_chr1 (223093…223258)	*DAS1*
PRKp/c	P_{FDH1}	Yes	PP7435_chr3 (423504…424503)	T_{RPP1B}	PP7435_chr4 (463560…464058)	*AOX1*
groEL	P_{PDC1}	No	PP7435_chr3 (1860841…1861824)	T_{RPS17B}	PP7435_chr2 (905111…905593)	*DAS1*
groES	P_{RPP1B}	No	PP7435_chr4 (462240…463233)	T_{DAS1}	PP7435_chr3 (636813…637362)	*DAS1*

*Supplementary Table 9: Primer sequences for verification of engineered loci, band sizes after PCR amplification are 4300 bp (AOX1), 3320 bp (DAS1) and 2420 (DAS2) for wt P. pastoris; Δaox1::TDH3, PRK, PGK1 (8700 bp); Δdas1::*RuBisCO*, groEL,gGroES (7200 bp), Δdas1 (3925 bp); Δdas2::TKL1, TPI1 (4700 bp)*

Primer	**Sequence**
AOX1_Seq1_fw	TAATGTACATAGATCTTGAGATAAATTTCACGTTTA
AOX1_Seq1_rev	ATGAGACTGAGGTTTCATGAGTC
DAS1_Seq1_fw	ATTCTGTCGAAAATGGAAGCG
DAS1_Seq1_rev	CACTTGCATCACTGGCT
DAS2_Seq1_fw	CCTTTCCTTCCTGTTCCATT
DAS2_Seq1_rev	TCCTGAGTGACCGTTGTGTT

Supplementary Table 10: Mass/charge ratios evaluated for sugar phosphate mass distribution analysis

Isotopologues of analytes	Retention time (min)	m/z	Mass extraction window (ppm left / right)
2PG_M0	15.59	475.1583	50 / 50
2PG_M1		476.1617	
2PG_M2		477.1650	
2PG_M3		478.1684	
3PG_M0	15.99	475.1583	50 / 50
3PG_M1		476.1617	
3PG_M2		477.1650	
3PG_M3		478.1684	
DHAP_M0	15.66	430.1661	50 / 50
DHAP_M1		431.1694	
DHAP_M2		432.1728	
DHAP_M3		433.1761	
F6P_M0	26.90	720.2851	50 / 50
F6P_M1		721.2884	
F6P_M2		722.2918	
F6P_M3		723.2951	
F6P_M4		724.2985	
F6P_M5		725.3018	
F6P_M6		726.3052	
GAP_M0	15.46	430.1661	50 / 50
GAP_M1		431.1694	
GAP_M2		432.1728	
GAP_M3		433.1761	
M6P_M0	27.04	720.2851	50 / 50
M6P_M1		721.2884	
M6P_M2		722.2918	
M6P_M3		723.2951	
M6P_M4		724.2985	
M6P_M5		725.3018	
M6P_M6		726.3052	
R5P_M0	23.00	618.235	50 / 50
R5P_M1		619.2383	
R5P_M2		620.2417	
R5P_M3		621.245	
R5P_M4		622.2484	
R5P_M5		623.2517	

Supplementary Sequences

>RuBisCO
ATGGACCAATCTGCTAGATACGCTGACTTGTCCTTGAAAGAAGAGGACTTGATCAAG
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TTGGAAGCTGCTGCTCATTTCGCTGCTGAATCTTCCACTGGAACTAACGTTGAGGTTT
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CTTCCGAGGACATGAGAATCGCTTACCCATTGGAGTTGTTCGACAGAAACGTTACTG
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GGGTGACATTGAGCACGCTAAGATGATCGACTTCTACGTTCCTGAGAGATGTATCCA
GATGTTCGACGGTCCAGCTACTGACATTTCCAACTTGTGGAGAATCTTGGGTAGACC
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GGTTTACGACGCTATGAAGAGAGCTCAAGACGACACTGGTCAGGCTAAGTTGTTCTC
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GGAGGTTTTCGGTCCAGATGCTGACAAGTTGGCTTTCTTGGTTGACGGTTACGTTGG
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CGAGAGAGATGAGTGTCAGGGTCCAGTTTACTTCCAAAAGTGGTACGGTATGAAGC
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>PRK
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>groEL
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A

>groES
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AAGATCGACAACGAAGAGGTTTTGATCATGTCCGAGTCCGACATCTTGGCTATCGTT
GAAGCT

Supplementary References

1. Roesslein, M., Wolf, M., Wampfler, B. & Wegscheider, W. A forgotten fact about the standard deviation. *Accredit. Qual. Assur.* **12,** 495–496 (2007).
2. Schada von Borzyskowski, L. *et al.* An engineered Calvin-Benson-Bassham cycle for carbon dioxide fixation in *Methylobacterium extorquens* AM1. *Metab. Eng.* **47,** 423–433 (2018).
3. Groher, A. & Weuster-Botz, D. General medium for the autotrophic cultivation of acetogens. *Bioprocess Biosyst. Eng.* **39,** 1645–1650 (2016).
4. Wang, S.-K., Hu, Y.-R., Wang, F., Stiles, A. R. & Liu, C.-Z. Scale-up cultivation of *Chlorella ellipsoidea* from indoor to outdoor in bubble column bioreactors. *Bioresour. Technol.* **156,** 117–122 (2014).
5. Chiu, S.-Y., Tsai, M.-T., Kao, C.-Y., Ong, S.-C. & Lin, C.-S. The air-lift photobioreactors with flow patterning for high-density cultures of microalgae and carbon dioxide removal. *Eng. Life Sci.* **9,** 254–260 (2009).
6. Krzemińska, I., Pawlik-Skowrońska, B., Trzcińska, M. & Tys, J. Influence of photoperiods on the growth rate and biomass productivity of green microalgae. *Bioprocess Biosyst. Eng.* **37,** 735–741 (2014).
7. Iluz, D. & Abu-Ghosh, S. A novel photobioreactor creating fluctuating light from solar energy for a higher light-to-biomass conversion efficiency. *Energy Convers. Manag.* **126,** 767–773 (2016).
8. Janoska, A. *et al.* A liquid foam-bed photobioreactor for microalgae production. *Chem. Eng. J.* **313,** 1206–1214 (2017).
9. Friedrich, C. G., Bowien, B. & Friedrich, B. Formate and Oxalate Metabolism in *Alcaligenes eutrophus*. *J. Gen. Microbiol.* **115,** 185–192 (1979).
10. Bernstein, H. C. *et al.* Effect of mono- and dichromatic light quality on growth rates and photosynthetic performance of *Synechococcus sp.* PCC 7002. *Front. Microbiol.* **5,** 488 (2014).
11. Yu, J. *et al.* S*ynechococcus elongatus* UTEX 2973, a fast growing cyanobacterial chassis for biosynthesis using light and CO_2. *Sci. Rep.* **5,** 8132 (2015).
12. Jahn, U., Huber, H., Eisenreich, W., Hugler, M. & Fuchs, G. Insights into the Autotrophic

CO_2 Fixation Pathway of the Archaeon *Ignicoccus hospitalis*: Comprehensive Analysis of the Central Carbon Metabolism. *J. Bacteriol.* **189,** 4108–4119 (2007).